Inter-individual physiological variation in the nematode *Caenorhabditis elegans*

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Acknowledgments

"All the conditions of happiness are realised in the life of the man of science."

- Bertrand Russell

My years in Barcelona have been the best of my life, and in large part this has been because someone was letting me earn my living spending my days playing with worms and enjoying their beauty. I would first like to thank Ben for accepting me in his lab – given my questionable CV - and for his excellent guidance throughout my PhD. I have learnt a lot from him and I regret that I will leave his lab with more that could have been learnt. I also extend my thanks to the members of my Thesis Advisory Committee – Bill Keyes, Salvador Aznar-Benitah and especially to James Sharpe – for their interest shown and feedback given in our annual meetings.

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Abstract

For unknown reasons, genetically identical animals often differ substantially in their phenotypic traits, even in a controlled environment. Here I investigate the causes of inter-individual physiological variation using a model organism, the nematode *Caenorhabditis elegans*. Isogenic nematodes vary in their size at hatching, speed of development, growth rate, starvation resistance and fecundity. I show that much of this variation is due to the age of an individual's mother, with young mothers producing progeny impaired for many traits. The underlying molecular mechanism for multiple traits is a progressive, age-dependent increase in the maternal provisioning of a lipoprotein complex, yolk/vitellogenin, to embryos. The production of sub-optimal progeny by young mothers likely reflects a trade-off between the competing fitness traits of a short generation time and progeny survival and fecundity. These results identify age-dependent changes in maternal provisioning to embryos as an important source of phenotypic variation throughout the life of an animal.

Resumen

Por causas desconocidas, animales idénticos genéticamente suelen variar sustancialmente en sus rasgos fenotípicos, aunque el ambiente en el que habiten sea el mismo. Aquí investigo las causas de variación fisiológica entre individuos utilizando un organismo modelo, el nematodo *Caenorhabditis elegans*. Nematodos isogénicos varían en su tamaño al nacer, en su velocidad de crecimiento y desarrollo, en su resistencia a la privación de alimento y en su fecundidad. Muestro que muchas de estas variaciones se deben a la edad de la madre del individuo, con las madres jóvenes engendrando a progenie peor en muchos respetos. Para muchos rasgos fenotípicos, el mecanismo molecular subyacente es un aumento progresivo con la edad de la madre de la provisión de un complejo lipoproteínico, yema/vitelogenina, a los embriones. Ecológicamente, es probable que la producción de progenie inferior por parte de las madres jóvenes esté compensada por la ventaja opuesta de tener un tiempo generacional corto. Los resultados presentados destacan los cambios en la provisión maternal de recursos a los embriones como una fuente significativa de variación fenotípica a lo largo de la vida de un animal.

Prologue

The aim of this study was to identify the causes of phenotypic variation observed among genetically identical individuals of a widely studied animal model, the nematode *Caenorhabditis elegans*. Here I demonstrate that in this species maternal age strongly influences progeny phenotypes, with the progeny of young mothers impaired for many traits. Maternal provisioning of yolk proteins, vitellogenins, to offspring increases with age and this differential provisioning underlies most of the phenotypic impairments observed in early progeny.

The introduction is divided into four parts. First I briefly highlight the significance of inter-individual phenotypic variation to biology and medicine. Second, I introduce *C. elegans*, the star of the show, giving the functional and ecological context required for proper interpretation of this work. Third, I provide an overview of how parental life history can shape progeny phenotype in animals and summarise the known molecular mechanisms. Finally I describe the important molecular player in the story, the vitellogenins, in terms of their function and regulation in this species and others.

The main body of results presented in this work is in the form a manuscript, of which I am joint first author, which was originally submitted to the journal *Nature* in May 2017 and resubmitted with revisions, in the presented form, in August 2017. The paper describes the work summarised above, in addition to the contribution of Mirko Francesconi, who discovered a novel regulated shift in relative developmental timing between tissues that is strongly influenced by maternal age. In addition to that manuscript, I provide an Appendix that contains further results which were not included in the initial manuscript submission but are relevant to the regulation of progeny phenotypes by maternal age.

Lastly, I offer my interpretation of the results presented and discuss the relevance of my findings to the understanding of *C. elegans* biology and ecology and to laboratory research in this species, past and future.

Abbreviations

A. mellifera	*Apis mellifera*
Ab	antibody
AMPK	AMP-activated protein kinase
ASDF	age-dependent somatic depletion of fat
ATP	adenosine triphosphate
BF	brightfield
BMI	body mass index
BODIPY	boron-dipyrromethane
BODIPY-FA	BODIPY-conjugated fatty acid
bp	base pairs
C. elegans	*Caenorhabditis elegans*
ChIP-Seq	chromatin immunoprecipitation sequencing
D. melanogaster	*Drosophila melanogaster*
D. rerio	*Danio rerio*
DAE	DAF-16 associated element
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
E. coli	*Escherichia coli*
GFP	green fluorescent protein
H3K20	histone 3 lysine 20
H3K9	histone 3 lysine 9
H3K9me3	histone 3 lysine 9 trimethylation
IIS	insulin/insulin-like signalling
IVF	*in vitro* fertilisation
kDa	kilodaltons
miRNA	micro RNA
mRNA	messenger RNA
MUFA	monounsaturated fatty acid
NGM	nematode growth medium
NS	not significant
nt	nucleotides
PAMP	pathogen associated molecular pattern
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PIKK	phosphatidylinositol kinase-related kinase
PIP_3	phosphatidylinositol (3,4,5)-trisphosphate
PTEN	phosphatase and tensin homologue
PUFA	polyunsaturated fatty acid
qPCR	quantitative reverse transcription PCR
ROS	reactive oxygen species
RNA	ribonucleic acid
RNAi	RNA interference
R-Smad	receptor-regulated Smad
S. mimosarum	*Stegodyphus mimosarum*
SAM	S-adenosyl methionine

siRNA	short interfering RNA
sncRNA	small noncoding RNA
TAG	triacylglyceride
TGF-β	transforming growth factor β
TOR	target of rapamycin
tRNA	transfer RNA
tsRNA	tRNA-derived small RNA
VPE	vitellogenin promoter element
YA	young adult
YP115	yolk protein (115 kDa), cleavage product of *vit-6*
YP170	yolk protein (170 kDa), product of *vit-1* to *vit-5*
YP170A	yolk protein (170 kDa) A, product of *vit-3* to *vit-5*
YP170B	yolk protein (170 kDa) B, product of *vit-2*
YP88	yolk protein (88 kDa), cleavage product of *vit-6*

Contents

Acknowledgments ... iii
Abstract ... vii
Prologue ... xi
Abbreviations ... xiii
List of figures ... xvii
1. INTRODUCTION ... 1
1.1 Predicting individual phenotype from genotype ... 1
1.2 Introduction to *C. elegans* ... 4
1.2.1 *C. elegans* anatomy and development ... 5
1.2.2 Alternative life cycles ... 8
1.2.3 The ecology of *C. elegans* ... 8
1.2.4 *C. elegans* as a domesticated animal ... 9
1.3 Parental effects on progeny phenotypes ... 10
1.3 1 Influences of parental life history on offspring phenotypes ... 12
1.3.2 Molecular mechanisms of parental effects ... 21
1.3.3 Summary ... 30
1.4. Vitellogenins, the ubiquitous yolk proteins ... 31
1.4.1 Vitellogenins across the tree of life ... 31
1.4.2 Vitellogenins in *C. elegans* physiology ... 34
1.4.3 Regulation of vitellogenins in *C. elegans* ... 48
1.4.4 Possible alternative functions of vitellogenins in *C. elegans* ... 69
1.4.5 Summary ... 71
2. RESULTS ... 73
Maternal age generates phenotypic variation in *C. elegans* ... 74
Abstract: ... 75
Main Text: ... 76
Acknowledgments ... 113
Materials and Methods ... 114
Extended Data Tables ... 126
APPENDIX ... 130
Genetic regulation of the maternal age effect on vitellogenin expression ... 130
Mechanisms by which vitellogenin affects L1 starvation resistance ... 134

Embryo size does not reflect yolk provisioning 140
Additional Methods 142
3. DISCUSSION 145
3.1 Vitellogenin titre is an intermediate phenotype that may predict individual phenotypic outcomes 145
3.2 The difficulty of overexpressing vitellogenins 146
3.3 The adaptive and ecological significance of maternal age effects in C. elegans 147
3.4 Potential mechanisms for the effect of yolk on L1 starvation resistance 151
3.5 Specific components of yolk may mediate progeny phenotypes 155
3.6 The difficulty of understanding vitellogenin regulation 161
3.7 Potential confounding effects of maternal age 164
3.8 Embryo size does not reflect maternal provisioning in *C. elegans* 165
4. CONCLUSION 167
5. REFERENCES 169

List of figures

Figure	Page(s)
Figure i	7
Figure ii	38
Figure iii	40
Figure iv	45
Figure v	47
Figure vi	52
Figure vii	56
Figure viii	61
Figure ix	70
Figure x	158-159
Figure xi	163
Figure 1	77-78
Figure 2	81-83
Figure 3	87-88
Figure 4	95-96
Figure 5	108-109
Figure 6	131-132
Figure 7	135-136
Figure 8	138-139
Figure 9	141
Extended Data Figure 1	78-79
Extended Data Figure 2	85
Extended Data Figure 3	88-90
Extended Data Figure 4	91-93
Extended Data Figure 5	96-99
Extended Data Figure 6	101
Extended Data Figure 7	103-106
Extended Data Figure 8	110

1. INTRODUCTION

1.1 Predicting individual phenotype from genotype

On the eve of the release of the first draft of the human genome in mid-2000, the excitement in the scientific and medical communities was palpable. Many heralded a coming revolution in medicine, whereby the genetic bases of the many complex diseases afflicting western society would be comprehensively identified. This was predicted to lead the way within little more than a decade to 'individualised' medicine, whereby extensive genetic testing of individual patients would profile the unique combination of gene variants that would allow medical professionals to accurately predict the risk of various pathologies and prescribe timely preventative interventions. Through the field of pharmacogenomics, these interventions would be tailored to the individual, reducing the risk of adverse drug reactions and efficiently minimising disease risk (Collins 1999).

I write in 2017 and so far the promised revolution has largely failed to materialise. The explosion of research effort on genomics and individualised medicine has not yet exerted any measurable effect on population health outcomes (Joyner et al. 2016). The unexpectedly complex genetic architecture of many disorders has frustrated the identification of major predisposing variants (Rich 2016), let alone the development of novel, widely applicable interventions based on individual genetics. In the case of oncology, several molecularly targeted treatments have indeed been developed that appear to have potential for future clinical relevance, among them PARP inhibitors for treatment of BRCA-mutant cancers (Sonnenblick et al. 2015), immunotherapy for cancers with high microsatellite instability (Gatalica et al. 2016) and BRAF inhibitors for late-stage melanoma (Ascierto et al. 2016). However, despite the high hopes for individualised

medicine based on tumour profiling (Prasad 2016), patient outcomes were not improved by molecularly targeted treatments in the only randomised controlled trial to date (Le Tourneau et al. 2015). Beyond the lessons for scientists in the morality of generating high societal expectations about ambitious and unproven technologies (Petersen 2009), there is another, biological lesson to be learned: predicting individual phenotype from genotype is difficult.

Biologists typically consider an individual's phenotype to be a product of their genotype and their environment - a dichotomous view held even by the general population and encapsulated in the maxim "nature versus nurture". Thus it seemed that with a complete knowledge of individual genetic makeup and a decent sketch of environmental and lifestyle conditions, the prediction of disease phenotypes should have been relatively straightforward. However, it is not only complex genetics that hinder such prediction; even genetically identical individuals in what is ostensibly the same environment can display phenotypic variability. This variability can be a result of stochastic biological processes or difficult-to-measure environmental factors (Geiler-Samerotte et al. 2013). Indeed, environmental factors affecting phenotype may be difficult to measure because they have been lost in the sands of time - that is, they acted upon an individual's parents, or even earlier ancestors. In such cases, an identification of the molecular traces left by such influences may aid phenotypic prediction. Such 'intermediate' phenotypes will be more useful for phenotypic prediction than raw genetic data, as they have the capacity to capture environmental influence, contemporary or ancestral, as well as the aggregate of the subtle individual influences of multiple genetic variants that may impinge on a particular process (Burga and Lehner 2013).

The study of the origin of inter-individual phenotypic variation is thus important not only to an understanding of basic biology but also of the limitations faced by much-hyped new technologies in medicine and biomedical research. Such

research requires appropriate model systems, in which genetic and environmental factors can be controlled so as to allow the study of the residual phenotypic variability, and in which the molecular mechanisms that drive phenotypic variability can be identified and modified. To that end, the aim of the present thesis project was to investigate phenotypic variation in genetically identical individuals reared in an identical environment in the important model organism *Caenorhabditis elegans*.

1.2 Introduction to *C. elegans*

C. elegans is a small, free-living roundworm of the phylum Nematoda. After extensive comparison of various 'animalcules', *C. elegans* was chosen by Sydney Brenner as an ideal model organism with which to tackle the formidable problem of understanding how an animal's genotype maps, through the process of development, onto the resulting phenotype. Brenner chose well - in the forty years since 'the worm' made its debut in Brenner's seminal paper 'The genetics of *C. elegans*' (Brenner 1974), research effort in the worm has increased exponentially to the point where we can uncontroversially describe the worm as one of the best understood animals today. Indeed, Brenner's 2002 Nobel Prize lecture was entitled 'Nature's gift to science' in homage to this humble creature (Brenner 2003).

The properties that make *C. elegans* exquisitely suited to laboratory research are numerous. The worm has a body plan that is simple, yet still contains many of the same organs found in vertebrates. Its tissues are transparent, making it extremely amenable to microscopic analysis. Its life-cycle is fast, at around 3 days in the laboratory, with a median lifespan of around 3 weeks. Brenner highlighted the worm's tractability for genetics on account of its 'beautiful sex life'; as a self-fertilising hermaphrodite each worm can produce hundreds of genetically identical copies of herself in a short space of time. However rare males also exist and can serve as vectors for the transmission of genes between lines. Hermaphrodites have two X chromosomes but males have only one, so males arise spontaneously in populations of hermaphrodites by chromosomal nondisjunction. Further useful properties discovered later, such as the almost indefinite survival of frozen worms and the ease of selective repression of specific genes via the technique of RNA interference (RNAi) by feeding, have compounded the worm's rightful place as a giant of modern biology. The ability to rear thousands of isogenic worms in a controlled environment makes *C.*

elegans the ideal organism for the study of phenotypic variation arising independent of genetic or environmental factors, as in the present work. Additionally, its amenability to microscopy facilitates the study of molecular events at the individual level, which is invisible in population average measurements using standard biochemical techniques (Geiler-Samerotte et al. 2013).

1.2.1 *C. elegans* anatomy and development

C. elegans undergoes a determinate pattern of growth and development, with a series of stereotyped cell divisions that have been painstakingly mapped (Sulston and Horvitz 1977). A newly-hatched larva contains exactly 558 cells, which increase to 959 somatic cells in the adult hermaphrodite, in addition to several thousand germline nuclei.

The worm has a fluid-filled body cavity (the pseudocoelom) that serves as a hydrostatic skeleton. Within the pseudocoelom lies an alimentary canal that consists of a pharynx that pumps and grinds bacteria and an intestine that runs almost the entire length of the body to the anus. The 20 cells of the intestine are extremely large and highly polyploid, often binucleate, with many mitochondria and ribosomes. The intestine functions for digestion but also assumes roles filled by other organs in vertebrates, such as lipid storage.

The worm has two tubular gonads that each develop from a single cell during post-embryonic development. The germline passes through a period of spermatogenesis during the fourth larval stage before switching sex in early adulthood, leading to the production of oocytes that are fertilised during adulthood using the stored self-sperm. A self-fertilised hermaphrodite produces around 300 progeny within around 4 days at 20 °C, but can produce around 1000 over a longer period if fertilised by a male. The quantity of self-sperm is therefore

the primary factor limiting brood size in self-fertilised hermaphrodites (Cutter and Pitnick 2004). The germline is constantly proliferating, with stacked oocytes in the proximal gonad passing sequentially through the spermatheca for fertilisation. After fertilisation a largely impermeable eggshell forms, and shortly afterwards the egg is deposited via an opening in the body wall, the vulva, to complete development independent of the mother.

The worm's nervous system consists of 302 cells in the adult hermaphrodite. Consisting of motor neurons, touch neurons and chemosensory neurons, the nervous system is the worm's window on the world, by which it can sense and react to its environment. *C. elegans* has a muscle-lined body cavity, which allows the worm to locomote fairly rapidly in solid or liquid environments via sinusoidal undulations of the body. The worm also has a skin, the hypodermis, which secretes a tough but flexible cuticle that provides protection from desiccation, injury and infection. To allow growth, the cuticle must be shed periodically - between hatching and adulthood the worm undergoes four cycles of moulting, which separate the larval stages from one another. These larval stages are labelled L1-4 (**Fig i**).

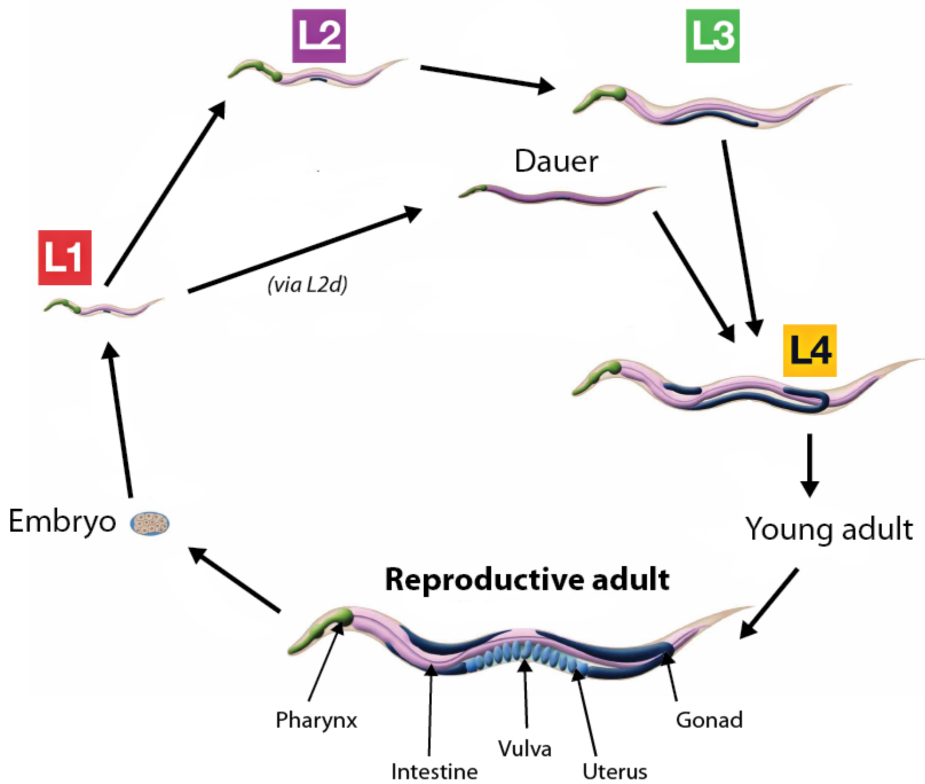


Figure i. Lifecycle and basic anatomy of *C. elegans* hermaphrodites.
At 20 °C, each adult produces around 300 embryos in 3-4 days. The generation time (from embryo to embryo) is around 65 h. Adapted from WormAtlas.com.

1.2.2 Alternative life cycles

Under challenging environmental conditions, the worm can opt for several alternative life histories, with the ability to arrest development at several stages. The best-studied, and probably most important ecologically, is the shift, under food limitation, high density and/or high temperature, to dauer, an alternative L3 stage that is motile, stress resistant and extremely long lived. Another, which is important for the interpretation of this work, is starvation-induced L1 arrest, whereby animals that hatch in the absence of food can survive for around 3 weeks, initiating post-embryonic development only when food becomes available. Time spent in L1 arrest does not decrease adult lifespan after recovery, initially leading to the belief that worms in L1 arrest do not age (Johnson et al. 1984). More recently it was shown that worms that undergo extended L1 starvation do accumulate molecular hallmarks of ageing, but that this apparent ‘ageing’ can be largely reversed upon feeding (Roux et al. 2016). Nonetheless, worms recovered for an extended L1 starvation are often worse for wear (Jobson et al. 2015, Lee et al. 2012). Starvation-induced L1 developmental arrest relies on the maintenance of cellular quiescence by insulin-like signalling and TOR signalling (both pathways reviewed in section **1.4.3.2**; Fukuyama et al. 2015, Fukuyama et al. 2006, Fukuyama et al. 2012).

1.2.3 The ecology of *C. elegans*

The ecology of *C. elegans* and related nematodes was long-neglected but in the last decade or so, there has been an increase in research effort with the acceptance that a working knowledge of the worm’s natural history is crucial to properly interpret the wealth of experimental data accumulated using this organism (Schulenburg and Félix 2017). The natural habitat of *C. elegans* seems to be bacteria-rich rotting fruit or plant stems. The patchy distribution of these rich resources likely leads to explosive population growth in proliferating populations,

followed by widespread entry into the dauer stage when food becomes exhausted. Dauers are key to dispersal to new resource patches, often by hitching a ride on passing invertebrates. When one or a few dauers succeed in reaching a benign environment, they can pass to reproductive adulthood and begin the cycle anew (Félix and Braendle 2010). This mode of explosive population growth, followed by strong bottlenecks upon colonising a new resource patch, fits well with the evolution in *C. elegans* of hermaphroditism. Hermaphroditism is enriched among species with this type of meta-population dynamics, as lone individuals can successfully colonise a new environment without requiring a mate (Pannell et al. 2015).

1.2.4 *C. elegans* as a domesticated animal

The standard laboratory strain of *C. elegans* should be considered a domesticated animal (Sterken et al. 2015). The environment of the laboratory agar plate is unnatural in many respects, such as its two-dimensionality, nearly continuous proliferative population growth and monoxenic culture with *Escherichia coli* OP50, likely a suboptimal food source originally chosen for its optical, rather than nutritional, qualities (MacNeil et al. 2013, Pang and Curran 2014, Stiernagle 1999). The commonly used 'wildtype' strain of *C. elegans*, N2, was found in a compost heap near Bristol, England, in 1951 and cultured in the laboratory for around 18 years before it was first frozen (Stiernagle 1999). As such, it had many generations to become adapted to its new environment. Large effects on multiple life-history traits that increase fitness in the laboratory have been reported resulting from several mutations absent in wild strains that must have arisen and been fixed during this period. Among them are genes involved in neuronal oxygen sensing, such as *npr-1* and *glb-1*, which alter numerous behavioural traits as well as body size and fecundity (Andersen et al. 2014, Persson et al. 2009). *nath-10*, another mutation fixed in N2, encodes an essential protein

acetyltransferase and has many pleiotropic effects, including delayed sexual maturation and increased fecundity (Duveau and Félix 2012).

1.3 Parental effects on progeny phenotypes

Through the process of embryonic and postnatal development, an animal's form and characteristics come to be defined as an interplay of the genetic information it carries and the myriad facets of the environment that the individual experiences. However, it is now appreciated that an individual's genotype and environmental experiences do not fully determine phenotype. The genotype, phenotype and experiences of one's ancestors can leave traces that can influence gene regulation, physiology and behaviour, sometimes for several generations. Here I will mostly limit consideration to the most proximal ancestors - the parents - to provide an overview of how, and in what ways, parental experience or phenotype even before conception can alter and influence an individual's phenotype.

We can define parental effects as the influence of parental phenotype on progeny phenotype, independent of their genetic contribution. Parents, especially mothers, can alter the quantity or quality of nutrients supplied to offspring, or potentially alter the supply of particular metabolites. Environmental influences can also be epigenetic, involving transmission of environmental information via transient modifications of genetic material (DNA methylation or chromatin modification) or transmission of RNA. Parental environmental experiences can also directly influence cellular processes in the gametes or developing embryo. In mammals, direct effects can persist for two generations, as the grandmaternal environment can also affect the primordial germ cells held within the developing embryo (Skinner 2008). Many genes can have maternal or ancestral effects, where maternal or ancestral genotype influences progeny phenotype independent of genotype (for example, Capowski et al. 1991 and Greer et al. 2011). However,

maternal genotype effects are outside of the scope of this review, which will focus on life history.

There is wide disagreement about the adaptive significance of parental effects. In some cases, e.g. toxin exposure, fitness effects on both parents and offspring are negative and are unlikely to be adaptive. However even when progeny fitness appears to be reduced, this could act to boost parental fitness in some contexts, for example if progeny number increase (Smith and Fretwell 1974). Some have argued that adaptive maternal effects can arise as a result of selection to maximise maternal fitness at the expense of offspring fitness (Marshall and Uller 2007) while others have asserted that maternal effects must be beneficial to offspring to be considered adaptive (Dey et al. 2016). Even when altered phenotypes of progeny appear intuitively advantageous, it is often difficult to firmly establish that they will be fitter in the context in which they emerge (Marshall et al. 2010). Here, I will largely omit discussion of the adaptive significance of parental effects, being most concerned with how parental experience can generate phenotypic diversity in offspring.

As mothers contribute the majority of the zygotic cytoplasm and supply nutrients to support embryonic development, maternal effects seem more likely to be pervasive than paternal effects. However, a multitude of paternal effects have been uncovered in diverse species (reviewed in Curley et al. 2011 and Rando 2012). As the cytoplasmic contribution of fathers is typically minimal, paternal effects are more easily attributed to epigenetic transmission. Correspondingly there has been extensive research effort in the study of paternal effects as the field of epigenetics has flourished. However, paternal effects are not always epigenetic effects - they can also act directly on the zygote, or act indirectly through modulating maternal physiology or behaviour (Crean and Bonduriansky 2014).

The effects of many parental experiences act differently according to gender, both in parents and in offspring. In mammals, male offspring are generally more sensitive to the effects of metabolic reprogramming by parental experience (Lane et al. 2014). However, sometimes sex differences affect which ancestor's experience is relevant; Pembrey et al. (2006) found that in a human population, the paternal grandmother's food availability affects mortality of granddaughters only, and likewise with paternal grandfathers and grandsons, although both parents influenced the mortality risk of daughters. Effects can also be sensitive to critical windows of development outside of which environmental inputs may have opposite effects; Pembrey et al. (2006) also found that grandparental effects of food availability on mortality were strikingly reversed within or outside the pre-pubertal slow growth period (approximately 8-12 years old).

1.3.1 Influences of parental life history on offspring phenotypes

1.3.1.1 Parental diet

As suggested by the impact of parental and grandparental food availability on mortality risk (Pembrey et al. 2006), diet is a major parental environmental influence with the potential to alter progeny phenotypes and metabolism (the latter reviewed in Rando and Simmons 2015). Dietary restriction leads to the production of larger offspring in many species that lack parental care, such as *C. elegans* (Harvey and Orbidans 2011, Hibshman et al. 2016), *Drosophila melanogaster* (Valtonen et al. 2012), *Daphnia magna* (Garbutt and Little 2017) and lecithotrophic poeciliid fishes (Reznick et al. 1996). Larger progeny will often have improved survival, for example by improved resistance to infection (Garbutt and Little 2017).

However, progeny responses to parental dietary inputs are complex. Often U-shaped responses are observed, whereby either scarcity or abundance of food, or a specific macronutrient, produce similar phenotypic perturbations. In *C. elegans* either very low or very high food concentration leads hermaphrodites to produce larger eggs (Harvey and Orbidans 2011) and fathers to sire progeny with reduced fat stores (Miersch and Döring 2012). There will also be interactions with progeny diet; in *D. melanogaster* either low or high paternal dietary sugar content leads to increases in progeny fat content, but only when progeny themselves were fed a high-sugar diet (Öst et al. 2014). Macronutrient ratios, rather than levels of individual macronutrients, can also affect progeny phenotypes, with strong but differing responses to macronutrient ratios in mothers or fathers (Bonduriansky et al. 2016).

Maternal diet seems likely to have strong direct effects on progeny outcomes but maternal and paternal dietary interventions often produce strikingly similar effects (Rando and Simmons 2015). In moths, the quality of both maternal and paternal diet affected progeny immunity to a similar magnitude, to an extent that approached the impact of the progeny's own diet (Triggs and Knell 2012). Likewise, in mice parental high-fat diets had roughly similar and additive impacts on progeny phenotypes (McPherson et al. 2015). Transient dietary insults, such as food deprivation, long before conception can influence progeny phenotypes. In *C. elegans*, a period of starvation-induced L1 arrest prior to the start of parental postembryonic development leads to smaller, slow-developing worms in the next generation, accompanied by multigenerational increases in resistance to L1 starvation (Jobson et al. 2015) and lifespan (Rechavi et al. 2014). In mice, starvation of males for just 24 hours 2 weeks prior to conception impacted offspring serum glucose levels (Anderson et al. 2006). These examples suggest epigenetic mechanisms often mediate the effects of parental diet. However, in mammals offspring are particularly sensitive to direct effects of maternal diet, and other factors, during gestation.

1.3.1.2 Environmental exposure in utero

In the autumn of 1944, Nazi-occupied Holland was placed under a food embargo, leading to famine and stringent food rationing - the infamous Hunger Winter. The famine was lifted by the following summer, but by then many pregnant women had been exposed to severe caloric restriction during gestation. This unique episode of famine in an otherwise well-nourished western population has enabled the study of the effects of maternal nutrient deprivation *in utero*. In adulthood, numerous metabolic phenotypes resulted, including an increased incidence of obesity, high cholesterol and type II diabetes - a suite of phenotypes sometimes called 'metabolic syndrome' (Alberti et al. 2005). However here too the effect of maternal nutrient deprivation depended on the period of exposure - famine during mid to late gestation produced perturbations in glucose-insulin metabolism, while exposure during early gestation led to altered lipid metabolism in adulthood (Painter et al. 2005). Similar effects are observed in rodents and can propagate indirectly across generations - male mice born to mothers deprived of calories in late gestation sire underweight, glucose intolerant pups (Jimenez-Chillaron et al. 2009).

The effects of the Dutch Hunger Winter are concordant with the 'thrifty phenotype' hypothesis of Barker (Hales and Barker 1992). The authors observed a greater predisposition to adulthood metabolic syndrome in individuals that had a low birth weight but subsequent rapid weight gain during childhood. They suggested that low birth weight caused by problems with placental nutrient transfer, mimicking *in utero* famine exposure, led to metabolic reprogramming of offspring in anticipation of harsh postnatal conditions - the 'thrifty phenotype'. Key to the hypothesis is an interaction between maternal and offspring environments; disease outcomes are produced only when ample nutrition is encountered during postnatal growth. This prediction is consistent with the divergent population outcomes of those exposed to the Hunger Winter with those

affected by famine during the Siege of Leningrad (Stanner et al. 1997), where nutritional conditions were poor before and after the Siege and little effect of *in utero* famine exposure could be detected (Hales and Barker 2001). Among the cohort of individuals affected by the Hunger Winter during gestation, the incidence of schizophrenia doubled (Susser et al. 1996), as in an analogous urban Chinese population during the Great Leap Forward famine (Song et al. 2009). Similarly, among offspring of Muslim women who were in early pregnancy during Ramadan, a 20 % increase in adult disability was observed, with an enrichment for mental disabilities (Almond and Mazumder 2011). These examples firmly establish the importance of maternal nutrition over the course of gestation in humans, perhaps unsurprisingly.

However, *in utero* conditions, dietary or otherwise, can affect progeny phenotypes from the point of conception or earlier (reviewed in Lane et al. 2014). Studies in sheep indicate that maternal overfeeding around conception increases fat mass in female offspring (Rattanatray et al. 2010), and that both maternal obesity and maternal weight loss around conception produce similar changes in offspring hepatic fatty acid metabolism (Nicholas et al. 2014). In mice it was shown that a halving of maternal dietary protein for 3.5 days after conception leads to hypertension in offspring (Watkins et al. 2011). Maternal immune responses at the point of conception can also impact progeny; the offspring of pregnant mice injected with bacterial lipopolysaccharide on the first day of pregnancy exhibited alterations in fat metabolism, behaviour and immune function (Williams et al. 2011).

The importance of the environment at conception is demonstrated by in vitro fertilisation (IVF). IVF presents an unnatural environment at conception that produces many effects on IVF-conceived individuals, which cannot be rescued even by very early embryonic transplantation. In mice, IVF leads to impaired glucose-insulin metabolism (Chen et al. 2014, Feuer et al. 2014), reduced growth,

accumulation of fat (Feuer et al. 2014), stiffer blood vessels and hypertension, and a 25 % reduction in lifespan on a high fat diet (Rexhaj et al. 2013). Data on human children conceived by IVF suggests that they share many of these phenotypes (Ceelen et al. 2008, Scherrer et al. 2012). Studies have shown that the IVF culture medium in which conception occurs affects embryo gene expression and cellular proliferation rate (Kleijkers et al. 2015) and weight and height of children at 2 years of age (Kleijkers et al. 2014).

The *in utero* environment at conception may appear to be a domain for exclusively maternal effects, but fathers can also influence progeny via seminal fluid, either directly or via its influence on the female reproductive tract (reviewed in Robertson 2005). Seminal fluid protects sperm from oxidative damage in the female reproductive tract, which may influence progeny traits. Excision of the male accessory sex glands in hamsters leads to progeny displaying abnormal patterns of growth, fecundity and behaviour (Wong et al. 2007). In mice, excision of the seminal vesicles causes altered fat and metabolic phenotypes in male progeny (Bromfield et al. 2014). Seminal fluids can also act more broadly on mothers - insect seminal fluid proteins can alter female post-copulatory behaviours (reviewed in Avila et al. 2011). Lastly, seminal fluid can provide direct antimicrobial defence (Lung et al. 2001) or directly provision offspring with specific metabolites, such as plant-acquired defensive alkaloids in moths (Dussourd et al. 1988).

1.3.1.3 Toxin exposure

Exposure to drugs or toxins even before conception can have negative impacts on progeny. Many effects will be direct effects of toxins; for example, maternal smoking during pregnancy is associated with adverse metabolic and behavioural outcomes (Behl et al. 2013, Wakschlag et al. 2002). However many indirect paternal effects have been observed; paternal smoking has been associated with

an increase in childhood body mass index (BMI) in sons (Pembrey et al. 2006) and an increased risk of childhood leukaemia (Lee et al. 2009). Likewise, alcoholism in fathers has been associated with reduced birth weight (Little 1987) and with hyperactivity and impaired cognition (Hegedus et al. 1984, Tarter et al. 1984). Experimental studies in rats confirm that pre-conception chronic exposure of male rats to alcohol causes low body weight, retarded development and altered behavioural and emotional responses (Ledig et al. 1998) - the same phenotypes produced by maternal pre-conception exposure to alcohol (Ledig et al. 1990). Even a single acute dose of alcohol administered to male mice prior to conception leads to low birth weight, slowing of development and an increase in aggressive behaviour in progeny (Meek et al. 2007). Paternal cocaine use causes reduced self-administration of cocaine, a sign of resistance to the drug, in male progeny (Vassoler et al. 2013). Effects of exposure to environmental toxins can last for several generations. Betel nuts (*Areca catechu*) are chewed across much of south-east Asia for their stimulant properties. It has been shown that chewing of betel nuts is associated with metabolic syndrome not only in the chewers but also in their progeny (Tony et al. 2006). In mice, feeding with betel nuts causes glucose intolerance, which can be transmitted to progeny and grandprogeny (Boucher et al. 1994), suggesting multigenerational transmission is likely in humans.

1.3.1.4 Parental physicochemical environment

Physicochemical conditions of the parental environment can influence progeny phenotypes. Frazier and Roth (2009) showed that in *C. elegans*, exposure of adults to high salt concentration leads to an increase in glycerol in embryos. As glycerol improves embryo survival and development in hyperosmotic conditions, this is likely an adaptive conditioning of offspring in response to maternal environment. However as glycerol levels increase, glycogen levels are reduced, increasing vulnerability of embryos to hypoxia. Dey et al. (2016) took advantage of this trade-off to expose a high salt-adapted *C. elegans* population to fluctuating

cycles of anoxia in an experimental evolution experiment. The authors found that under a predictable regime of fluctuating conditions, mothers evolved the ability to produce high glycogen embryos when their progeny were likely to develop under anoxic conditions, underscoring the potential for adaptive, anticipatory maternal effects when maternal conditions can reliably predict offspring conditions. However they were unable to find any evidence for bet-hedging or diversification strategies evolving under unpredictably fluctuating conditions.

Temperature can also produce heritable responses in gene expression. Schott et al. (2014) showed that mild heat stress in *C. elegans* is associated with heritable changes in mRNA expression. Klosin et al. (2017) demonstrated that ancestral temperature could influence the transcriptional output of a repetitive transgene array, an effect that was observed for a remarkable 14 generations after the initial environmental perturbation. Increased expression of endogenous repetitive elements, such as transposons, was also observed.

1.3.1.5 Parental social environment

The social environment of parents, both before conception and after birth, has the capacity to alter progeny phenotypes. Maternal care, such as licking and grooming, directly influence cognitive and emotional development in rodents (reviewed in Fish et al. 2004). Surprisingly, the social context of fathers prior to conception can also influence progeny. Male mice that have endured chronic social defeat stress by living with a dominant male exhibit depressive behaviours that can be inherited by offspring, even when the males are removed shortly after mating (Dietz et al. 2011). Likewise, if male mice have been raised in social isolation, offspring have reduced body weight at weaning (Mashoodh et al. 2012). This effect was shown to occur indirectly, through modification of maternal care behaviour. Similarly, the inheritance of depressive behaviours after paternal chronic social defeat stress was largely abrogated when progeny were fertilised

by IVF (Dietz et al. 2011), suggesting an indirect mechanism requiring parental contact is at play. However, Rodgers et al. (2013) found that paternal stress alters microRNAs (miRNAs) in sperm (reviewed in section **1.3.2.1**) and affects stress reactivity of offspring. Progeny can also inherit acquired fear responses. Male mice that were conditioned to fear a specific odour sired progeny and grandprogeny that also displayed the same fear response, which was associated with morphological changes of neuroanatomical features in the brain (Dias and Ressler 2014).

1.3.1.6 Parental age or size

There is a widespread relationship between maternal age or size and progeny size or quality, although it is not universal (reviewed in Marshall et al. 2010). Sometimes seasonal trends also influence progeny quality, as seen in the increase in egg mass of brown anole lizards during the breeding season (Warner and Lovern 2014). Maternal age effects on offspring developmental and reproductive traits have the potential to influence population structure for several generations (Benton et al. 2008). In most species older individuals are also larger and in many studies it is unclear if maternal age affects progeny characteristics independent of size effects, although sometimes this has been established (e.g. Burton et al. 2016). It has been argued by some that size *per se* is not the causal factor impacting progeny phenotypes, but rather maternal condition, which correlates with size (Rollinson and Rowe 2016). Although it would seem obvious that maternal size affects progeny primarily through direct investment, in humans the body fat of the father has a greater influence on fat and metabolism of young children than does the body fat of the mother, implying that more complex modes of inheritance are possible (Figueroa-Colon et al. 2000).

In many species advanced parental age affects progeny phenotypes negatively, a phenomenon known as reproductive ageing. In mothers this is mostly a product

of declining oocyte quality, with a concomitant increase in rates of chromosomal nondisjunction and associated abnormalities in progeny (reviewed in Nagaoka et al. 2012). Furthermore, oocytes accumulate mutations at an increasing rate with maternal age (Wong et al. 2016). *C. elegans* hermaphrodites also undergo reproductive ageing, with a loss of germline integrity and embryo viability and a sharp increase in the frequency of spontaneous males, an indicator of chromosomal nondisjunction (Luo et al. 2010). Advanced paternal age in humans has been linked to increased incidence of schizophrenia (Malaspina et al. 2001), autism (Lundström et al. 2010) and non-verbal cognitive function (Malaspina et al. 2005). Curiously, a U-shaped relationship to parental age can be observed for many traits, with worse outcomes among post-pubescent but immature parents. Even when various socioeconomic factors are controlled for, teenage mothers have a greater risk of poor birth outcomes (Chen et al. 2007). Strikingly, the risk of poor birth outcomes is also elevated in progeny of teenage fathers (Chen et al. 2008). Adult offspring of both teenage mothers and older mothers display poorer non-verbal cognition (Malaspina et al. 2005), while rates of autism increase in offspring of very young or old fathers (Lundström et al. 2010). In human studies it is difficult to be confident that all complex socioeconomic factors have been adequately addressed. However in mice too, progeny of post-pubescent fathers have poorer behavioural outcomes than progeny of mature males (Auroux et al. 1998). Maternal reproductive history can also be important. Interestingly, number of older biological siblings, especially brothers, has been strongly associated with sexual orientation in humans (Bogaert and Skorska 2011, Yule et al. 2014).

1.3.2 Molecular mechanisms of parental effects

The mechanisms by which parental life history can affect progeny phenotypes are diverse. The burgeoning field of epigenetics has provided the most detailed mechanistic characterisation of how traces of parental experience or condition are inherited by progeny and possibly further generations. However parental effects need not be epigenetic; they may be direct effects of environmental exposure on embryos or germ cells; they may be due to differential maternal investment or provisioning of specific nutrients or hormones; or they may be mediated by other maternal cellular contributions, such as mitochondria. Ironically, they can also be genetic. In the case of the effects of both paternal and maternal age, accumulation of *de novo* germline mutations at an increasing rate may increase the likelihood of various pathologies. Likewise, chromosomal nondisjunction in aged oocytes underlies many pathologies that increase in the progeny of older mothers (Nagaoka et al. 2012). Similarly, while toxin exposure, such as paternal smoking, can have substantial epigenetic impact, it can also induce *de novo* germline mutations (Yauk et al. 2007) that could contribute to the observed increase in frequency of progeny childhood cancers. The likelihood of genetic vs epigenetic mechanisms can be inferred by the penetrance and duration of phenotypic consequences; effects which act through *de novo* mutation are unlikely to cause consistent, highly penetrant phenotypic changes in progeny, while epigenetic effects, while occasionally extremely persistent (e.g. Klosin et al. 2017), eventually ‘wear off’ after a number of generations (Klosin and Lehner 2016).

1.3.2.1 Epigenetic mechanisms

Epigenetic inheritance is that which takes place independent of DNA sequence. A cellular memory of gene transcription can be sustained by any positive feedback mechanism (Ptashne 2013), such as autoregulation by transcription factors in bacteria (Ptashne 1986) or in *C. elegans* neurons (Hobert 2011). Rightly

or wrongly, however, the word 'epigenetic' has come to be understood as referring to effects mediated by DNA methylation, histone modifications or transfer of RNA populations (Miska and Ferguson-Smith 2016, Rando 2012). In plants, epigenetic inheritance via these mechanisms has been clearly established (Henderson and Jacobsen 2007). These epigenetic mechanisms also appear to be the most likely explanations for transgenerational effects of ancestral life history in animals, defined as those which cannot be direct effects, occurring more than two generations from environmental exposure (Skinner 2008). Inheritance via epigenetic mechanisms is typically short term, lasting up to 3 or 4 generations. However longer lasting inheritance could arise from feedback involving multiple epigenetic pathways (Klosin and Lehner 2016). Crosstalk between epigenetic mechanisms is extensive; DNA methylation can affect chromatin, directly or by recruitment of chromatin modifiers, while histone modifications can in turn recruit DNA methylases or demethylases. Both DNA methylation and chromatin structure affect transcription, while diverse classes of RNAs guide methylation and histone modification (Rando 2012). Individual life history influence on epigenetic modifications is likely to be extensive; methylation is altered at multiple loci in sperm with increasing paternal age (Flanagan et al. 2006), while both DNA methylation and histone acetylation patterns in monozygotic twins become increasingly divergent with age (Fraga et al. 2005). Sperm genomes are largely depleted of histones (Balhorn 2007) and both maternal and paternal genomes undergo extensive demethylation early in development (Reik 2007). Nonetheless, it is apparent that many loci escape this epigenetic reprogramming, thereby potentially influencing gene expression in multiple tissues throughout development.

Chromatin modifications

Histones, the core protein of the nucleosomes that comprise chromatin, have long N terminal tails that can undergo a variety of covalent modifications, such as

methylation or acetylation, which are associated with active or repressed genomic regions (reviewed in Berger 2007). Histone modifications have been implicated in many examples of inheritance of environmental influence on gene expression or phenotype. The inherited resistance to cocaine in the male offspring of cocaine-exposed male rats is associated with histone acetylation at the promoter of the *Bdnf* gene in paternal sperm. BDNF reduces the behavioural effects of cocaine, and is upregulated in the brain of the next generation, providing cocaine resistance that can be reversed with a pharmacological BDNF inhibitor (Vassoler et al. 2013). Impairment of metabolism observed in mice conceived by IVF has been associated with histone 4 acetylation of the promoter of *Txnip*, a gene involved in metabolic responses to oxidative and nutritional stress, that persists from the early embryogenesis into adulthood in adipose tissues (Feuer et al. 2014). The U-shaped response of progeny body fat to paternal dietary sugars in *D. melanogaster* was shown to require the histone 3 lysine 9 (H3K9) methyltransferase Su(var)3-9 and the H3K20 methyltransferase Su(var)4-20 (Öst et al. 2014). Klosin et al. (2017) found that the enduring effect of temperature on the expression of a repetitive transgene array in *C. elegans* was mediated by the H3K9 methyltransferase SET-25 and associated with loss of the repressive H3K9 trimethyl (H3K9me3) histone modification on the array at high temperature, followed by a gradual replacement over multiple generations.

DNA methylation

Methylation of cytosine residues of DNA is an important mechanism of epigenetic regulation in many species. Methylation of gene promoters is normally associated with transcriptional repression. Not all species methylate their DNA to a significant extent and in *C. elegans* cytosine methylation is undetectable (Simpson et al. 1986). However it was recently discovered that adenine methylation is widespread throughout the genome (Greer et al. 2015); as yet the broad significance of this for gene regulation in the worm remains unclear (Luo

and He 2017). In mammals, many ‘imprinted’ genomic loci have a methylation status that varies according to the parent of origin (Wood and Oakey 2006). Either maternal or paternal loss of imprinted loci can lead to distinct developmental abnormalities (Buiting et al. 1995).

DNA methylation can function within a generation as a memory of life history experience. For example, the behavioural impact of maternal grooming in rats is thought to stem from an induced persistent differential methylation state at the locus encoding the glucocorticoid receptor in the hippocampus (Weaver et al. 2004). Reduced methylation in highly groomed offspring is also associated with an increase in histone acetylation and transcription factor binding at the same locus. Germline DNA methylation can transmit a memory of experience to offspring. A heritable conditioned fear response to a specific odour in mice is associated with hypomethylation in sperm of a locus encoding the corresponding odour receptor (Dias and Ressler 2014). In this case the differential methylation state does not persist in the neuroanatomical structures that are altered in the progeny of conditioned mice, suggesting that sperm methylation may direct other epigenetic marks that do persist during development.

DNA methylation may also mediate the effects of periconception environment or parental condition. In IVF-conceived mice, vascular dysfunction is associated with increased promoter methylation and correspondingly reduced expression in the aorta of a gene involved in cardiovascular regulation (Rexhaj et al. 2013). In humans, paternal obesity is associated with hypomethylation of the *IGF2* gene (encoding insulin-like growth factor 2, a major foetal growth factor) in umbilical cord blood of progeny (Soubry et al. 2013).

DNA methylation has been shown to provide a direct link between diet, metabolism and epigenetic inheritance. It was found that seasonal variation in maternal intake of methyl groups around conception in rural Gambia, reflected in

maternal blood biomarkers, predicted methylation at metastable ‘epialleles’ in newborn offspring (Dominguez-Salas et al. 2014). Likewise, in mice maternal dietary methyl supplementation affects the offspring phenotypic outcomes of the A^{vy} allele, which most prominently affects coat colour but can also cause obesity, diabetes and tumour susceptibility (Yen et al. 1994), via differential methylation and thus activation of a transposable element harboured within the *agouti* gene (Waterland and Jirtle 2003). Although it has not been demonstrated, it is possible that dietary availability of methyl donors similarly affects histone methylation (Sharma and Rando 2017).

Despite the impact of maternal diet on DNA methylation, it is not clear if methylation plays a significant role in mediating the many reported phenotypic effects of paternal diet. Although paternal undernourishment in utero has been reported to induce hypomethylation in various regions of the mouse sperm genome (Radford et al. 2014) and the sperm genome of obese human men is differentially methylated (Donkin et al. 2016), it was found that sperm methylation in mouse was unaffected by a variety of paternal postnatal feeding regimes that induce phenotypic effects in the next generation (Shea et al. 2015).

Small RNA transfer

Small noncoding RNAs (sncRNAs) are regulatory molecules with diverse structure and biological function (reviewed in Sarkies and Miska 2014). In *C. elegans*, application of exogenous double-stranded RNA (dsRNA) can lead to strong, stable and heritable silencing of complementary genes. dsRNA is processed to form primary and secondary short interfering RNAs (siRNAs) that are required to produce silencing (reviewed in Grishok 2013). These siRNAs, and the associated silencing, can be inherited for several generations via either the male or female line (Alcazar et al. 2008, Buckley et al. 2012). In addition to its contributions to this highly artificial paradigm of environmentally induced trait

acquisition and inheritance, the RNAi pathway has a role in endogenous gene regulation (reviewed in Billi et al. 2014). The endogenous RNAi pathway can be affected by the environment, with heritable consequences for gene expression. (Schott et al. 2014) found that heritable alterations in mRNA levels following mild transient heat stress were due to disruption of the endogenous RNAi pathway in the germline. (Houri-Ze'evi et al. 2016). The endogenous and exogenous RNAi pathways are tough to compete for common enzymatic components, such as DCR-1 (Duchaine et al. 2006) and RDE-1 (Sarkies et al. 2013). This competition can be relevant for transgenerational inheritance of exogenous RNAi silencing. (Houri-Ze'evi et al. 2016) found that a second, unrelated exogenous RNAi stimulus extended the generational duration and strength of RNAi silencing from previous generations, proposing a model by which negative autoregulation of endogenous RNAi pathways components is disrupted by application of exogenous dsRNA, thus potentiating the RNAi response.

sncRNAs are emerging as an important mediator of environmental information transmission via sperm (reviewed in Chen et al. 2016b). microRNAs (miRNAs) are short RNA molecules (around 22 nt in length) that bind to 3' untranslated regions (UTRs) to negatively regulate their target mRNAs (Bartel 2004). In mice, heritable paternal mental stress alters sperm miRNA content (Rodgers et al. 2013); when a cocktail of 9 differentially regulated miRNAs were injected into zygotes, the impact of paternal stress on progeny behaviour was recapitulated (Rodgers et al. 2015). In mice, sperm miRNAs are also influenced by a high-fat diet (Fullston et al. 2013) or a high-fat, high-sugar diet (Grandjean et al. 2015). In the latter case, a single, specific miRNA injected into zygotes phenocopied acquired paternal metabolic pathologies and remarkably could be inherited by further generations, suggesting that this miRNA guided the formation of other, stable epigenetic marks.

An exciting recent discovery is that tRNA-derived small RNAs (tsRNAs) may play a major role in transmitting environmental information through sperm in mammals. tsRNAs comprise the majority of the sncRNA pool in mature sperm, with miRNAs a distant second (Chen et al. 2016a). tsRNAs have been shown to be altered by diet (Chen et al. 2016a) or exposure to an endocrine disruptor (Schuster et al. 2016) in rodents, and by obesity in humans (Donkin et al. 2016). Zygotic injection of tsRNA fragments from the sperm of fathers raised on a high fat diet partially recapitulated acquired paternal metabolic disorders (Chen et al. 2016a). A single tsRNA derived from a glycine tRNA specifically regulated a small subset of genes in the mouse zygote - those regulated by the endogenous retroviral element MERVL, which is important for pluripotency in the very early embryo (Sharma et al. 2016). It was shown that sperm tsRNAs do not originate from degraded sperm tRNAs but rather are likely to be acquired via transfer of extracellular vesicles from the epididymis (Sharma et al. 2016). This suggests direct transfer of environmental information from somatic to germline tissues.

1.3.2.2 Cytoplasmic contributions

Organelles supplied by gametes can influence progeny phenotypes and could mediate the effects of parental life history. The most obvious cytoplasmic contribution is mitochondria, which are exclusively maternally supplied by the oocyte; paternal mitochondria are subject to degradation by autophagy upon fertilisation (Sato and Sato 2013). Mitochondrial function in oocytes is thought to contribute to the phenomenon of reproductive aging (reviewed in Bentov et al. 2011). It has been shown that mitochondrial function in oocytes is sensitive to maternal condition and environmental exposure (reviewed in Grindler and Moley 2013). Maternal obesity can affect levels of reactive oxygen species (ROS), mitochondrial membrane potential, induction of autophagy and mitochondrial DNA levels (Igosheva et al. 2010, Wu et al. 2015). Likewise a maternal high-fat diet can impact mitochondria (McPherson et al. 2015). Tobacco use can deplete

the pool of healthy oocytes by promoting apoptosis via the stimulation, by smoke-borne polycyclic aromatic hydrocarbons, of the pro-apoptotic mitochondrial protein Bax (Jurisicova et al. 2007).

Although in most taxa the cytoplasmic contributions of sperm to the zygote are minimal, they could still be significant. In *C. elegans*, paternal centrioles are remarkably persistent, surviving multiple cell cycles to be still detectable in the embryo at the 550 cell stage, shortly before hatching (Balestra et al. 2015). It has been suggested that centrioles could contribute to epigenetic events of developmental significance (Tajbakhsh and Gonzalez 2009). Additionally, paternal diet has been reported to impact maternally inherited mitochondria in the early mouse embryo (McPherson et al. 2015).

1.3.2.3 Nutrient provisioning

The relationship of maternal age or size with progeny size or quality is often assumed to be a result of increased maternal investment or provisioning of progeny (e.g. Plaistow et al. 2007). Egg or progeny size itself is often taken as a proxy for maternal investment or provisioning (e.g. in *C. elegans* by Harvey and Orbidans 2011 or Hibshman et al. 2016), although several studies have demonstrated that egg size does not always correlate with egg quality or offspring size (McIntyre and Gooding 2000, Uusi-Heikkilä et al. 2012). Few studies attempt any experimental interventions to specifically identify differentially supplied provisions or to provide a direct causal link between provisioning and progeny phenotypes. There are some exceptions; for example, Berkeley et al. (2004) showed that the larvae of older marine fish receive a greater investment of triacylglycerides (TAG) and have phenotypic outcomes superior to those of younger mothers, independent of size. Warner and Lovern (2014) surgically removed yolk from the eggs of a species of lizard with no parental care and found that, like the smaller eggs produced by dietary restricted mothers, they produced

smaller progeny with poor growth and survival. While this study established yolk provisioning as a plausible mechanism underlying the effect of maternal dietary regime on progeny phenotypes, the volume of yolk removed far exceeded the observed physiological range and the phenotypic effects were more modest than those observed by dietary intervention.

In some cases, what is assumed to be maternal provisioning may in fact be maternally directed synthesis by offspring. Frazier and Roth (2009) assumed that the increased embryonic glycerol concentration under maternal high salt concentrations was a result of direct provisioning. However it was later found that the glycerol build-up required the biosynthetic enzyme GPDH-2 in progeny and was a result of transmission of environmental information by maternal signalling to the germline via the IIS pathway (Burton et al. 2017).

In addition to bulk nutrient provisioning, maternal supply of micronutrients or hormones can influence progeny phenotypes. Zinc deficiency in the maternal diet for only 5 days before conception leads to developmental abnormalities in mice foetuses (Tian et al. 2014). Maternal zinc deficiency leads to a global decrease in the H3K4me3 histone modification in oocytes and aberrant overexpression of repetitive elements (Tian and Diaz 2013). Birds are known to deposit androgens such as testosterone into the yolk of their eggs (reviewed in Groothuis et al. 2005). Under conditions of strong intraspecific competition (Bentz et al. 2016) or in response to encounters with intruders to the nest (Navara et al. 2006) mothers can increase the androgen supply to offspring, which leads to increased aggression, survival and growth rates but a suppression of immune function (Groothuis et al. 2005).

1.3.3 Summary

Effects of parental life history in animals, including humans, are widespread and pervasive. Parental diet and toxin exposure are particularly prominent, with many demonstrated cases of phenotypic consequences for progeny of environmental exposures prior to conception, at conception or during gestation. Other environmental conditions experienced by parents, such as social environment or physiochemical conditions, can impact progeny phenotypes. Additionally, ontogenetic factors such as parental age can be important. Both maternal and paternal life history are important, often having similar effects on progeny. However the impact of parental environment is not simple, and often exhibits complex, nonlinear relationships with progeny outcomes or sex-specific effects.

Parental life history can influence progeny phenotypes via various mechanisms. First, influences can be genetic, where environmental exposure or age, for example, create *de novo* germline mutations. Most attention has been paid to epigenetic effects - histone modifications, DNA methylation or small RNA transfer have all been shown to mediate the influence of a variety of parental environmental exposures on progeny phenotypes. However parents, especially mothers, can also affect progeny outcomes via cytoplasmic contributions such as organelles or by provisioning of bulk nutrients or micronutrients.

1.4. Vitellogenins, the ubiquitous yolk proteins

1.4.1 Vitellogenins across the tree of life

Although various other yolk proteins exist (Tufail et al. 2014), vitellogenins are the principal yolk proteins by which oviparous animals supply nutrients to support the embryonic development of their progeny. Vitellogenins are large lipoglycophosphoproteins that are expressed in somatic tissues, such as the vertebrate liver (Romano et al. 2004) or the insect fat body (Tufail et al. 2014). They recruit lipids and other nutrients before they are secreted into circulation (e.g. vertebrate blood or insect hemolymph) and are taken up by oocytes via receptor-mediated endocytosis. In many taxa, once taken up and stored in crystalline yolk platelets they are known as vitellins (Raikhel and Dhadialla 1992).

Vitellogenins form a gene superfamily found in all oviparous taxa (Sappington and Raikhel 1998), including the monotreme mammals (Brawand et al. 2008). While vitellogenins display a high degree of structural conservation, there is extensive divergence in their regulation and in the number of paralogues that exist in each species (Tufail et al. 2014), with 3 vitellogenins found in *Gallus gallus*, 4 in *Xenopus laevis* and 7 in *Danio rerio* (Sun and Zhang 2015). Although eutherian mammals retain only pseudogenic vitellogenin sequences (Brawand et al. 2008), vitellogenins are believed to be the ancestor of human apoB, the principal component of low density lipoprotein (LDL), on the basis of sequence similarity (Baker 1988b).

1.4.1.1 Molecular characteristics of vitellogenins

Vitellogenins are secreted as large oligomeric complexes, typically 400-600 kDa homodimers with a lipid and phospholipid content of around 20 % by weight

(Raikhel and Dhadialla 1992, Tufail and Takeda 2008). In most taxa they undergo proteolytic cleavage and post-translational modifications, such as glycosylation, phosphorylation and occasionally sulphation (Raikhel and Dhadialla 1992), which enable them to carry their cargo of carbohydrates, lipids and other nutrients. In vertebrates, vitellogenins are additionally cleaved after uptake within yolk platelets by cathepsins to form lipovitellins and phosvitins. Lipovitellins are larger hydrophobic subunits that carry lipids, while phosvitins are smaller subunits with a high degree of phosphorylation.

1.4.1.2 Regulation of vitellogenins

While vitellogenin regulation is diverse, they are usually regulated in a sex-, tissue- and stage-specific manner, being expressed in specific somatic tissues of adult females (Raikhel and Dhadialla 1992). However, vitellogenins are often expressed in male animals to some extent, e.g. in the male honeybee *Apis mellifera* (Piulachs et al. 2003). Vitellogenins are subject to hormonal regulation at the transcriptional level in insects and vertebrates (Tufail et al. 2014). In insects, regulation is by neuropeptides, ecdysone and juvenile hormone, the latter being regulated in turn by insulin/insulin-like signalling (IIS) in *D. melanogaster* (Tatar et al. 2003). In *Aedes aegypti* mosquitoes, insulin signalling acts on vitellogenesis through the nutrient-sensing TOR (target of rapamycin) complex (Roy et al. 2007). In vertebrates vitellogenins are under estrogenic control. Expression in male animals can be induced by exogenous application of 17-β estradiol (Skipper and Hamilton 1977). Vitellogenin expression is often affected by heavy metals, such as cadmium or lead, or estrogenic compounds, such as bisphenol A (Tufail et al. 2014), making them a popular choice of biosensor for endocrine disruptors in aquatic species, both vertebrate and invertebrate (Campbell et al. 2006, Rodriguez-Mozaz et al. 2005).

1.4.1.3 Alternative functions of vitellogenins

The presence of multiple paralogues in many species raises the possibility of functional divergence or specialisation of vitellogenins (Tufail and Takeda 2008). In fact, a wealth of studies indicate that vitellogenins may indeed have alternative roles to play in organismal physiology, for example their involvement in the hormonal dynamics of sterile worker honeybees (Guidugli et al. 2005, Nelson et al. 2007).

Multiple functions of vitellogenins in inflammation and the immune response have been reported. Vitellogenins from a variety of fish (and the more distantly related scallop) display broad-spectrum antibacterial activity (Sun and Zhang 2015). They are capable of binding directly to bacterial pathogens and inducing their aggregation. Vitellogenins also function as an opsonin, facilitating phagocytosis by macrophages (Liu et al. 2009). Vitellogenins can also directly kill bacteria by binding to cell-wall components (Li et al. 2009). In the queen of the honeybee *A. mellifera*, vitellogenin has also been reported to directly bind and carry pathogen cell wall fragments into eggs, implying involvement in transfer of specific immunity across generations (Salmela et al. 2015). In support of these reported immune functions, vitellogenins are upregulated in male *Puntius conchoniu* fish in response to injection of *E. coli* (Shi et al. 2006). Likewise, in male zebrafish *D. rerio* vitellogenins have been characterised as acute phase proteins due to their rapid upregulation in response to injection of bacterial cell wall components (Tong et al. 2010). It has been suggested that the rapid rate of evolution observed in hymenopteran insects is due to vitellogenins' role in defence against pathogen attack (Salmela 2017).

Vitellogenin purified from the amphioxus, *Branchiostoma japonicum,* has hemagglutinating properties against erythrocytes from chick, toad and carp (Zhang et al. 2005). Similarly, a plasma-clotting protein found in the crustacean

Pacifastacus leniusculus was found to be homologous to vitellogenins (Hall et al. 1999b). Indeed, von Willebrand factor, a human blood glycoprotein involved in clotting, is also homologous to vitellogenins from chicken, frog and *C. elegans* (Baker 1988a).

Vitellogenins have been proposed to function as an antioxidant. In the honeybee *A. mellifera*, the resistance of individual sterile workers to oxidative stress correlated with vitellogenin levels in hemolymph. Vitellogenin is preferentially carbonylated, with high vitellogenin levels associated with reduced carbonylation of other proteins. Vitellogenin RNAi also increased sensitivity to paraquat, a strong oxidating agent (Seehuus et al. 2006). Three homologous genes derived from an ancient duplication of vitellogenin in *A. mellifera* seem to have acquired specialised functions in oxidative stress response and inflammation (Salmela et al. 2016).

The highly charged phosvitin domain allows vitellogenins to function as the main carrier of zinc ions in many species, such as *A. mellifera* (Amdam et al. 2004), *G. gallus* (Mitchell and Carlisle 1991) and *X. laevis* (Falchuk 1998). In the bony fish *Sciaenops ocellatus*, vitellogenin additionally carries calcium, magnesium, iron and copper (Ghosh and Thomas 1995).

1.4.2 Vitellogenins in *C. elegans* physiology

1.4.2.1 Molecular characteristics of *C. elegans* vitellogenin

Four yolk polypeptides are found in *C. elegans* - two larger polypeptides with a molecular weight of around 170 kDa (YP170A and YP170B) and two smaller polypeptides at around 115 kDa and 88 kDa (YP115 and YP88, respectively). These polypeptides associate to form 2 distinct large oligomeric lipoprotein complexes. The B complex, with a molecular weight estimated at 437,000 kDa,

contains only YP170B as a simple dimer, as is typical of vitellogenins in other species. The A complex, with an estimated weight of 439,000 kDa, is an oligomer composed of YP170A, YP115 and YP88. The diameter of these yolk complexes is estimated at 12.8 - 14.6 nm (Sharrock et al. 1990), although *in vitro* they have been observed to form clusters up to 100 nm across (Kuo et al. 2013).

C. elegans yolk proteins are synthesised in the intestine of the adult hermaphrodite in order to provision her abundant embryos (Kimble and Sharrock 1983). The process of activating abundant yolk production at sexual maturation is often referred to as vitellogenesis. Yolk complexes are secreted into the pseudocoelom, from where they pass through the gonadal basal lamina and through the 500 nm sheath pores of the somatic gonad (Hall et al. 1999a) before uptake by maturing oocytes via receptor-mediated endocytosis (Grant and Hirsh 1999).

Vitellogenins have been found in multiple proteomic studies to be associated with lipid droplets, likely recruiting lipids during yolk biogenesis (Vrablik et al. 2015, Zhang et al. 2012). Yolk complexes purified from embryos contain around 15 % lipid by weight (Sharrock et al. 1990), although the likelihood of lipids disassociating from yolk complexes after endocytosis (Matyash et al. 2001) or being utilised by rapidly developing embryos suggests this may be an underestimate of the total lipid content of yolk upon endocytosis. Kubagawa et al. (2006) profiled the lipid composition of yolk complexes that had accumulated in the pseudocoelom of receptor-mediated endocytosis deficient *rme-2* mutants. In broad agreement with (Sharrock et al. 1990), they found that the phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) comprise over half of the total lipid content. Neutral lipids constitute around 30 %, with free fatty acids and small amounts of cholesterol making up the remainder (**Table i**).

C. elegans is unable to synthesise cholesterol, which it likely obtains in the wild from consumption of yeast or decomposed eukaryotic matter and faeces (Schulenburg and Félix 2017). Although worms do not deploy cholesterol as a component of cell membranes (Kurzchalia and Ward 2003), cholesterol is essential to normal growth as the precursor of the dafachronic acids used by worms to regulate crucial life history decisions (Butcher 2017). Employing fluorescent and photoactivatable cholesterol analogues, Matyash et al. (2001) found sperm and oocytes to be major sites of cholesterol deposition, with significant maternal provisioning of cholesterol via yolk. They also suggested that the YP170B dimer encoded by *vit-2* may have a higher affinity for cholesterol binding than the YP170A/YP115/YP88 oligomer. This maternal supply of cholesterol is physiologically relevant and sufficient to support normal growth and development under laboratory conditions, with widespread defects in growth and development failing to become apparent until the third generation grown under conditions of cholesterol deprivation (Yochem et al. 1999). Eventually cholesterol deprivation leads to impairments in growth, development and reproduction (Shim et al. 2002).

Lipid distribution (percentage)	
Phosphatidylcholine	23.0
Phosphoethanolamine	28.2
Other phospholipids	2.8
Diacylglycerides	3.2
Free fatty acids	16.2
Triacylglycerides	26.4
Cholesterol	<1.0

Table i Composition of lipid fraction of yolk isolated from hermaphrodite body cavity. Adapted from Kubagawa et al. (2006).

1.4.2.2 Vitellogenin genes in *C. elegans*

There are 6 vitellogenin genes in the *C. elegans* genome (**Fig ii**). *vit-2*, the best-studied of the vitellogenins (Goszczynski et al. 2016), encodes YP170B. *vit-1* is 82 % identical to *vit-2* but may be a pseudogene. Spieth et al. (1985) reported a

single nucleotide deletion in the *vit-1* coding sequence causing a frameshift and a premature stop codon. It has been noted that while the other vitellogenins are 85-90 % conserved between *C. elegans* and the closely related species *C. briggsae*, *vit-1* is only 73 % conserved (Blumenthal et al. 1984), supporting the notion that it is a pseudogene. However, *vit-1* may be translated; VIT-1 protein has been reported to be found in multiple proteomics studies (Depuydt et al. 2013, Liang et al. 2014, Zhang et al. 2012).

vit-3, *vit-4* and *vit-5* encode YP170A. *vit-3* and *vit-4* are 99 % identical and appear in tandem on the X chromosome. *vit-3* and *vit-4* are derived from a recent duplication event, as *C. briggsae* lacks *vit-4* (Blumenthal et al. 1984). *vit-5* is 96 % identical to *vit-3* and *vit-4*, and 67 % identical to *vit-2* (Spieth et al. 1985).

vit-6 is the single divergent member of the *C. elegans* vitellogenins, sharing only 50 % identity with *vit-2* (Spieth et al. 1985). *vit-6* encodes a single polypeptide of around 180 kDa that is cleaved after secretion into the pseudocoelom but before endocytosis into the oocyte to form YP115, derived from the C-terminal portion of VIT-6, and YP88, derived from the N-terminal portion (Sharrock 1984, Spieth and Blumenthal 1985). Despite being cleaved into two separate polypeptides, YP115 and YP88 appear to behave as a single monomeric unit and are linked covalently to YP170A via disulphide bonds (Sharrock et al. 1990). *vit-6* is the only *C. elegans* vitellogenin that is not on the X chromosome, being found instead on chromosome IV.

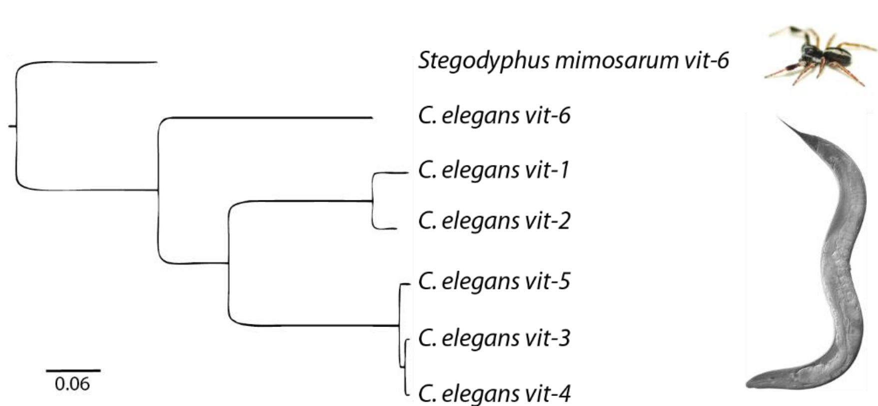


Fig ii. The vitellogenin family in *C. elegans* is comprised of 6 genes.
The outgroup is the *vit-6* gene from the African social velvet spider, *Stegodyphus mimosarum*. Arachnid vitellogenins are the sister group to nematode vitellogenins (Tufail et al. 2014). Scale bar represents nucleotide substitutions per site. *C. elegans* image credit: I Chin Sang, *S. mimosarum* image credit Martin Forman.

Although nematode vitellogenins are homologous with vertebrate and other vitellogenins (Nardelli et al. 1987, Spieth et al. 1985), all nematode vitellogenins lack the serine-rich phopshvitin domain common to vitellogenins of other taxa (Nardelli et al. 1987, Spieth et al. 1991). Although it is considerably diverged, *vit-6* is much more closely related to the other nematode vitellogenins than to vertebrate vitellogenins, indicating that this divergence likely occurred within nematodes (Spieth et al. 1991).

vit-1, *vit-2*, *vit-3*, *vit-4* and *vit-5* all have exceptionally short 5' UTRs (Spieth et al. 1985). This may be related to the presence of potential stem-loop forming structures at the 5' end of all the vitellogenins. The potential stem-loop forming bases are highly conserved, with only 1 % divergence between *C. elegans* and *C. briggsae* observed within these regions, compared to approximately 15 % throughout the genes. Such structural features of vitellogenin mRNAs could serve to impede translation (Zucker-Aprison and Blumenthal 1989), potentially mediating post-translational regulation of vitellogenin expression.

Although *vit-6* shares only around 21 % identity at the protein level with other vitellogenins, their amino acid compositions are strikingly similar (Spieth et al. 1991), likely reflecting their role in providing developing embryos with an optimal pool of amino acids. In all vitellogenins, there has also been strong conservation of cysteine residues, particularly present in pairs near both termini of the genes (Spieth et al. 1991). These residues are likely required for the formation of disulphide bonds. The *vit* genes also show evidence of strong selection at the level of codon choice (Spieth et al. 1991). Such a strong preference for certain codons is typical of other abundantly expressed nematode genes, such as collagen, actin or myosin.

1.4.2.3 Secretion and uptake of vitellogenins

Although it was assumed that yolk in *C. elegans* was loaded into oocytes by receptor-mediated endocytosis (Sharrock et al. 1990), as in other taxa and as suggested by ultrastructural analysis (Hall et al. 1999a), the precise mechanism was unknown until a landmark study by Grant and Hirsh (1999). Using a transgenic strain expressing a *vit-2::gfp* translational fusion under control of the *vit-2* promoter, the authors could observe correct sex-, stage- and tissue-specific regulation of *vit-2*, in addition to VIT-2::GFP secretion from the intestine into the pseudocoelom and subsequent uptake by the 3 proximal oocytes immediately before fertilisation (**Fig iii**), as seen with immunofluorescence labelling by Hall et al. (1999a). The authors used RNAi to knock down *in silico* predicted components of clathrin-coated pits and showed them to be necessary for yolk uptake by oocytes. Additionally, they confirmed that homologues of the Rab

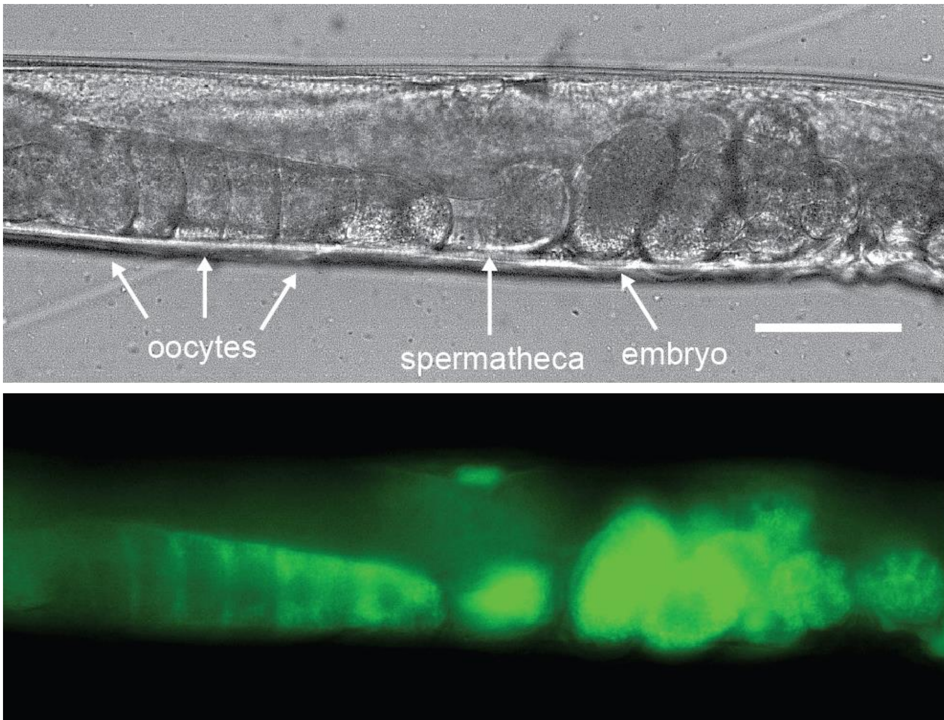


Fig iii. Uptake of aVIT-2::GFP translational fusion protein by proximal oocytes prior to fertilization. Scale bar, 50 µm.

proteins of the endocytic sorting pathway were necessary for normal yolk uptake and sorting. Phenotypes varied according to the gene; for example RNAi knockdown of *rab-7*, required for trafficking of cargo from the early to the late endosome, led to the presence of abnormally large yolk granules that persisted throughout the whole body of hatched larvae, instead of displaying the usual segregation to the intestine (reviewed below). They also demonstrated a requirement for the secretory COPI pathway, likely involved in the rapid recycling of receptors to the cell surface.

In addition to confirming yolk uptake by receptor-mediated endocytosis using reverse genetics, Grant and Hirsh performed a forward genetic screen, looking for mutants that accumulated pseudocoelomic VIT-2::GFP, suggestive of defects in endocytosis. In doing so they identified the *C. elegans* yolk receptor, *rme-2*. *rme-2* is necessary for yolk uptake, as embryos of *rme-2* null mutants contain no detectable yolk. A member of the low-density lipoprotein related (LDLR) superfamily, *rme-2* is expressed exclusively in oocytes, first appearing in cytoplasmic puncta near the gonad bend. Later RME-2 is strongly localised to the cell surface in maturing oocytes, before rapid removal after fertilisation and subsequent lysosomal degradation during embryogenesis. Ectopic expression of *rme-2* under a muscle-specific promoter causes accumulation of VIT-2::GFP in that tissue, confirming that *rme-2* is sufficient for yolk uptake. It is worth noting that although the *vit-2::gfp* strain used in this study appears to secrete and uptake yolk normally, by immunofluorescence labelling Hall et al. (1999a) found that the same strain seemed to accumulate less pseudocoelomic yolk and exhibited larger intestinal yolk particles, suggesting that the fluorescent tag may interfere somewhat with proper yolk secretion.

After endocytosis, yolk appears in oocytes and embryos in a membrane-bound compartment, often referred to as yolk granules, as opposed to unbounded, extracellular yolk particles (Hall et al. 1999a). Various non-protein components

taken up with yolk, such as the fluorescent fatty-acid analogue BODIPY-FA (Kubagawa et al. 2006), the cholesterol analogue dehydroergosterol (Matyash et al. 2001) and fluorescently labelled dsRNA (Marré et al. 2016), have been found to undergo a rapid relocalisation away from the yolk granules to the cytoplasm or other cellular organelles. This implies that yolk is actively sorted into various components shortly after uptake.

The process of vitellogenin secretion and uptake is very fast. Bossinger and Schierenberg (2003) reported that yolk labelled with the fluorescent dye Lucifer yellow by maternal feeding appeared in oocytes within minutes of introducing mothers to the dye. A thorough quantitative analysis was provided by Kuo et al. (2013), who used fluorescent nanodiamond particles coated with purified VIT-2::GFP. After injection into the anterior intestinal cells next to the pharynx, the nanodiamond particles were detected in the pseudocoelom within 5 minutes, in the gonad within 12 minutes, in the oocytes within 20 minutes and finally in fertilised embryos within 55 minutes. Interestingly, the particles were detected almost immediately in the posterior intestine, implying that yolk-sized particles can move freely and rapidly between the cells of the intestine.

In the course of embryogenesis, yolk accumulates in the gut primordium (Sharrock 1983). Yolk deposition in the gut by various mechanisms, such as prelocalisation or segregation, is common in other taxa (Bossinger and Schierenberg 2003). In the case of *C. elegans*, yolk appears to be secreted from other cells and taken up again by the gut cells by receptor-mediated endocytosis (Bossinger and Schierenberg 2003); yolk labelled by Lucifer yellow injection into the AB cell (which does not contribute to the gut) at the 2-cell stage is depleted during mid-embryogenesis from AB descendants and is transferred to the gut. Breaking the impermeable embryonic vitelline layer by means of laser cutting in a medium containing Lucifer yellow, the authors confirmed specific uptake of yolk from the extracellular space into the gut primordium. Other dyes were not

taken up. This uptake could be blocked by various pharmacological inhibitors of receptor-mediated endocytosis. Grant and Hirsh (1999) failed to find any expression of *rme-2* during embryogenesis, implying the existence of another receptor capable of mediating yolk uptake, possibly in a processed form, in the worm.

1.4.2.4 *C. elegans* vitellogenins are highly expressed

The gonad comprises around one quarter of the adult worm's body volume and turns over every 6.5 hours at the peak of the reproductive period (Hirsh et al. 1976, Jung et al. 2012). Therefore in one day, the worm produces a quantity of embryos roughly equivalent to her own body weight. It has been estimated that yolk comprises 37 % of the total protein in the embryo (Kimble and Sharrock 1983). Van Nostrand et al. (2013) found that around 3 % of all RNA-seq reads in young adult worms mapped to vitellogenins. This is despite the fact that they are expressed exclusively in the 20 cells of the intestine, and despite the fact that the present work suggests this proportion is only set to increase throughout the reproductive period. The level of expression was such that when the authors knocked down *unc-62*, a necessary transcriptional activator of vitellogenins, the level of transcription of almost all other genes expressed in the intestine rose as a result. Kimble and Sharrock (1983) found that YP170 alone comprised 25 % of de novo protein synthesis from dissected adult hermaphrodite intestines *in vitro*. With these figures in mind, we might therefore be justified in proposing that the primary purpose of the adult hermaphrodite worm is to produce prodigious quantities of yolk to provision her embryos, at a presumably huge metabolic cost.

It has been often noted in the literature that yolk accumulates to pathological levels in ageing worms (Herndon et al. 2002; **Fig iv**). *vit* RNAi has been reported to extend lifespan (Murphy et al. 2003, Seah et al. 2016), and it has been suggested that it does so by preventing lipotoxicity associated with the

accumulation of yolk during ageing. However, knockdown of the yolk receptor *rme-2*, which leads to extensive build-up of yolk in the extracellular spaces of the worm, has been found not to influence lifespan, casting doubt on the significance of yolk toxicity in ageing (Seah et al. 2016). The accumulation of yolk has been understood as at least partly a consequence of the cessation of reproduction, causing the absence of the main yolk sink by which the massive quantities of yolk produced leave the body (McGee et al. 2011). However, ageing worms do continue to synthesise yolk *de novo*, even up to day 10 of adulthood (Liang et al. 2014). As to why worms continue to produce yolk well into old age, it has been suggested to be compatible with the hyperfunction theory (Blagosklonny 2012), which argues that pathological features of ageing are the result of quasi-programmed molecular changes that serve a key purpose during development and reproduction. In this case, proponents of the theory argue that pathological production of yolk is merely a result of the inappropriate continuation of the programmed massive yolk synthesis during the reproductive period, exacerbated by the loss of the yolk sink; a case of the tap being left on causing the bathroom to flood (Ackerman and Gems 2012, Gems and de la Guardia 2013).

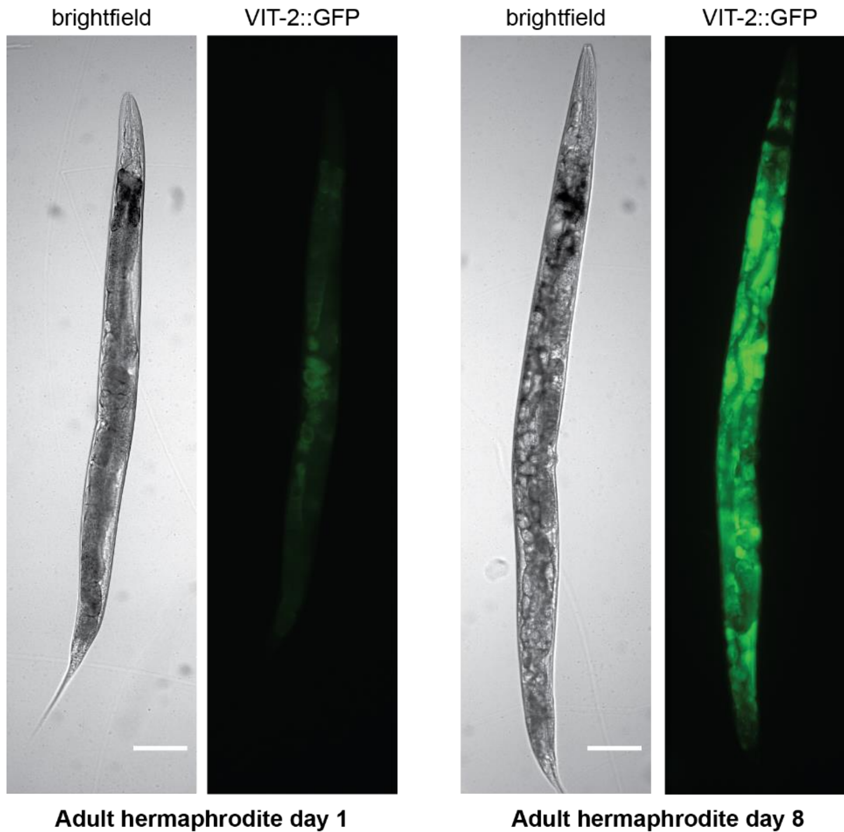


Fig iv. Yolk accumulates to pathological levels in post-reproductive hermaphrodites. Brightfield and fluorescence images are shown of hermaphrodites on adult day 1 or adult day 8 carrying a VIT-2::GFP translational fusion. Scale bar, 50 µm.

1.4.2.5 Vitellogenins are dispensable for development in *C. elegans*

Although it has been widely assumed in the literature that abundant vitellogenin expression is required for adult fertility and embryonic viability (e.g. Khanna et al. 2014), a body of evidence suggests this is not the case. Van Rompay et al. (2015) identified 3 mutants that almost completely abrogate synthesis of all vitellogenins in the adult hermaphrodite but that have little to no effect on her brood size, reproductive timing or the viability of her embryos. Indeed, in *rme-2(b1008)* mutants that lack the yolk receptor, embryos appear completely devoid

of any detectable yolk but 23 % are still viable (Grant and Hirsh 1999). r*me-2(b1008)* mutants do have a severely reduced brood size (78 compared to ~300 in N2; Grant and Hirsh 1999), but in the light of the full brood size of almost yolkless mutants it seems more likely that this is due to defects in ovulation and fertilisation caused by a total absence of vitellogenin uptake. Oocytes require polyunsaturated fatty acids (PUFAs) supplied by yolk in order to produce an unknown signal to attract sperm and complete successful fertilisation (Kubagawa et al. 2006); indeed many *rme-2* oocytes are destroyed during the passage through the spermatheca (Grant and Hirsh 1999). This is also consistent with the maintenance of abundant males in *rme-2(b1008)* populations (personal observation); the higher motility of male sperm (LaMunyon and Ward 1998) probably provides it with a selective advantage over the hermaphrodite's own sperm when successful fertilisation relies on a blind search in the absence of any attractive signal. We can speculate that in mutants that almost abrogate yolk synthesis, the small amount of PUFAs delivered by the little remaining yolk suffices to produce the necessary signal to attract sperm to the oocyte.

If yolk is largely dispensable for embryogenesis, why does the worm expend so much energy producing it in staggering quantities? The answer, supported by the findings of this study, seems to be that abundant yolk supports postembryonic progeny survival and development. This has been suggested on the basis of the significant proportion of yolk left in the larvae after embryogenesis has been completed. Sharrock (1983) found abundant immunofluorescence staining with anti-vitellogenin antibodies in the larval intestine immediately prior to hatching (**Fig v**). Bossinger and Schierenberg (2003) tracked yolk stained by maternal feeding with the fluorescent dye Lucifer yellow and observed significant intestinal yolk retention at hatching that was lost during L1 starvation, leading them to suggest that yolk's primary purpose is postembryonic survival under adverse conditions.

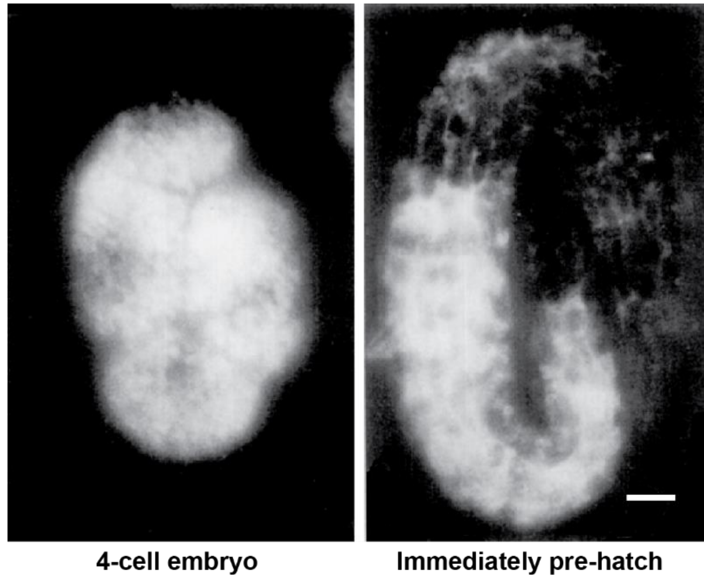


Fig v. Vitellogenin by immunofluorescence in an early embryo and in an embryo immediately prior to hatching. Scale bar, 5µm. Adapted from Sharrock (1983).

It has been reported that mutants defective in yolk synthesis (Van Rompay et al. 2015), endocytosis or appropriate utilisation (Chotard et al. 2010) have impaired survival of larval starvation, although no other phenotypic consequences have been reported.

The 'boom-and-bust' mode of rapid population growth on ephemeral, dispersed resource patches (Félix and Braendle 2010) means few individuals will survive to proliferate outside their 'home' patch. The scale of yolk production in *C. elegans* indicates that the worm places a premium on the ability of its progeny to survive and thrive in harsh environmental conditions; it must be a hard knock life, for a worm.

1.4.3 Regulation of vitellogenins in *C. elegans*

The vitellogenins are subject to tight regulatory constraints; their abundant expression needs to be confined in a tissue-, stage- and sex- specific manner, limited to the intestine of the adult hermaphrodite worm. As the metabolic cost of abundant vitellogenin expression must be staggering, fine-tuning of their expression is paramount; hence it is to be expected, as seems to be the case, that multiple regulatory inputs exist, integrating signals stemming from nutritional status, environmental conditions and extra-intestinal tissues. It has often been stated in the literature that the promoters of the *vit* genes are simple (e.g. MacMorris et al. 1992), but recent extensive genetic dissection has found multiple regulators and signalling pathways impinging directly and indirectly on only a small section of the promoter of *vit-2* (Goszczynski et al. 2016), the most extensively studied of the vitellogenins.

Many studies have claimed to find differential regulation of vitellogenins, but in the light of the principal finding of the present work, namely that vitellogenin expression is highly dynamic, caution is required as the likelihood of artefacts is high in any genetic or environmental context that alters developmental timings. With noteworthy exceptions (e.g. Goszczynski et al. 2016 and Van Rompay et al. 2015) few studies appear to have controlled for this in any way.

To begin, I will look at the basic logic of the regulation of vitellogenins, which determines the localisation and timing of their expression. To continue, I will look at the major signalling pathways that have been identified as regulating vitellogenins. I will also briefly touch on how other tissues, through these pathways or others, can exert influence on vitellogenin expression in the intestine. Lastly, I will mention a few of the environmental influences that have been shown to influence vitellogenin expression.

1.4.3.1 Tissue-, stage- and sex-specific regulation

The primary regulatory logic of high yolk expression in a tissue-, stage- and sex-specific manner can be recapitulated with only 247 bp of the *vit-2* promoter (MacMorris et al. 1992). Even a minimal 44 bp enhancer region from the *vit-2* promoter 145-188 bp upstream of the initiation codon allows for full recapitulation of sex- and tissue-specificity and partial recapitulation of stage-specificity, with only weak expression evident in developing larvae (Goszczynski et al. 2016).

Three functionally associated sequence elements are found in the vitellogenin promoters. Two have been christened *vit* promoter elements, VPE1 (TGTCAAT) and VPE2 (CTGATAA; MacMorris et al. 1992), while the third is a direct binding site for the transcription factor MAB-3 (AATGTTGCGA(T/A)NT; Shen and Hodgkin 1988, Yi and Zarkower 1999). Remarkably there is considerable conservation of these promoter elements across vast phylogenetic distances - the exact VPE2 sequence is found upstream of vitellogenin genes in *Xenopus laevis* and *Gallus gallus*, with very similar sequences also found in *D. melanogaster* (Spieth et al. 1985).

VPE2 confers abundant expression in a tissue-specific manner. VPE2 is directly bound by the intestinal master regulator ELT-2 (McGhee et al. 2007). Knockdown of *elt-2* in young adult worms strongly reduces expression from a 44 bp enhancer region of the *vit-2* promoter (Goszczynski et al. 2016).

VPE1 is bound by an isoform of the transcription factor UNC-62, which is necessary for vitellogenin expression. Isoform-specific RNAi targeting *unc-62a* reduces vitellogenin expression between four- and ten-fold (Van Nostrand et al. 2013). As *unc-62a* undergoes a dramatic increase in intestine-specific expression between the L3 stage and adulthood (Van Nostrand et al. 2013) it likely confers

some stage-specificity, although Goszczynski et al. (2016) found that a 44 bp promoter element that does not contain VPE1 recapitulated stage-specificity to some extent.

Sex-specificity is conferred by the MAB-3 binding site, as *mab-3* mutant males accumulate yolk (Yi and Zarkower 1999). *mab-3* is homologous to the *Doublesex* gene of *D. melanogaster*, which also represses yolk protein transcription (Yi and Zarkower 1999). MAB-3 has been shown experimentally to act directly on the *C. elegans vit-2* promoter, but all vitellogenins contain potential MAB-3 binding sites and all are deregulated in *mab-3* loss of function mutants (Yi and Zarkower 1999). Direct MAB-3 binding may inhibit activation by *elt-2* or *unc-62* (Goszczynski et al. 2016). *mab-3* acts in the sex determination pathway downstream of *tra-1*, which blocks *mab-3* activity in hermaphrodites and thus relieves the repression of the vitellogenins. Curiously, in a *mab-3* mutant background different *tra-1* alleles have different effects on the relative abundances of the yolk proteins, suggesting that *tra-1* also acts independently of *mab-3* to influence synthesis of specific vitellogenins (Shen and Hodgkin 1988). Surprisingly, Goszczynski et al. (2016) found that removal of the MAB-3 binding site abolished expression driven by a 44 bp enhancer element of the *vit-2* promoter in both sexes, suggesting an additional unknown regulator necessary for expression also binds this sequence element.

1.4.3.2 Regulation by signalling pathways

In this section I discuss regulation of vitellogenins at the level of individual signalling pathways, although in so doing I gloss over the extensive and complex interactions that can exist between various pathways. As an example, there is extensive cross-talk between the insulin-like signalling and TOR pathways (Narasimhan et al. 2009), with TOR acting both downstream and parallel to insulin-like signalling (Baumeister et al. 2006). Interaction between signalling

pathways can be relevant in some tissues or processes but not others (Qi et al. 2017), with various pathways converging on the same downstream targets, such as *sgk-1* and *skn-1* (Robida-Stubbs et al. 2012).

Insulin/insulin-like growth factor signalling (IIS)

The widely-studied insulin/insulin-like growth factor signalling (IIS) pathway plays a crucial role in the transcriptional response of *C. elegans* to environmental conditions, such as nutrient availability and various stressors. In turn, IIS regulates pivotal life-history decisions for individual worms, notably L1 and dauer arrest (Baugh 2013, Murphy and Hu 2005). *daf-2*, encoding the single orthologue of the human insulin receptor (Kimura et al. 1997), is the most-studied gene in *C. elegans* owing to the remarkable extension of lifespan and healthspan observed in *daf-2* mutants (Kenyon et al. 1993). This lifespan extension, and many other phenotypes of *daf-2* mutants, can be suppressed by loss of function of the transcription factor *daf-16*, the sole *C. elegans* forkhead box O (FOXO) homologue (Kenyon et al. 1993, Murphy and Hu 2005). DAF-2 is bound by a plethora of insulin-like peptides, both agonists and antagonists (Murphy 2006). DAF-2 activity, via a phosphatidylinositol(3,4,5)-trisphosphate (PIP_3) signalling cascade, leads to phosphorylation and consequent cytoplasmic localisation of DAF-16. In the absence of signalling by DAF-2, DAF-16 enters the nucleus and directly or indirectly modulates the expression of thousands of downstream target genes (Murphy 2006) (**Fig vi**).

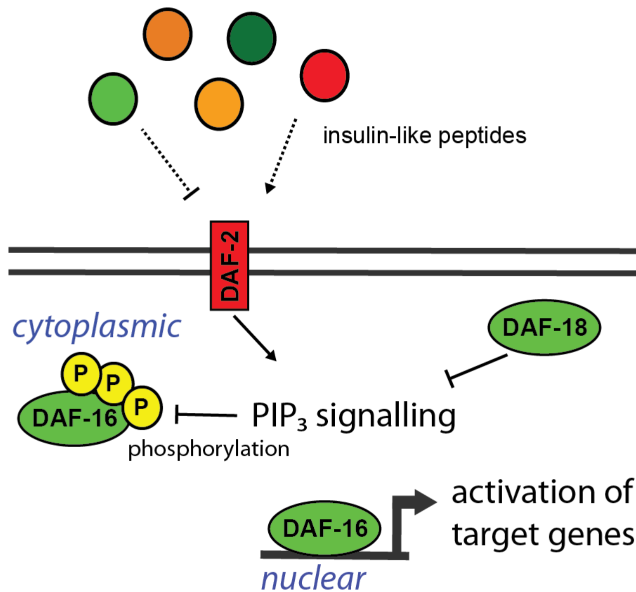


Fig vi. Schematic of the IIS pathway in *C. elegans*.
Components that promote IIS are shown in red. Components that antagonise or are antagonised by the pathway are shown in green. Although the pathway is shown here acting within a cell, IIS acts systemically and different components may be active in different tissues. After Murphy and Hu (2005).

The IIS pathway has widely been reported as a repressor of vitellogenins. Multiple studies, particularly of the ageing process in *daf-2* mutants, have found that IIS mutants exhibit reduced vitellogenin transcription and accumulation in older worms (DePina et al. 2011, Murphy et al. 2003). This has even been suggested to partially mediate the longevity of *daf-2* worms, given that vitellogenin knockdown by RNAi can extend lifespan (Murphy et al. 2003).

DePina et al. (2011) tackled the subject of IIS regulation of vitellogenesis most directly. They found a 4-fold increase in yolk protein stained by Coomassie Blue in bulk lysates of hermaphrodites from adult day 1 to day 4 at 25 °C run on denaturing acrylamide gels, despite measuring a 7-fold decrease in vitellogenin transcription from adult day 1 to day 6 by quantitative reverse transcription PCR (qPCR). In worms carrying the pleiotropic class II *daf-2* mutant allele *e1370*

(Gems et al. 1998), yolk protein levels were observed to be lower than wildtype and to not increase from day 1 to day 4. Both results were *daf-16* dependent. However, they measured the level of vitellogenin transcription to be similar in *daf-2* mutant and wildtype worms during the self-fertile reproductive period. The authors infer *daf-16* dependent post-transcriptional regulation of yolk protein production.

However a number of caveats colour the interpretation of this study. The choice of development at 25 °C will have accelerated the self-fertile reproductive period, which should be completed in wildtype worms by adult day 4. Likewise, the use of the total soluble protein fraction of bulk lysates of hermaphrodites presents some troubling issues; yolk may not be completely solubilised, and results may be complicated by the presence or absence of embryos *in utero*. In particular, the reproductive spans of *daf-2* mutants are substantially extended (Murphy et al. 2003). Worms that no longer produce embryos may accumulate yolk to higher levels due to the absence of the yolk sink that they represent (McGee et al. 2011). It is unclear to what extent the lack of accumulation of yolk proteins in *daf-2* mutants between adult day 1 and day 4 in this study, and its apparent *daf-16* dependence, may be due to their extended reproductive span and the reversal of this phenotype by *daf-16* loss of function.

In their thorough dissection of regulatory inputs impinging on a 44 bp 'enhancer' element of the *vit-2* promoter, Goszczynski et al. (2016) found that *daf-2* loss of function abolished transcriptional output from the enhancer element in a *daf-16* dependent manner. Furthermore, a *daf-16* null mutant had higher expression driven by the enhancer, suggesting constitutive repression of this element by the IIS pathway. Indeed, they found that both DAF-16 DNA-binding isoforms bound directly to the 44 bp enhancer sequence *in vitro*, although no prior studies had identified vitellogenins as sites of DAF-16 binding *in vivo* using either ChIP-seq (Oh et al. 2006) or DNA adenine methyltransferase identification (DamID;

Schuster et al. 2010). *daf-2* was found to exert its influence in a cell-nonautonomous manner, as intestine-specific *daf-2* RNAi did not abolish transcription; this is consistent with the reported expression pattern of *daf-2*, which is expressed mainly in neurons (Li et al. 2014) and the germline (Honnen et al. 2012). In contrast, intestinal *daf-16* RNAi partially restores expression in the context of *daf-2* loss of function. This indicates that while there is likely some direct repression of vitellogenins by *daf-16*, some degree of repression comes from *daf-16* activity in other tissues, presumably via cross-talk with other signalling pathways. These results support the notion that IIS is a major regulator of vitellogenesis in *C. elegans*.

However, the same authors also found that despite abolishing expression from the 44 bp enhancer, *daf-2* RNAi had virtually no effect on expression driven by the full 2.7 kb *vit-2* promoter region. The authors conclude that although IIS acts strongly on the 44 bp enhancer, there are likely numerous other signalling pathways converging on the full promoter region, leading to the influence of IIS on vitellogenesis in early adulthood being modest, at best. How to reconcile this with their own results, and the previous consensus of vitellogenins as IIS targets? The authors point out that the characterisation of vitellogenins as IIS targets has been largely based on expression studies of ageing worms. The notion that IIS plays a major role in the regulation of vitellogenins in the context of post-reproductive yolk accumulation is thus compatible with the idea that it has a minor role, as one of many regulatory players, in vitellogenesis during the reproductive period that we are principally concerned about in the present work. Indeed, the strongest effects on vitellogenin levels of *daf-2* mutants in the work of DePina et al. (2011) were found in post-reproductive worms. Also consistent with the modest role IIS plays is a meta-analysis of *daf-16* loss of function expression studies by Tepper et al. (2013), which found the regulatory association with vitellogenins to be weak and inconsistent.

TGF-β signalling

The TGF-β superfamily is an extensive class of secreted ligands that play fundamental roles in animal physiology, development and growth. The canonical TGF-β signalling pathway consists of two transmembrane serine/threonine kinase receptors (type I and type II) and two or three intracellular SMAD signal transducers. Upon ligand binding, the type I and type II receptors form a complex that results in the phosphorylation and activation of the type I receptor, which in turn phosphorylates receptor Smads (R-SMADs). Phosphorylated R-SMADs form a complex with Co-SMADs, which enter the nucleus to directly regulate transcription (ten Dijke and Hill 2004).

In *C. elegans* two parallel, distinct branches of TGF-β signalling have been characterised - the dauer pathway and the Sma/Mab pathway. Both pathways share the single type II receptor, DAF-4, with specificity of input provided by distinct ligands and type II receptors and specificity of output provided by distinct suites of downstream SMADs and associated transcription factors (Gumienny and Savage-Dunn 2013).

Alongside other signalling pathways, the dauer TGF-β pathway plays a key role in the determination of whether or not to enter the dauer diapause, a decision based on environmental and nutritional status in early development. Loss of signalling results in dauer-constitutive phenotypes. The ligand DAF-7, one of five identified TGF-β homologues in C. elegans, binds to DAF-4 and the type II receptor DAF-1, leading to signal transduction by the SMADs DAF-8, DAF-14 and DAF-3 (Gumienny and Savage-Dunn 2013).

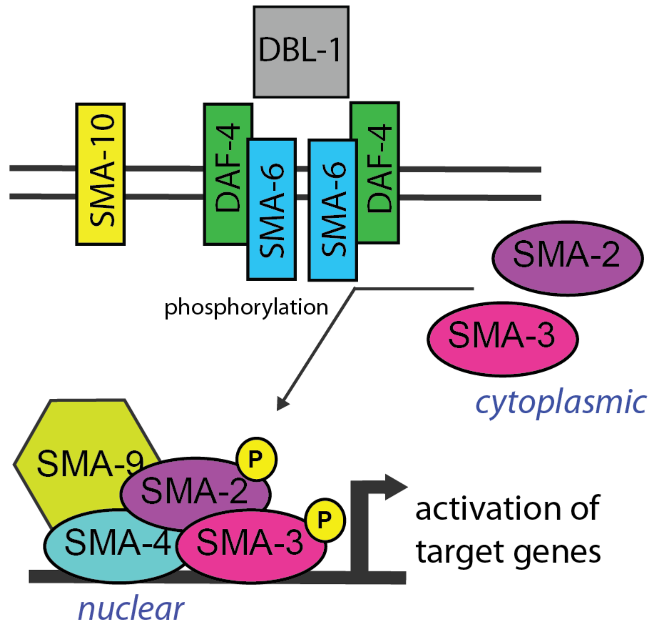


Fig vii. Schematic of the Sma/Mab TGF-β signalling pathway.
After Gumienny and Savage-Dunn (2013).

The Sma/Mab TGF-β signalling pathway regulates postembryonic growth (Savage-Dunn et al. 2000), germline maintenance and reproductive ageing (Luo et al. 2010) and some aspects of male development (Savage et al. 1996). The ligand for this pathway is DBL-1; *dbl-1* loss of function leads to small mutants with reduced body size, while *dbl-1* overexpression results in abnormally long worms (Suzuki et al. 1999). *dbl-1* is expressed principally in neurons (Ramakrishnan et al. 2014). DBL-1 signalling is potentiated by a transmembrane protein SMA-10, which binds to the type I and type II receptors DAF-4 and SMA-6 (Gumienny et al. 2010). SMA-6 phosphorylates the R-SMADs SMA-2 and SMA-3, which associate with the Co-SMAD SMA-4 to influence transcription (**Fig vii**). No activity of *sma-2*, *sma-3* or *sma-4* can be detected in the absence of any one of the three genes, suggesting that they form a heterotrimeric complex (Savage-Dunn et al. 2000). A subset of Sma/Mab targets require the transcriptional co-factor *sma-9*, encoding a large zinc finger transcription factor

homologous to *Schnurri* from *D. melanogaster* (Liang et al. 2003). Transcriptional targets include *lon-1*, which when mutated suppresses the body size phenotype of *sma* mutants (Maduzia et al. 2002). Activity of the pathway in the hypodermis is necessary and sufficient for determining body size phenotypes (Wang et al. 2002).

In an attempt to identify additional transcription factors that interact with the MAB-3 binding site of the 44 bp enhancer element from the *vit-2* promoter, Goszczynski et al. (2016) performed an RNAi screen, targeting 167 of the approximately 200 transcription factors thought to be active in the *C. elegans* intestine. They found a strong repression of transcription resulting from knockdown of the Sma/Mab Co-SMAD *sma-4*, implying that TGF-β signalling promotes vitellogenesis. Indeed, subsequent knockdown of *dbl-1*, *daf-4*, *sma-6*, *sma-10*, *sma-2*, *sma-3* and *sma-9* all produced a strong repression of transcriptional output from the enhancer element. Intestine-specific RNAi indicated that all of these components acted within the intestine, except for *dbl-1*, consistent with its neuronal expression pattern. Importantly these experiments were conducted with concurrent *lon-1* RNAi, in order to exclude body size as a confounding factor. Similar results were obtained using either 247 bp or 2.7 kb stretches of the *vit-2* promoter. The authors were unable to find a direct association of SMADs or SMA-9 with the 44 bp enhancer element, demonstrating that this regulation is likely to be indirect. The suppression of transcriptional output from the full *vit-2* promoter by knockdown of *sma-9* was ~40 %, suggesting that while important, TGF-β signalling exerts a quantitative influence on vitellogenesis in concert with other signalling pathways.

Target-of-rapamycin (TOR) signalling

Originally discovered as the protein inhibited by the potent antifungal compound rapamycin that was originally discovered on Rapa Nui (also known as Easter

Island), TOR (target of rapamycin) kinase, a serine/threonine kinase of the phosphatidylinositol kinase-related kinase (PIKK) family, is a ubiquitous central regulator of growth in eukaryotes. Every eukaryotic genome examined, bar a handful of fungal pathogens (Shertz et al. 2010), contains a TOR gene (Wullschleger et al. 2006). TOR kinase integrates inputs from growth factors, nutrient (especially amino acid) availability, energy levels and stress conditions, and in turn coordinates transcription and translation, ribosome biogenesis, metabolism and autophagy (Wullschleger et al. 2006).

TOR kinase participates in two protein complexes, TORC1 and TORC2, defined in part by TOR's interaction with two mutually exclusive binding partners, Raptor and Rictor respectively (Jacinto et al. 2004, Sarbassov et al. 2004). TORC1 controls temporal aspects of growth in yeast and mammalian embryogenesis (Wullschleger et al. 2006); TORC2, in contrast, is known to affect spatial aspects of growth by regulation of the actin cytoskeleton (Sarbassov et al. 2004). Among other targets, TORC2 phosphorylates and activates PDK-1, AKT-1 and AKT-2 (Jones et al. 2009, Soukas et al. 2009), components of the PIP_3 signalling cascade linking DAF-2 signalling to DAF-16 activity (Murphy and Hu 2005). However the principal downstream effector of most of the phenotypes that manifest in *rictor* mutants with compromised TORC2 activity is the serum and glucocorticoid-induced kinase, SGK-1 (Jones et al. 2009, Soukas et al. 2009), which also acts in the IIS pathway downstream of PIP_3 signalling (Murphy and Hu 2005). SGK-1 is also capable of phosphorylating DAF-16 *in vitro* (Hertweck et al. 2004).

In a reverse genetic screen for activators of transcriptional output from the *vit-3* promoter at the L4 to adult transition, Dowen et al. (2016) identified mutations in *alg-1*, an Argonaute protein involved in microRNA (miRNA) biogenesis that acts in the hypodermis to promote vitellogenesis (reviewed in section **1.4.3.3**). The screen also identified a mutation in *sgk-1* that led to repression of the *vit-3*

promoter. *sgk-1* is an excellent candidate for a regulator of vitellogenesis, as it is expressed exclusively in the adult intestine and neurons (Hertweck et al. 2004). It was found that an *sgk-1* gain-of-function mutation could rescue the vitellogenesis defects of mutants in the hypodermal miRNA pathway. As mutations in the PIP_3 signalling modulator *daf-18* (the *C. elegans* orthologue of human phosphatase and tensin homologue, PTEN) could not rescue expression from the *vit-3* promoter in a hypodermal miRNA pathway mutant, it was determined that this pathway did not act via the IIS pathway. Likewise, the influence of *sgk-1* on the *vit-3* promoter was *daf-16* independent.

Intestinal RNAi against *let-363*, encoding the *C. elegans* homologue of TOR kinase, strongly repressed the *vit-3* promoter. Knockdown of the TORC2 components *rict-1* (*rictor*) and *sinh-1*, but not TORC1-specific components, repressed vitellogenesis, suggesting that TORC2 promotes vitellogenesis through its primary target, SGK-1. Via a forward genetic screen in the *sgk-1* mutant background, it was found that SGK-1 acts through PQM-1, a nematode-specific zinc finger transcription factor.

pqm-1 was previously characterised in a search for the mechanism by which *daf-16* appeared to repress a large class of downstream targets, despite being a direct transcriptional activator. These targets are strongly enriched for intestinal genes and share a DNA motif, dubbed the *daf-16* associated element (DAE), that is bound by PQM-1 (Tepper et al. 2013). Like DAF-16, PQM-1 undergoes a shift in subcellular localisation under stress conditions, but curiously exhibits an opposite and mutually antagonistic shift; when DAF-16 is largely cytoplasmic PQM-1 is found in the nucleus, while nuclear DAF-16 promotes the cytoplasmic localisation of PQM-1 (Tepper et al. 2013). PQM-1 undergoes a progressive shift from nuclear to cytoplasmic localisation as worms reach adulthood (Dowen et al. 2016) that continues during adult ageing (Tepper et al. 2013). ChIP-seq data

indicate that PQM-1 binds upstream of *sgk-1* (Tepper et al. 2013), suggesting that feedback mechanisms modulate the activity of the signalling pathway.

Dowen et al. (2016) found that when the function of *sgk-1* or hypodermal miRNA pathway components was lost, PQM-1 was inappropriately maintained in the nucleus during adulthood. These knockdowns also caused reduced intestinal fat stores, as determined by staining with the lipophilic dye Oil Red O, suggesting a wider disruption of lipid metabolism as a result of the perturbation of hypodermis to TORC2 signalling.

sgk-1 was independently identified as an activator of *vit-2* in response to high concentrations of iron (Wang et al. 2016).

Regulation by SKN-1

skn-1 encodes a transcription factor, distantly homologous to mammalian Nrf proteins, which coordinates systemic detoxification responses under normal conditions or in response to acute oxidative stress (An and Blackwell 2003, Oliveira et al. 2009). *skn-1* may also play a broader role in coordinating lipid homeostasis (Steinbaugh et al. 2015). *skn-1* is known to be a downstream target of both the IIS and TOR pathways (Robida-Stubbs et al. 2012, Tullet et al. 2008). *skn-1* likely plays a role in regulating the vitellogenins, and vice versa.

Lynn et al. (2015) described how, in *skn-1* gain-of-function mutants, lipid stores are transferred to the germline from the intestine rapidly after the cessation of reproduction, a phenomenon christened ‘age-dependent somatic depletion of fat’ or ASDF (**Fig viii**). RNAi against vitellogenins prevents ASDF, suggesting that vitellogenins act as the mechanism by which *skn-1* causes intestinal depletion and germline accumulation of lipid stores.

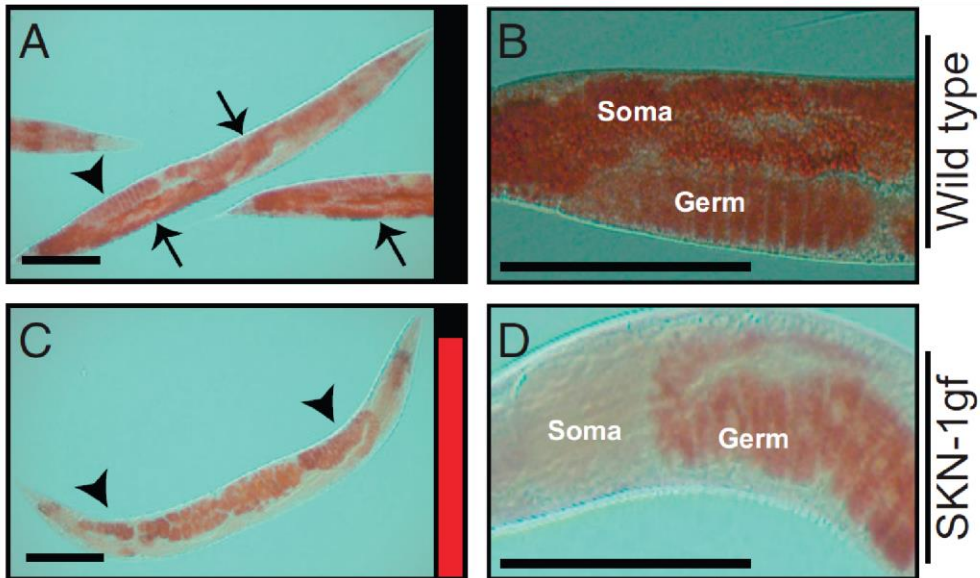


Figure viii. Age-dependent somatic depletion of fat in *skn-1* gain of function mutants. Fixed post-reproductive worms are stained with Oil Red O, a lipid stain. Arrows indicate soma and arrowheads indicate germline. Scale bars, 100um. Reprinted from Lynn et al. (2015).

Specific lipids could also regulate ASDF - dietary supplementation of the monounsaturated fatty acid (MUFA) oleic acid, lacking in *skn-1* gain-of-function mutants, could suppress ASDF.

In turn, vitellogenin accumulation in the worm may influence *skn-1* activity. The longevity phenotype of germline-less *glp-1* mutants requires *skn-1*. Steinbaugh et al. (2015) suggested that the pseudocoelomic accumulation of yolk in *glp-1* mutants induces a stress response mediated by *skn-1*, resulting in the longevity phenotype. Indeed, knockdown of the yolk receptor *rme-2*, causing yolk build up in the pseudocoelom, induced nuclear accumulation of SKN-1 and increased stress resistance. Strikingly, they also found that *fat-6* and *fat-7*, two fatty acid desaturases required for biosynthesis of oleic acid, were required for the activation of *skn-1* and associated longevity in a *glp-1* mutant background.

Other regulators

mafr-1 encodes a conserved corepressor RNA pol III transcription. By its repression of tRNA and ribosomal RNA synthesis it affects the biosynthetic capacity of an organism - indeed in worms *mafr-1* RNAi increases body size, while overexpression reduces it (Khanna et al. 2014). *mafr-1* can act downstream of TORC1 signalling (Pradhan et al. 2017). *mafr-1* also regulates selected RNA pol II transcripts, among them the vitellogenins that are repressed by *mafr-1* (Khanna et al. 2014, Pradhan et al. 2017). *mafr-1* knockdown or overexpression altered the total lipid storage of worms. Mutations in either *daf-18* or *daf-16* abrogate the effect of *mafr-1* alteration on lipid stores, so *mafr-1* likely acts upstream of the IIS pathway to regulate lipid homeostasis. However in a *daf-16* mutant background *mafr-1* RNAi still upregulated *vit-2*, *vit-4* and *vit-5* but curiously not *vit-6*, suggesting differential regulation by IIS of the most diverged member of the vitellogenins (Khanna et al. 2014).

kri-1 is a conserved gene with ankyrin repeats originally identified in reverse genetic screens for genes required for *daf-16* dependent lifespan extension in response to germline loss. In this capacity *kri-1* functions to promote DAF-16 nuclear localisation in the intestine (Berman and Kenyon 2006). Goszczynski et al. (2016) found that *kri-1* knockdown in the intestine strongly repressed the *vit-2* promoter and endogenous *vit-2* transcripts in a *daf-16* independent manner.

Goszczynski et al. (2016) also identified a forkhead-domain transcription factor, FKH-9, as a direct transcriptional activator for the 44 bp enhancer element of the *vit-2* promoter, with a binding site between the ELT-2 and MAB-3 binding sites. However, the effect of *fkh-9* RNAi on the full 2.7 kb promoter region was undetectable.

Van Rompay et al. (2015) identified *lrp-2* as a necessary activator of *vit-2* expression in a forward genetic screen. *lrp-2* is a member of the LDL receptor superfamily with a wide expression pattern, detected in body wall muscle, hypodermis and neurons. *lrp-2* has a largely flat expression profile during development, with a spike in expression evident around the L3-L4 moult. *lrp-2* mutants retain eggs, resulting in internal hatching and a 'bag-of-worms' phenotype.

1.4.3.3 Regulation by extra-intestinal tissues

Many signalling pathways act systemically, with various components of the pathway, as constructed by classical genetics, actually operating within different tissues (Libina et al. 2003). Here I briefly highlight control of vitellogenin expression in the intestine by other tissues, operating through signalling pathways already reviewed or as-yet undiscovered mechanisms.

Nervous system

As the proximal sites of environmental sensing, neurons are likely to be important in the fine-tuning of vitellogenesis by various pathways in response to environmental conditions. Indeed, the expression of the Sma/Mab TGF-β pathway ligand *dbl-1* and the insulin receptor homologue *daf-2* are largely neuronal (Li et al. 2014, Ramakrishnan et al. 2014). *dbl-1* has been shown to influence vitellogenin expression in a cell-nonautonomous manner (Goszczynski et al. 2016).

Another neuronal regulator of vitellogenins was described by Van Rompay et al. (2015) and independently verified by Dowen et al. (2016). *ceh-60* encodes a DNA binding homeobox protein whose expression appears confined to the chemosensory amphid neurons (note however that as this expression pattern was characterised by using a *ceh-60::gfp* transgene, additional expression in the germline, where this transgene is likely to be silenced, cannot be ruled out). Van Rompay et al. (2015) also identified a novel protein, *vrp-1* (*vitellogenin regulating protein 1*), which is expressed in intestinal nuclei and is regulated by, and in turn regulates, *ceh-60*. Both genes display expression spikes around the L3-L4 and L4-adult moults, and both mutants fail to activate vitellogenesis at the latter moult. *ceh-60* mutants have very strongly reduced survival of L1 starvation, likely mediated by lack of maternal vitellogenin production.

Hypodermis

The hypodermis is important for regulating the timing of developmental events in *C. elegans* - many so-called ‘heterochronic’ mutants act in the hypodermis to control the timing of adult cell fate determination (Vella and Slack 2005). The hypodermis is also important for the initiation of vitellogenesis in the intestine at the L4-adult moult.

In a forward genetic screen for regulators of the *vit-3* promoter, Dowen et al. (2016) found *alg-1*, an Argonaute protein that is an essential cofactor for miRNA biogenesis. miRNAs are short RNA molecules (around 22 nt in length) that bind to 3’ untranslated regions (UTRs) to negatively regulate their target mRNAs (Bartel 2004). This led them to investigate the role of miRNAs in the initiation of vitellogenesis. *lin-4* and *let-7* are miRNAs that regulate the L1-L2 moult and L4-adult moult, respectively. While *let-7* has a broader expression pattern, *lin-4* expression is confined to neurons and the hypodermis. Loss-of-function mutants for both *lin-4* and *let-7* fail to initiate vitellogenesis at the L4-adult moult. Knockdown of the downstream mRNA targets negatively regulated by these miRNAs could partially rescue expression from the *vit-3* promoter.

lin-29 encodes a zinc transcription factor normally expressed in the hypodermis during the L4 stage as the culmination of sequential miRNA expression during development. *lin-4* and *let-7* do not express *lin-29* at the L4 stage. RNAi against *lin-29* in the hypodermis, but not the intestine, caused failure to initiate vitellogenesis at the L4-adult moult. Likewise, expression of *let-7* under a hypodermal promoter, but not an intestinal promoter, could rescue the vitellogenesis defect of a *let-7* null mutant. Surprisingly hypodermal or intestinal expression of *lin-29* could rescue a *lin-29* null mutant, implying that although *lin-29* is only expressed in the hypodermis, it acts through a regulator that can influence vitellogenesis in either a cell-autonomous or cell-nonautonomous

manner. The authors ultimately found that *lin-29* in the hypodermis activates the *sgk-1* kinase through TORC2 signalling in the intestine, as reviewed in section **1.4.3.2**.

Germline

The germline can exert a profound influence on somatic tissues, as demonstrated by the longevity of germline-less *glp-1* mutants. We have already seen that a component of the 'germline pathway' required for this longevity, *kri-1*, acts in the intestine to promote vitellogenesis, although it is not clear whether it responds in this context to extra-intestinal signalling (Berman and Kenyon 2006, Goszczynski et al. 2016). However some instances of regulation of vitellogenesis by the germline have been described.

In a forward genetic screen for receptor-mediated endocytosis mutants that accumulate yolk at high levels in the pseudocoelom, Balklava et al. (2016) identified *pitr-1*, encoding an inorganic phosphate membrane transporter. Surprisingly, rather than disrupting yolk endocytosis, this mutant caused yolk build-up by increased vitellogenin expression at both the mRNA and protein levels. This astonishing effect was corroborated with a second null allele and systemic RNAi against *pitr-1*. *pitr-1* mutants also had a low brood size and partial penetrance of embryonic inviability.

Examining the expression pattern of *pitr-1*, the authors found the bulk of expression occurred in the germline at all developmental stages. Exclusive expression in the germline under a *pie-1* promoter could rescue the brood size defect and, incredibly, restored normal expression levels of vitellogenin, although embryonic viability was unaffected. This result suggests the existence of an uncharacterised pathway that requires this phosphate transporter in order to

transduce signals from the germline to the intestine, thereby repressing vitellogenin expression.

DePina et al. (2011) also found that the germline can affect vitellogenin transcription. In *fem-1* mutants with a feminised germline, vitellogenin transcription was repressed, although protein levels appeared to be unaffected. *fem-3* mutants with a masculinised germline exhibited normal levels of transcription. This suggests the existence of a sperm-derived signal that promotes vitellogenin transcription.

1.4.3.4 Environmental regulation

Mating

DePina et al. (2011) found that mating increased transcription of vitellogenins and prevented the age-related decline of mRNA levels in a *daf-2* independent manner. Protein levels did not appear to be affected, although this may be a result of the extended reproductive span of outcrossed hermaphrodites, as suggested above for *daf-2* mutants. These results are consistent with the repression of vitellogenin transcription in the absence of sperm in *fem-1* mutants (reviewed in section **1.4.3.3**). However as other physiological effects apparently caused by mating can be induced merely by male-conditioned media (Maures et al. 2014), this stimulation of vitellogenin transcription upon mating may be due to the presence of males rather than a sperm-derived signal, as the authors suggest.

Surprisingly, mating can affect vitellogenin expression in males. Shi et al. (2017) demonstrated that, as in hermaphrodites (Shi and Murphy 2014), mating causes males to shrink and die. Both expression profiling by microarrays and a *vit-2::gfp* transgene indicated germline-dependent ectopic expression of vitellogenin in mated males. The reduced lifespan of mated males was found to be dependent on

unc-62 and *pqm-1*, both established regulators of vitellogenins (reviewed in section **1.4.3.2**), although the effect of mutations in these genes on ectopic vitellogenin expression was not shown directly.

Ancestral experience

Rechavi et al. (2014) found that ancestral experience of starvation can influence expression of vitellogenins. The authors starved L1 larvae for 6 days before recovery and sequenced small RNAs in these worms and in subsequent generations. Strikingly, differentially expressed heritable small RNAs aligned antisense to all 6 vitellogenin genes in the F3 progeny, indicating that a transgenerational memory of starvation influences vitellogenin expression. The authors suggested that double stranded RNA normally required for biogenesis of small RNAs could be derived from transcription of genes or non-coding RNAs lying on the opposite DNA strand near the 5' or 3'ends of all of the vitellogenin coding sequences. Inheritance of differential small RNA expression required the genes *hrde-1* and *rde-4*. Despite the remarkable volume of inherited small RNAs complementary to all of the vitellogenins, only *vit-4* appeared to be differentially expressed in the parental and F3 generations, suggesting that the effect of transgenerational memory of environmental experience on vitellogenin expression is modest.

Oxidative stress

As reviewed in section **1.4.3.2**, in *skn-1* gain-of-function mutants vitellogenins mediate the age-dependent depletion of somatic fat, or ASDF. In wildtype worms a full-scale ASDF response is induced within 12 hours of exposure to hydrogen peroxide. Conversely, antioxidant treatment leads to accumulation of excess somatic fat (Lynn et al. 2015). These results suggest that *skn-1* regulates vitellogenin mobilisation as part of a response to acute oxidative stress.

1.4.4 Possible alternative functions of vitellogenins in *C. elegans*

The presence of multiple vitellogenin paralogues in the *C. elegans* genome, and the substantial sequence divergence of *vit-6*, suggests the possibility of their co-option for other functions (Tufail and Takeda 2008). Another indication that this may be the case is the expression of vitellogenins in males to a similar degree as in hermaphrodites at the L4 stage (Celniker et al. 2009). In the light of the various alternative functions known in other taxa (reviewed in section **1.4.1.3**), it is tempting to speculate on what other physiological or regulatory roles *C. elegans* vitellogenins may play. However, few papers have described any secondary functions for vitellogenins.

As in the honeybee *A. mellifera* (Seehuus et al. 2006), vitellogenins may provide an antioxidant capacity in worms. Nakamura et al. (1999) found that *vit-6* was preferentially carbonylated in ageing worms, suggesting a protective role. The idea of vitellogenins as antioxidants is also consistent with the dramatic mobilisation of somatic fat in response to hydrogen peroxide, which may indicate secretion of vitellogenins as part of an acute oxidative stress response (Lynn et al. 2015). Other studies lend some support to this notion (Fischer et al. 2014, Fischer et al. 2013).

Only a single paper has suggested that vitellogenins act directly against pathogens in worms (Fischer et al. 2013). Since in adult hermaphrodites vitellogenins are found in pseudocoelomic circulation, vitellogenin function as an opsonin, as in fish (Liu et al. 2009), could facilitate phagocytosis of pathogens by the coeolomocytes, scavenging cells anchored in the pseudocoelom. Additionally, vitellogenin uptake could serve as an intergenerational immunity signal. Marré et al. (2016) found that exogenous dsRNA can be exported from the intestine and

taken up along with yolk in an *rme-2* dependent manner, although this effect was not necessarily due to binding to vitellogenins as fluorescent dye injected into the pseudocoelom was also taken up. This nonspecific uptake of pseudocoelomic contents could include pathogen-associated molecular patterns (PAMPs) or pathogen-derived nucleic acids in systemic circulation, thereby priming progeny innate immunity, as in *A. mellifera* (Salmela et al. 2015). Early embryos oddly contain abundant vitellogenin mRNA (**Fig ix**), which is presumably maternally derived. This suggests the possibility of transfer of intestinal matter, including RNA, along with the abundant yolk flow from intestine to germline. As the intestine is a likely site of pathogen infection, this could also serve to prime progeny against maternally encountered pathogens. It would be interesting to see if other maternally expressed intestinal genes are detectable in early embryos, which would indicate that this hypothesis merits further investigation.

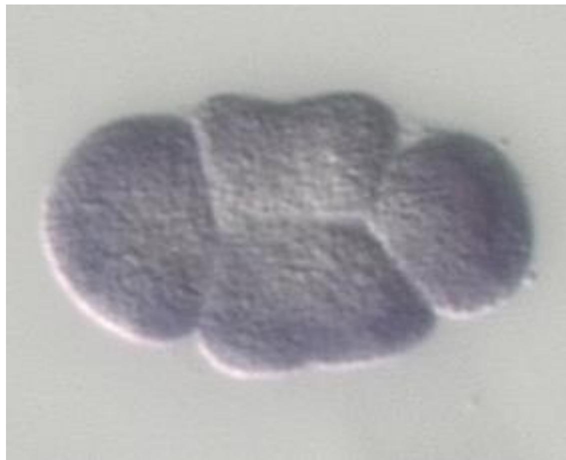


Figure ix. **Early embryos contain abundant vitellogenin mRNA.**
4-cell embryo with in situ hybridization (dark staining) against *vit-4* mRNA. Image reproduced from NextDB (Shin-i and Kohara 1998).

1.4.5 Summary

Vitellogenins are yolk proteins found ubiquitously in oviparous taxa. They transport a variety of lipids and micronutrients from adult tissues to oocytes. In addition, they can have other roles, in immune function, oxidative damage protection or social regulation. In *C. elegans*, 6 vitellogenin genes are very highly expressed in the intestine, although only a fraction of what is produced appears to be strictly required for embryonic development. *C. elegans* vitellogenins are regulated by various signalling pathways, such as the IIS, TOR and TGF-β pathways, acting in the intestine or in extra-intestinal tissues, and can also be regulated by environmental experience. In *C. elegans*, alternative functions of vitellogenin, besides nutrient provisioning to progeny, are unknown.

2. RESULTS

The main body of the results presented in this thesis is in the form of a manuscript that was resubmitted after a first round of revisions to the journal *Nature* in August 2017. I share first authorship of this manuscript with Mirko Francesconi, who discovered the novel shift in the relative speed of germline and somatic tissues which is regulated by, among other environmental conditions, maternal age.

Mirko contributed **figures 1** and **2**, **figure 4 panel a**, **extended data figures 1** and **2** and **extended data figure 7 panels g-i**.

Cristina Hidalgo contributed **extended data figure 5 panels b and c**.

I contributed **figures 3** and **5**, **extended data figures 3**, **4**, **6** and **8**, **figure 4 panels b-d**, **extended data figure 5 panels a** and **d-i**, and **extended data figure 7 panels a-f** and **j-n**.

In addition, I include an Appendix that contains further results that were not included in the manuscript but are relevant to the regulation of progeny phenotypes by maternal age.

Perez MF, Francesconi M, Hidalgo-Carcedo C, Lehner B. Maternal age generates phenotypic variation in Caenorhabditis elegans. Nature. 2017 Nov 7;552(7683):106–9. DOI: 10.1038/nature25012

APPENDIX

Genetic regulation of the maternal age effect on vitellogenin expression

I asked whether the progressive increase in embryonic yolk loading with maternal age is specifically regulated or whether these expression changes are more general to worms as they age. In order to address this question, I crossed three genetic mutants known to slow the rate of ageing to my *vit-2::gfp* reporter to see whether the dynamics of embryonic yolk loading is altered in any or all of these mutants. As development is also slowed in these mutants, mutant worms were not gravid at the timepoint corresponding to day 1 in wildtype mothers and so the examined timepoints were shifted to reflect this.

The aging regulators *eat-2*, a genetic model of dietary restriction, and *clk-1*, with impaired mitochondrial function, both seem to affect embryonic yolk levels, but both maintain a steady increase with maternal age (**Fig 6a & b**). However mutants for *daf-2*, the sole *C. elegans* orthologue of the human insulin receptor, strongly affect yolk loading dynamics. The class II reference allele *daf-2(e1370)* strongly repressed the progressive increase in embryonic yolk (**Fig 6c**). The weaker, class I allele *daf-2(e1368)* (Gems et al. 1998) had little effect when worms were cultured at 20 °C, but when mothers were shifted to 25 °C during the L4 stage the increase in yolk loading was completely abolished (**Fig 6d & e**). These effects were not due to growth differences as *daf-2* mutant adults, like wildtype adults, increased substantially in size during the period examined (**Fig 6f**). Surprisingly, despite the reported role of the insulin-like signalling pathway in blocking repression of vitellogenesis by the FOXO orthologue *daf-16* (DePina et al. 2011, Murphy et al. 2003), embryonic yolk levels in both *daf-2* mutants were not strongly reduced relative to wildtype controls and were even higher in early progeny.

Fig 6. The progressive increase in yolk provisioning to embryos is not a general feature of ageing but involves non-canonical insulin-like signalling and TGF-β signalling.

Plots show mean and 95 % confidence intervals for total VIT-2::GFP fluorescence intensity in early embryos across a range of maternal ages for different genotypes relative to wildtype controls (except panel **f**). Allele names are indicated in the relevant panel.

(**a**) *eat-2*, a genetic model of dietary restriction. n = 22, 77 (WT); 32, 45, 32 (*eat-2*).

(**b**) *clk-1*, with alterations in mitochondrial electron transport chain function. n = 44, 35, 31 (WT); 14, 37, 35 (*clk-1*).

(**c**) *daf-2(e1370)*, the strong, highly pleiotropic class II reference allele of the human insulin receptor orthologue. n = 55, 53, 56 (WT); 55, 57, 56 (*daf-2*).

(**d**) *daf-2(e1368)*, a weaker class I allele, cultured at 20 °C. n = 22, 44, 37, 38 (WT); 21, 35, 36, 26 (*daf-2*).

(**e**) *daf-2(e1368)*, with adults shifted to the non-permissive temperature of 25 °C before the L4-adult moult and the onset of vitellogenesis. n = 41, 64, 48, 45 (WT); 33, 48, 47, 52 (*daf-2*).

(**f**) Body length of *daf-2(e1370)* adults.

(**g**) *daf-2(e1370)*, a null allele (mu86) of the FOXO orthologue *daf-16* and *daf-2(e1370);daf-16(mu86)* double mutant. n = 29, 32, 28 (WT); 26, 14, 35 (*daf-2*); 30, 37, 26 (*daf-16*); 47, 45, 42 (*daf-2;daf-16*).

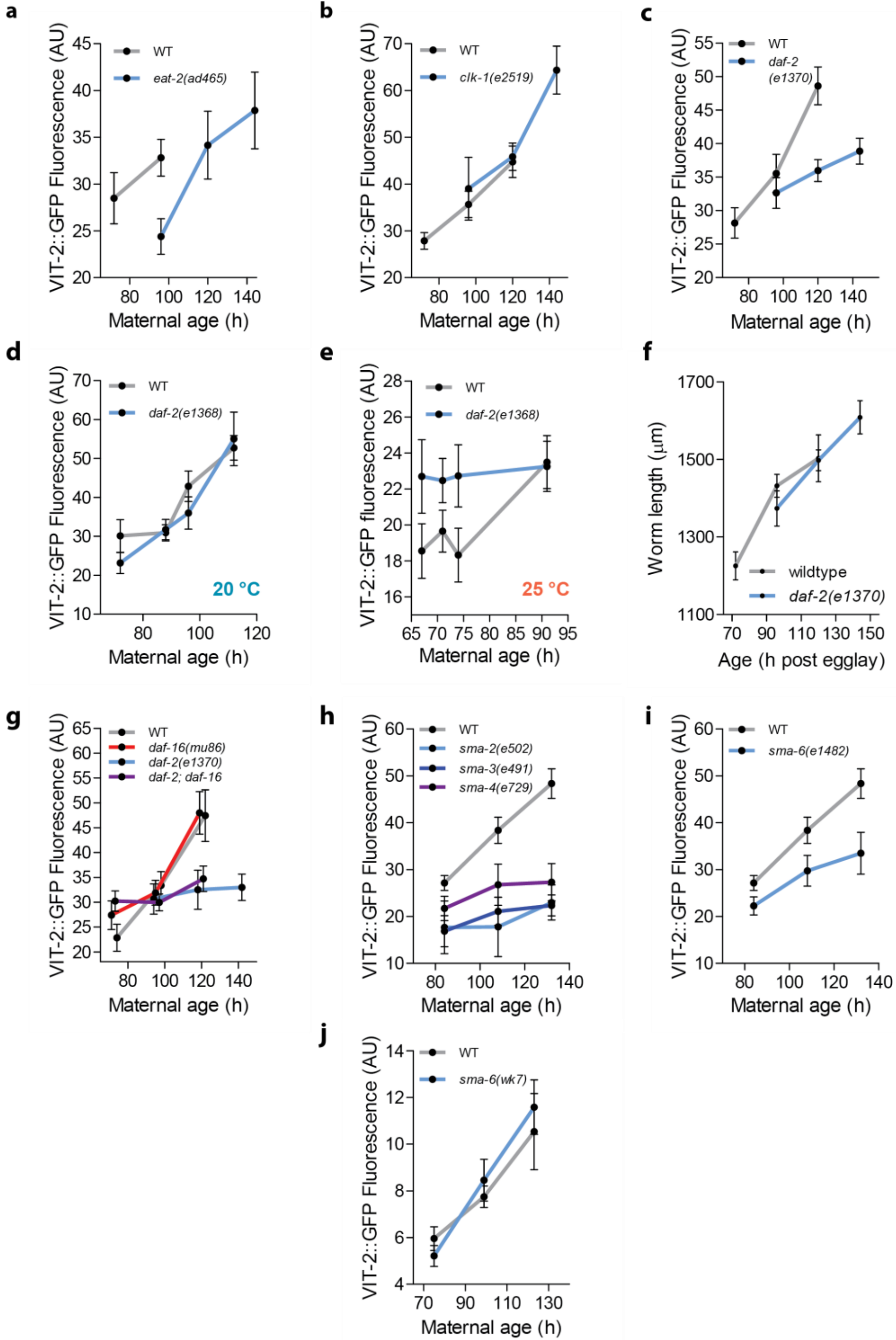
(**h**) The Sma/Mab TGF-β Co-SMADs *sma-2* and *sma-3* and the R-SMAD *sma-4*. n = 40, 36, 35 (WT); 21, 11, 27 (*sma-2*); 27, 35, 36 (*sma-3*); 32, 35, 35 (*sma-4*).

(**i**) A partial loss-of-function allele of the Sma/Mab-specific type I receptor *sma-6*. n = 40, 36, 35 (WT); 35, 38, 32 (*sma-6*).

(**j**) A null allele of the Sma/Mab-specific type I receptor *sma-6*. n = 32, 30, 30 (WT); 33, 33, 48 (*sma-6*).

Data shown in panels **h** and **i** were generated in the same experiment and so the wildtype control is the same and data from mutants are comparable between panels. Data from panel **j** comes from an experiment where a lower exposure time was used for imaging, hence the discrepancy in the range of the Y axis. AU, arbitrary units. WT, wildtype (N2) background.

Figure 6



In order to see if the master stress regulator *daf-16* mediates the effect of *daf-2* loss-of-function on embryonic yolk content, I examined yolk levels in embryos from mothers of different ages in a *daf-2(e1370);daf-16(mu86)* double mutant background. Interestingly, although most gross phenotypic effects of the *daf-2(e1370)* mutation were visibly rescued by the introduction of the null *daf-16(mu86)* allele, embryonic yolk levels remained unchanged by maternal age at a level similar to those of *daf-2(e1370)* (**Fig 6g**). I conclude that *daf-2* regulates the progressive increase in embryonic yolk loading via a *daf-16* independent mechanism.

As the Sma/Mab TGF-ß pathway has also been reported to regulate the *vit-2* promoter (Goszczynski et al. 2016), I crossed mutants for genes in this pathway, including the Co-SMADs *sma-2* and *sma-3*, the R-SMAD *sma-4* and the type I receptor *sma-6,* to the *vit-2::gfp* transgenic strain. In SMAD mutants, embryonic VIT-2::GFP levels are severely repressed (**Fig 6h**), confirming that this pathway is required for normal levels of yolk provisioning to offspring. Additionally these mutants display very little progressive increase in embryonic VIT-2::GFP levels with maternal age. However, the effect of a partial loss-of-function allele for the Sma/Mab-specific type I receptor *sma-6* on both overall yolk levels and the progressive increase in yolk provisioning was weaker than that of SMAD mutants (**Fig 6i**). I reasoned that this was due to residual function in this allele and repeated the experiment with a *sma-6* null allele. Surprisingly, in this mutant no difference was observed relative to wildtype at any stage (**Fig 6j**). I conclude that the Sma/Mab pathway is implicated in the genetic control of the observed progressive increase in embryonic yolk provisioning. However the role of the Sma/Mab SMADs *sma-2*, *sma-3* and *sma-4* in regulating yolk provisioning likely does not lie downstream of *sma-6* and occurs in response to cross-talk from another signalling pathway.

Mechanisms by which vitellogenin affects L1 starvation resistance

Roux et al. (2016) described a suite of progressive changes that occur during L1 diapause that resemble aging and are reversed upon feeding. I hypothesized that the reason early progeny appear more sensitive to the effects of L1 starvation is an accelerated rate of these aging-like changes. One such change is mitochondrial fragmentation - upon hatching larval mitochondria are tubular, but over time they break up to form small, round fragments. Using SJ4103, a transgenic strain expressing a mitochondrially-targeted *gfp* reporter under the control of the body wall muscle specific *myo-3* promoter (**Fig 7a**), I observed that the mitochondria of day 1 progeny after 10 days of starvation are shorter (**Fig 7b**) and more circular (**Fig 7c**) than those of day 2 progeny, indeed suggesting a greater degree of fragmentation in early progeny.

Roux et al. (2016) also reported a progressive increase in protein aggregation, akin to that seen in ageing adults. I examined the effect of yolk depletion by maternal feeding of *vit-5* and *vit-6* RNAi on the rate of protein aggregation, which would imply that this trait may also change with maternal age. I used a strain (AM140) with a *polyQ35:yfp* reporter under the control of the *unc-54* promoter, which expresses an aggregation-prone polyglutamine protein (Morley et al. 2002) (**Fig 7d**). Yolk depleted and control progeny both had a complete absence of any aggregation at hatching (data not shown). After 7 days (**Fig 7e**) or 10 days (**Fig 7f**) of starvation-induced L1 arrest, levels of polyglutamine aggregates did not differ between yolk-depleted and control progeny. After 14 days a small increase in aggregation was evident in yolk-depleted progeny (**Fig 7g**). Overall, the effect of severe yolk depletion was very mild and I expect that any differences between progeny of younger and older mothers in aggregation rates due to differential yolk loading would be undetectable.

Figure 7. Molecular hallmarks of ageing during starvation-induced L1 arrest are altered in yolk-depleted progeny and progeny of young mothers.

(**a**) Epifluorescence image of starved L1 worms expressing mitochondrial GFP from the *myo-3* promoter in body wall muscle. Scale bar, 20 μm.

(**b**) Mean mitochondria length after 10 days of starvation-induced L1 arrest of progeny from day 1 or day 2 mothers. Each point represents the mean length of 60-177 individually identified mitochondrial fragments in a single worm. n = 22 (day 1), 15 (day 2).

(**c**) Mean mitochondria form factor, a measure of circularity, after 10 days of starvation-induced L1 arrest of progeny from day 1 or day 2 mothers. A circle would have a value of 1 and a straight line, 0. Sample sizes as in panel **b**.

(**d**) Epifluorescence image of starved L1 worm expressing polyQ35::YFP. Aggregates of polyQ::YFP are indicated by white arrows. Scale bar, 20 μm.

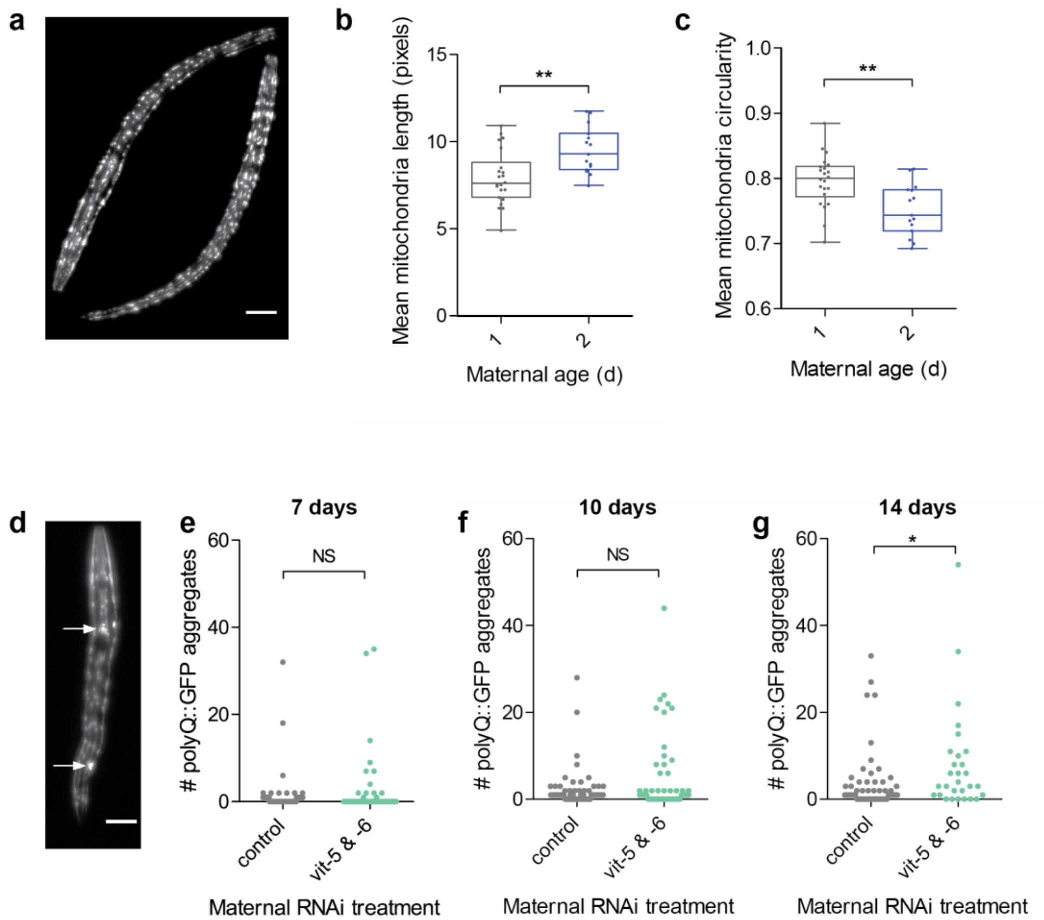
(**e**) Effect of maternal *vit-5* and *vit-6* or empty vector control RNAi on number of aggregates per larva after 7 days of starvation-induced L1 arrest. n = 67 (EV), 52 (*vit*).

(**f**) Effect of maternal *vit-5* and *vit-6* or empty vector control RNAi on number of aggregates per larva after 10 days of starvation-induced L1 arrest. n = 101 (EV), 58 (*vit*).

(**g**) Effect of maternal *vit-5* and *vit-6* or empty vector control RNAi on number of aggregates per larva after 14 days of starvation-induced L1 arrest. n = 56 (EV), 29 (*vit*).

Boxplots represent median values, interquartile ranges and Tukey whiskers with individual data points superimposed. * $P < 0.05$, ** $P < 0.01$. GLM analysis (panels **b** and **c**), two-tailed Mann Whitney test (panels **e**, **f** and **g**). NS, not significant.

Figure 7



An alternative hypothesis for the increased sensitivity of progeny of young mothers to starvation-induced L1 arrest was that an increased induction of autophagy in response to reduced nutrient stores caused a greater incidence of irreversible tissue damage. Kang et al. (2007) found that over-induction of autophagy in the pharyngeal muscle can negatively impact recovery from L1 starvation. I examined the expression of LGG-1::GFP, a common marker for autophagy induction that localises to the autophagosome from the cytoplasm. LGG-1 expression is upregulated by elevated autophagy (Palmisano and Meléndez 2016). In order to accurately identify the pharynx, I crossed a single-copy *pmyo-2:mcherry* transgene, which is expressed exclusively in pharyngeal muscle, into the LGG-1::GFP strain. Additionally, I could use the pharyngeal expression of mCherry as a directional marker to digitally straighten worms and quantify the mean fluorescence level in segments along the anterior-posterior axis.

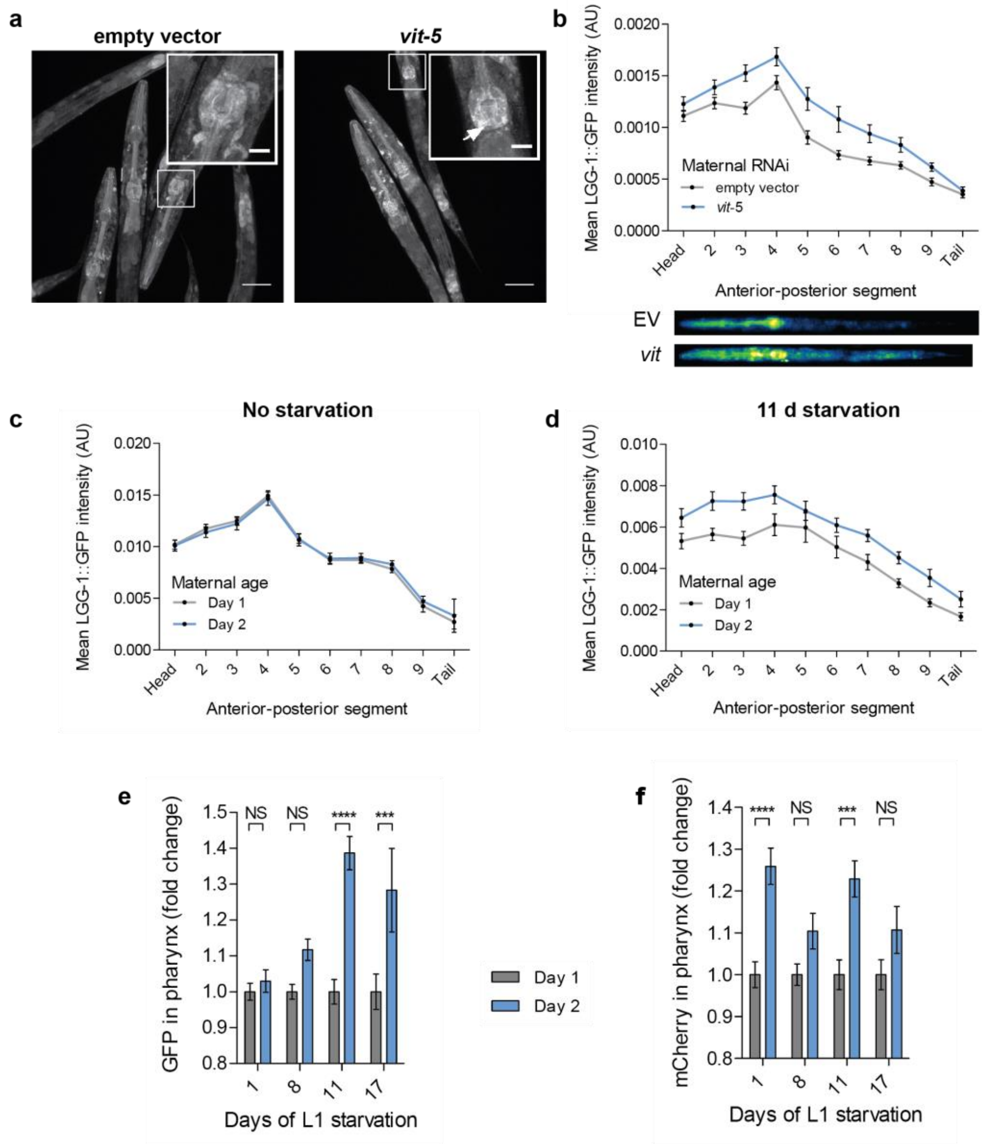
First, I examined the expression of LGG-1::GFP during starvation-induced L1 arrest in yolk-depleted progeny. At hatching the expression of LGG-1::GFP appeared mildly elevated in yolk-depleted progeny, especially in the pharynx (**Fig 8a**). After a 7 day starvation the expression of LGG-1::GFP was higher in both pharyngeal and intestinal tissues (**Fig 8b**). I conclude that severe resource deprivation during L1 starvation leads to the induction of autophagy.

To see if autophagy is also upregulated in progeny of young mothers, I starved L1 larvae from day 1 or day 2 hermaphrodites and periodically sampled them by imaging to assay the induction of autophagy. At hatching, no difference in the expression of LGG-1::GFP was detected (**Fig 8c**). After 11 days of starvation, differences emerged but, contrary to expectations, it was the progeny of day 2 mothers that exhibited elevated LGG-1::GFP expression along the length of the body (**Fig 8d**). Total pharyngeal LGG-1::GFP intensity increased over the course of L1 starvation but more so in day 2 progeny, and was around 40 % higher after

11 days of starvation (**Fig 8e**). I also quantified the total pharyngeal mCherry fluorescence. Surprisingly, although the level of expression of the *pmyo-2:mCherry* transgene remained approximately constant over the course of starvation (data not shown), it was more highly expressed in progeny of day 2 mothers (**Fig 8f**).

Figure 8. Autophagy is more strongly induced by L1 starvation in yolk-depleted progeny but less strongly induced in progeny of young mothers.
(**a**) Confocal microscopy images of L1 larvae from mothers treated with empty vector control or *vit*-5 RNAi expressing the autophagy marker LGG-1::GFP. Inset is an expanded section of the posterior bulb of the pharynx, indicated by the white frame in the main image. The arrow marks a fluorescent punctum in the pharynx of a yolk-depleted larva. Scale bars, 20 μm main image, 5 μm inset.
(**b**) LGG-1::GFP expression after 7 days of starvation-induced L1 arrest in progeny from mothers treated with empty vector control (grey) or *vit-5* (blue) RNAi. Mean fluorescence of 10 transverse segments spanning the length of the body is shown. Underneath are two representative, digitally straightened images aligned to the corresponding segments. Worms were starved for 7 days. n = 70 (control), 71 (*vit-5*).
(**c**) Mean LGG-1::GFP fluorescence along length of the body of newly hatched L1 larvae from day 1 or day 2 mothers. n = 159 (day 1), 139 (day 2).
(**d**) Mean LGG-1::GFP fluorescence along length of the body of L1 larvae from day 1 or day 2 mothers after 11 days of starvation. n = 89 (day 1), 88 (day 2).
(**e**) Total LGG-1::GFP intensity in the pharynx of L1 larvae from day 1 or day 2 mothers over the course of L1 arrest. Fluorescence is normalised to the mean of the control group. n = 230, 103, 65, 59 (day 1); 146, 50, 59, 34 (day 2).
(**f**) Total mCherry intensity from the *myo-2* promoter in the pharynx of L1 larvae from day 1 or day 2 mothers over the course of L1 arrest. Fluorescence is normalised to the mean of the control group. Sample sizes as in panel **e**.
Error bars in panels **b**, **c** and **d** show 95 % confidence intervals for mean fluorescence of each segment. Error bars in panels **e** and **f** represent standard error of the mean. *** $P < 0.001$, **** $P < 0.0001$, 2-way ANOVA with Bonferroni multiple comparisons tests. EV, empty vector. NS, not significant.

Figure 8



Embryo size does not reflect yolk provisioning

Hibshman et al. (2016) found that embryo size, and in particular embryo length, increased under maternal dietary restriction, taken to be reflective of the extent of maternal provisioning. The authors found that embryo length under various degrees of dietary restriction correlated strongly with length at hatching, and also found that length at hatching increases with maternal age. Indeed, strong reductions in embryonic vitellogenin by maternal RNAi against *rme-2* or *vit-5* and *vit-6* substantially reduces embryo size (**Fig 9a**). I asked whether the increase in vitellogenin provisioning and offspring length that I observe with increasing maternal age are associated with a similar change in the size of embryos. To address this question, I processed brightfield images collected alongside fluorescent images for the experiment shown in **Fig 4b** to accurately measure the length, width and cross-sectional area of embryos (**Fig 9b**). I could thus compare these parameters across a range of 10 maternal ages in embryos where an increase in vitellogenin content was confirmed. Although some minor differences between groups were evident, likely attributable to batch effects, linear regression indicated no relationship between maternal age and embryo length, width or area (**Fig 9, c, d and e**). In the data shown embryos are not stage-matched, assuming that embryo size does not change over the course of development. To validate, a post-hoc selection of early embryos also found no relationship of maternal age to embryo size (data not shown). Likewise, manual measurements of embryo length indicated no relationship to maternal age (data not shown).

Figure 9

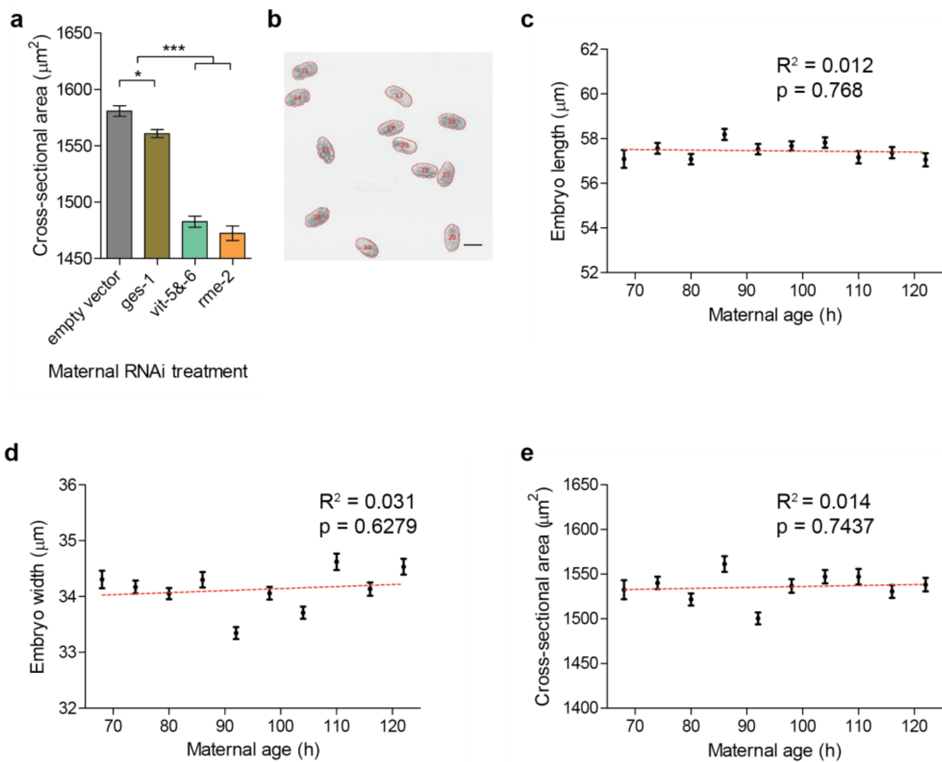


Fig 9. Embryo size does not change with maternal age.

(**a**) Embryo cross-sectional area after maternal RNAi treatments that strongly reduce vitellogenin loading. *ges-1* RNAi is included as an additional control. n = 398, 496, 311, 197.

(**b**) Illustration of thresholding of brightfield images to measure embryo size parameters. Outlines of thresholded embryos are shown in red. Numbers correspond to unique identifying labels automatically assigned to each embryo. Scale bar, 40 µm.

(**c**) Maximal embryo length against maternal age (hours post egg-lay). n = 250, 396, 561, 516, 527, 688, 627, 464, 705, 616.

(**d**) Maximal embryo width against maternal age. Samples sizes as in b.

(**e**) Embryo cross-sectional area against maternal age. Samples sizes as in b.

Error bars in panel **a** indicate standard error of the mean and in panels **c**, **d** and **e** indicate 95 % confidence intervals. * $P < 0.05$, *** $P < 0.001$. Kruskal Wallis test with Dunn's multiple comparison tests. In panels **c**, **d** and **e** R^2 and p values are from linear regression analysis. The red dashed line indicates the regression line.

Additional Methods

Strains used

The following strains were used; CB4856 (wild isolate), PB306 (wild isolate), BCN9070 *vit-2(crg9070[vit-2::gfp])*, BCN9077 *eat-2(ad465)*;*vit-2(crg9070[vit-2::gfp])*, BCN9086 *clk-1(e2519)*;*vit-2(crg9070[vit-2::gfp])*, BCN9089 *daf-2(e1370)*;*vit-2(crg9070[vit-2::gfp])*, BCN9077 *daf-2(e1368)*;*vit-2(crg9070[vit-2::gfp])*, BCN9078 *daf-16(mu86);vit-2(crg9070[vit-2::gfp])*, BCN9091 *daf-2(e1370)*;*daf-16(mu86);vit-2(crg9070[vit-2::gfp]),* BCN9081 *sma-2(e502);vit-2(crg9070[vit-2::gfp]),* BCN9082 *sma-3(e491);vit-2(crg9070[vit-2::gfp])*, BCN9084 *sma-4(e729);vit-2(crg9070[vit-2::gfp])*, BCN9085 *sma-6(e1482);vit-2(crg9070[vit-2::gfp]),* BCN9093 *sma-6(wk7);vit-2(crg9070[vit-2::gfp]),* MAH236 *sqIs13 [lgg-1p::GFP::lgg-1 + odr-1p::RFP]*, BCN9074 *sqIs13 [lgg-1p::GFP::lgg-1 + odr-1p::RFP];pmyo-2:mcherry,* AM140 *rmIs132 [unc-54p::Q35::YFP].*

Imaging and image analysis

Worms were imaged by epifluorescence microscopy as described previously with a 20x objective. Z stacks were acquired at 500 nm intervals. For analysis, Z stacks were subjected to a 50 pixel rolling ball background subtraction before maximum intensity projection of fluorescence images and standard deviation projection of brightfield images on ImageJ. Using CellProfiler, worms were segmented using the brightfield standard deviation projection (Ali et al. 2012) and mitochondrial fragments were segmented from the corresponding fluorescence max intensity projections, thresholding separately within each individual worm. Adjacent mitochondrial fragments were automatically separated by GFP intensity.

Worms were digitally straightened and mean fluorescence quantified in transverse segments using the Worm Toolbox package in CellProfiler.

polyQ35::YFP aggregates were counted by eye using epifluorescence microscopy and a 20x objective lens.

Statistical analysis

Lipid staining data for wild isolate embryos was analysed by generalised linear model analysis with the ‘lme4’ package in R, with the following terms included in the model: maternal age, embryo size, embryo nuclei count as fixed effects and replicate as random effects. Mitochondrial fragmentation was analysed with the ‘lme4’ package in R using measurements for individual mitochondria, with the following terms included in the model: maternal age as a fixed effect and individual worm as a random effect. For all generalised linear model analysis p values for pairwise comparisons were obtained by ANOVA comparison of a model including maternal age with a model omitting it, restricting the dataset to the two samples under comparison. p values were subsequently Bonferroni corrected. Linear regression analysis was performed with Graphpad Prism 5.04.

3. DISCUSSION

3.1 Vitellogenin titre is an intermediate phenotype that may predict individual phenotypic outcomes

In this work I have found that a major driver of phenotypic differences between genetically identical individuals in *C. elegans* is maternal age. As much of the effect of maternal age is mediated by embryonic levels of maternally-supplied yolk, I have identified a measurable 'intermediate phenotype' that provides a degree of prediction of the phenotypic outcome of an individual embryo. In this work I have only shown that increasing vitellogenin mediates the phenotypic effects of maternal age. There exists considerable variation in embryonic vitellogenin content within cohorts derived from synchronised mothers. Indeed, Mirko Francesconi has shown in the presented manuscript that vitellogenin expression is unusually variable between individual adults, even controlling carefully for age. Notably, I have not shown that inter-individual differences in embryonic vitellogenin in a single cohort causes phenotypic variation within that cohort. Such an effort has been hampered by the technical difficulty of tracking single worms from embryo to adulthood and phenotyping individuals without affecting development, and doing both in sufficient animals to afford a valid comparison. Success likely requires a high-throughput method of sorting individual embryos according to yolk content, such as FACS. However it is probable that the variation in adult vitellogenin production and consequent provisioning to embryos underlies a substantial fraction of the phenotypic variation within a cohort, and could have the potential to mediate the phenotypic impacts of other parental life history factors.

3.2 The difficulty of overexpressing vitellogenins

I have shown that a quantitative reduction of yolk, of a magnitude similar to the difference observed between day 1 and day 2 embryos, is sufficient to produce phenotypic impairments in day 2 progeny to a degree similar to that observed in day 1 progeny. Ideally, I would also show that an increase in yolk in the progeny of young mothers results in an improvement of offspring quality. However to perform this experiment is likely impossible.

An established approach for overexpressing a particular gene product is to add one or more additional copies to the genome. There are many reasons to think this approach will be fruitless in this particular case. There are already 6 vitellogenin genes in the *C. elegans* genome, including several that are almost identical copies. The intestinal cells of the worm undergo several rounds of endoreduplication, the last occurring at the L4-adult moult shortly before the onset of vitellogenesis (Hedgecock and White 1985). Most of the highly polyploid, binucleate intestinal cells of the adult hermaphrodite contain 64 haploid genomes, or almost 400 copies of *vit* genes per cell. It seems that with respect to the strategy of adding extra gene copies, evolution has managed to get there ahead of us.

As discussed in the introduction (section **1.4.2.4**), even a young adult worm churns out prodigious quantities of yolk. Consequently it is reasonable to assume that at least in a young adult, vitellogenin production may be near saturation and thus limited by the biosynthetic capacity of the intestine. Such an interpretation is supported by the observation of a global increase in non-yolk intestinal transcription upon abolition of vitellogenin transcription by *unc-62* RNAi (Van Nostrand et al. 2013). In such a scenario, adding additional copies of vitellogenin will have little impact on the overall levels of yolk production, although it may bias the composition of yolk towards one or another isoform according to which gene has been added. In the literature, this appears to be the case at the level of

transcription (Spieth et al. 1988) and translation (Van Rompay et al. 2015). Where overexpression of vitellogenins has been claimed (e.g. by Seah et al. 2016), the evidence provided has been wholly inadequate - in this example they show only that addition of extra copies of *vit-2* increases the level of *vit-2* transcripts, not total vitellogenin transcription or yolk protein production. Fischer et al. (2012) claim that overexpression of vitellogenins can be induced by application of phytoestrogens, but I was unable to replicate their results (data not shown).

3.3 The adaptive and ecological significance of maternal age effects in C. elegans

As vitellogenins are so highly expressed, it is likely that the increase in yolk production observed with mother's age could not be possible without an overall increase in biosynthetic capacity of the worm during adulthood. Indeed adult hermaphrodites, while not undergoing any further cell divisions, do increase substantially in volume from day 1 to day 3. This being the case, the worm could produce better provisioned, higher quality progeny by delaying sexual maturity. The contribution of Mirko Francesconi to the presented manuscript establishes firmly that the timing of maturation of somatic tissues and the germline can be uncoupled and indeed subject to seemingly regulated shifts in response to environmental (or maternal) conditions. This only makes it more plausible that if she so desired, a hermaphrodite worm could slow down the rate of germline maturation and progeny production and thus produce larger and healthier offspring. Why is the worm so impatient? Is the production of suboptimal progeny adaptive, the result of physiological constraints, or both? To answer this question we must consider both the ecology and ontogeny of *C. elegans*.

Although not ubiquitous, increases in progeny size and apparent quality due to increases in maternal age or size are widespread in many taxa, such as fish

(Marshall et al. 2010). Selection generally favours smaller offspring in benign environments and larger offspring in harsher environments. For a maternal effect of age or size to be adaptive, it is important that maternal phenotype serves as a reliable indicator of the likely environment of progeny (Kindsvater and Otto 2014). Marshall et al. (2010) argue, on the basis of simple modelling, that selection for production of suboptimal progeny is unlikely to occur, except in a few cases. These include constraints that promote reproduction at a suboptimal size, such as high mortality or fitness advantages in rapidly exploiting ephemeral habitats, or physiological constraints. The authors also argue that such physiological constraints are unlikely to exist, although Sakai and (Harada 2001) suggest a possible mechanism, christened 'terminal stream limitation', whereby resource extraction by offspring occurs at a rate independent of the number of offspring developing in a mother.

In ecological theory it is generally assumed that there is a trade-off between progeny number and progeny size, due to a limited pool of maternal resources (Smith and Fretwell 1974). However in *C. elegans* this relationship does not hold true. The principal trade-off governing reproductive traits in hermaphrodites undergoing self-fertilisation, which seems to be the norm in an ecological setting (Félix and Braendle 2010), is between progeny number and the speed of (germline) development, through its influence on the length of time devoted to spermatogenesis and so the total quantity of self-sperm (Cutter and Pitnick 2004). To this we may add progeny quality alongside progeny number, which may be expected to tip the balance. Nonetheless, we find that wild worm isolates prefer early reproduction - indeed in response to the luxurious conditions of the lab the N2 strain has evolved, via a mutation in *nath-10*, to have a larger brood size at the cost of a delay in reproduction (Duveau and Félix 2012). This implies that, outside of the lab, speedy reproduction brings a large competitive advantage that outweighs that of producing more and larger offspring. This may also be promoted by high rates of extrinsic mortality, as is likely the case for worms in

more natural settings (Laakso and Setälä 1999, Van Voorhies et al. 2005). If a hermaphrodite is unlikely to survive to use all her sperm it does her little good to spend precious time accumulating more.

In the most complete study of *Caenorhabditis* meta-population dynamics to date, Félix and Duveau (2012) find that worm populations usually proliferate for only 2-3 generations before resource depletion occurs. In this scenario, a small acceleration of reproduction in founding members may pay large dividends in terms of the final proportion of descendants in the mature, food-depleted colony. At least initially, the environment will likely be benign for progeny of young mothers - they will be born into a relatively empty resource patch that contained ample food to support their mother's development to reproductive adulthood only a short while ago. Meanwhile, for the progeny of older mothers in any generation, the situation will not be so rosy, as the environment will likely contain less food and be populated with numerous older siblings with which they will have to compete. Maternal age may thus serve as a reliable indicator of progression of colonisation and so progeny environment.

As for physiological constraints, the sequential production of progeny in *C. elegans*, whereby single oocytes pass in procession through yolk provisioning in the proximal gonad and onwards through the spermatheca for fertilisation, may indeed represent a form of 'terminal stream limitation'. In such a case, once reproduction begins a worm may be unable to slow this procession to strategically increase provisioning to individual offspring - this mode of sequential reproduction combined with a progressive increase in intestinal biosynthetic capacity may be a sort of physiological constraint that facilitates the adaptive production of sub-optimal progeny by young mothers.

Despite the general improbability of adaptive production of sub-optimal progeny by young mothers, the peculiar case of *C. elegans* thus appears to closely coincide

with the conditions postulated for just such an effect: 1) accelerated reproduction would provide an advantage in colonising ephemeral habitats; 2) maternal age likely predicts progeny environment by indicating colonisation progression; 3) rates of extrinsic mortality are likely high; 4) sibling-sibling competition is severe in extremely dense populations; and 5) sequential reproduction may place physiological constraints that favour production of sub-optimal progeny.

To evaluate the significance of my findings outside of the laboratory, it will be necessary to further establish their impact on *C. elegans* ecology and population dynamics. Importantly, I have shown that my findings are not limited to the lab-adapted N2 strain and that in wild *C. elegans* isolates, progeny of young mothers are also smaller and grow slowly. Maternal vitellogenin transcription and intensity of embryo lipid staining also increase with mother's age, suggesting that the underlying mechanism, of differential yolk provisioning, is the same. However, laboratory culture of *C. elegans* is unlike the worm's natural history in many respects besides the genetic background commonly used. It should be established that the effect of maternal age can be observed when worms are fed alternative diets. If these diets do affect the rate of maternal provisioning over the course of the reproductive period, as we might expect, it will be important to test the effects and interactions produced by complex mixtures of bacterial species, such as encountered in the wild (Samuel et al. 2016). It would also be informative to test how advantageous a developmental acceleration might be in the colonisation of an ephemeral habitat, which might be achieved experimentally by competing progeny derived from younger or older mothers for 2-3 generations, or computationally by modelling the meta-population dynamics of *C. elegans*. Given that passage through the dauer state is likely to be crucial to the success of a lineage in repeated colonisations of short-lived resource patches, it will be imperative to understand how maternal age interacts with this life history strategy; whether rates of dauer entry differ among progeny of younger and older mothers, whether dauer larvae from older mothers are, like L1 larvae, more

resistant to starvation and other hardships and whether the growth acceleration of the progeny of older mothers in maintained despite passage through the dauer state.

3.4 Potential mechanisms for the effect of yolk on L1 starvation resistance

As I and others have shown (Jobson et al. 2015, Lee et al. 2012), animals that undergo extended L1 starvation prior to recovery exhibit a variety of phenotypic impairments, including slow growth, errors in vulval development and rupture through the vulva. Most commonly and most importantly, long-starved worms often exhibit gross abnormalities in the development of one or both gonads. Gonads often undergo uncontrolled proliferation leading to the formation of germline 'tumours' (Berry et al. 1997, Lee et al. 2012). The common 'protruding vulva' phenotype observed in recovered, long-starved worms also appears to be caused by hyper-proliferation of the gonad and extrusion into an appendage that juts from the body. In some cases this may precede vulval rupture and death. Occasionally, gonads fail to develop and appear to be lacking entirely. All of these modes of aberrant gonad development are associated with sterility and thus complete failure of the organism to replicate itself. In this work we have found that the penetrance of these phenotypes, both somatic and germline, is increased in the progeny of young mothers, which we show is due to reduced provisioning with vitellogenin. Consideration of what might be the more proximal causes of these failures of development may provide clues as to the mechanism by which reduced yolk provisioning can affect progeny phenotypes.

Normal germline development relies on positional signals from the somatic gonad to regulate the migration and proliferation of germ cells (Killian and Hubbard 2005). Perturbation of the coordination between germ cells and the somatic gonad

could lead to the germline hyper-proliferation commonly observed in recovered, long-starved animals. One way this could happen is by the failure to maintain germ cell quiescence during L1 arrest. This quiescence is regulated by AMP-activated protein kinase (AMPK; Demoinet et al. 2017, Fukuyama et al. 2012), a conserved protein complex that directly senses energy levels in cells and acts under nutrient stress to promote pathways that generate ATP and repress those that utilise it (Hardie et al. 2012). AMPK acts upstream of TORC1 signalling to maintain quiescence (Fukuyama et al. 2012). Similar mechanisms, also involving TORC1 signalling in addition to insulin-like signalling, regulate the quiescence of various somatic progenitors (Fukuyama et al. 2015), including hypodermal seam cell progenitors (Baugh and Sternberg 2006). Defects in seam cell development could undermine the structural integrity of the hypodermis and increase the likelihood of vulval rupture (Grishok et al. 2001).

Various gross developmental abnormalities of both the germline and somatic tissues caused by extended L1 starvation might therefore result from a loss of quiescence and inappropriate cellular division during starvation. If this is the case, it would suggest that maintenance of quiescence is affected by the maternally provided nutrient stores of the worm. Ordinarily the presence of nutrients signals that the worm has encountered food and promotes the activation of proliferation in these cells (Fukuyama et al. 2015). However, it may be that some unknown metabolic program initiated by the dwindling of nutrient reserves to critical levels during a long starvation leads to the inappropriate activation of proliferation.

To address this hypothesis, the first step would be to establish whether such inappropriate cellular divisions during extended starvation occur at an appreciable frequency in a wildtype genetic background, and whether they occur at higher frequency in progeny of young mothers or yolk-depleted progeny. To follow aberrant germ-cell divisions in various mutants, Fukuyama et al. (2012) used immunofluorescence staining against the germline-specific protein PGL-1,

while Baugh and Sternberg (2006) followed seam cell divisions using Nomarski microscopy. I attempted to follow germ cell divisions over the course of larval starvation using GFP driven by the germline *pie-1* promoter; however I found that leaky expression of the promoter in other tissues became increasingly widespread as starvation proceeded, making this transgene unfit for purpose (data not shown). It would be interesting to repeat this effort by other methods such as those used in the aforementioned studies to evaluate this hypothesis. Given that the signalling inputs maintaining quiescence are well characterised, a role for loss of quiescence may be informative as to the mechanisms by which maternally-supplied yolk can exert control over progeny phenotypes.

An alternative hypothesis for the effects of long L1 starvation on progeny phenotypes is that irreversible damage occurs that compromises the future development and function of various tissues, such as the germline. Prolonged L1 arrest induces progressive changes that mimic those found in ageing individuals, including a slowing of movement, fragmentation of mitochondria and increased protein aggregation (Roux et al. 2016). The rate of 'ageing' during L1 arrest may be accelerated in progeny of young mothers, such that they are more likely to incur irreversible damage prior to recovery. To test this hypothesis, it would first be necessary to show these molecular changes occur at an accelerated rate in progeny of young mothers or yolk-depleted progeny. Preliminary results presented here indicate that mitochondria may indeed be more fragmented in starved progeny of young mothers. Protein aggregation also appears exacerbated in yolk-depleted progeny, although the effect of a strong maternal vitellogenin knockdown is mild and may not be expected to underlie any appreciable difference in the progeny of younger or older mothers.

Another process that might cause irreversible tissue damage under starvation is autophagy, whereby under nutrient stress cellular components are degraded and recycled. Autophagy is essential for proper survival of starvation-induced L1

arrest, with either too much or too little induction of the pathway increasing sensitivity to starvation (Kang et al. 2007). I hypothesised that autophagy is induced more strongly in progeny of young mothers in response to their limited nutrient stores and may lead to impairment of tissue function over the course of starvation. Yolk-depleted progeny did show higher, more punctate expression of LGG-1::GFP, a common marker for autophagy that localises to the autophagosome (Palmisano and Meléndez 2016). Surprisingly the pattern was reversed in the progeny of young mothers, with reduced expression of LGG-1 implying that autophagy is more strongly induced in the progeny of older mothers. It may be that autophagy mediates the release of nutrients from yolk granules and thus is mildly upregulated in the progeny of older mother with more ample stores of vitellogenin. The discrepancy with the strong induction of autophagy in yolk-depleted progeny may be interpreted as a symptom of an extreme nutritional deficiency, rather than a modest quantitative reduction as in younger progeny. As too much or too little autophagy can impact starvation survival, it may be that the elevated autophagy in day 2 supports survival. In any case these results do not support the straightforward hypothesis that yolk deficiency leads to elevated autophagy and tissue damage in the progeny of young mothers. The elevated expression of the *pmyo-2:mcherry* transgene in day 2 progeny in this experiment is surprising and may represent an effect specific to this promoter or a more general effect of maternal age on transgenes, which may merit further investigation. I do not believe that the elevated expression of *pmyo-2:mcherry* casts doubt on the results obtained concurrently with the LGG-1::GFP transgene, as they show quite distinct patterns of differences; unlike *pmyo-2:mcherry*, *lgg-1::gfp* expression is not different between day 1 and day 2 progeny at hatching and unlike *lgg-1::gfp, pmyo-2:mcherry* expression does not increase over the course of L1 starvation.

Given that we show that the difference between day 1 and day 2 progeny in sensitivity to larval starvation is due to differential vitellogenin loading, it is

puzzling that day 3 progeny, with still higher yolk titres, have a higher penetrance of sterility after 14 days than day 2 progeny. Although after 10 days the frequency of abnormal developmental phenotypes is similar in day 2 and day 3 progeny, after a 17 day starvation the day 3 progeny fare significantly worse (data not shown). Of the phenotypes shown in this work to be affected by maternal age, some show a more linear response (e.g. length at hatch) while others show a pattern of diminishing effect, with day 3 outcomes only slightly better than day 2 (e.g. the rate of post-embryonic development). Only sensitivity to extended starvation appears to show a peaked response to maternal age within the examined range, with the frequency of sterility and phenotypic abnormalities increasing from day 2 to day 3. It is likely that these phenotypes, unlike post-embryonic growth under immediate and continuous feeding, are confounded by another factor, such as reproductive ageing (Luo et al. 2010), which outweighs the influence of higher maternal provisioning.

3.5 Specific components of yolk may mediate progeny phenotypes

In this work I have established that reduced yolk provisioning to offspring of young mothers mediates many of the phenotypic impairments observed in these worms in comparison to older siblings. These impairments may result simply as a consequence of a broad reduction in energy availability during embryogenesis and early development. However as yolk complexes are composed of a complex cocktail of protein, phospholipids, neutral lipids, rare fatty acids (e.g. MUFAs), cholesterol, sugars and potentially vital micronutrients, it is worth discussing which components of yolk may be responsible for the phenotypes observed. While it is very likely that deficiencies in all of the various components of yolk will have some impact on offspring phenotypes, we can also imagine that a dearth

of some specific components may disproportionately impact the observed phenotypes, or at least a subset of them.

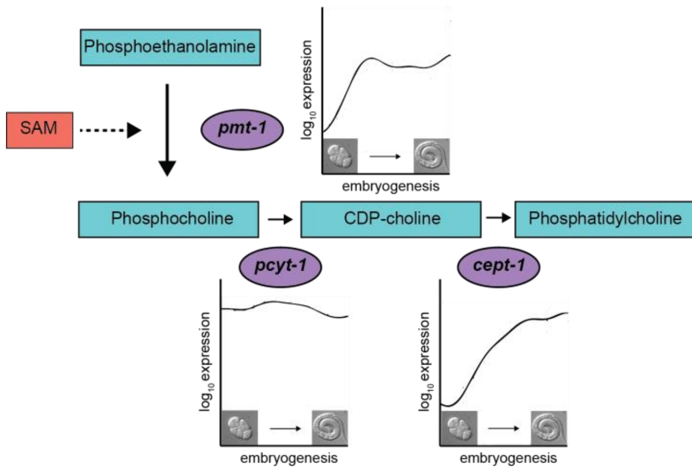
An important candidate for mediating some of the phenotypic impacts of yolk are phospholipids, especially phosphatidylcholine (PC). While phospholipids do not contribute much to energy resources, they are a critical resource during embryonic development as the principal constituent for the plasma membranes of the 558 cells into which the egg must divide before feeding can begin. Indeed, phospholipids constitute the majority of the lipid content of yolk (Kubagawa et al. 2006), comprised mostly of phosphatidylethanolamine (PE, 28 %) and PC (23 %). In worms, PC synthesis from phosphoethanolamine requires the essential cofactor S-adenosyl methionine (SAM) and the enzymes encoded by the genes *pmt-1*, *cept-1* and *pcyt-1* (Walker et al. 2011). All three of these genes are expressed during embryogenesis (**Fig x, a**), implying active synthesis of PC by the developing embryo and underscoring the importance of PC to embryogenesis.

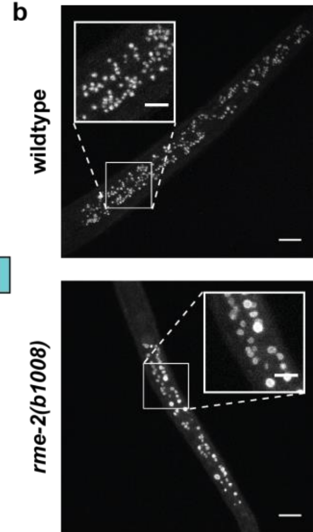
Decreased phospholipid availability to the developing embryo could limit cell size and thus body size at hatching, as I observed for progeny of young mothers. Mutants for PC production exhibit small body sizes (Ehmke et al. 2014). I hypothesise that decreased phospholipid availability for membrane formation also explains the observed enlargement of lysosome-related organelles, gut granules, in the intestines of hatching larvae from young mothers. Larger granules will have a reduced surface-area-to-volume ratio and so require less phospholipid to generate an equal organelle volume, although possibly affecting the (as yet mysterious) function of the gut granules. Mutants in SAM synthesis, such as *sams-1* mutants, display a 'bubble' phenotype of grossly enlarged intestinal lipid droplets (Ehmke et al. 2014, Walker et al. 2011) as a result of the impact on PC production. In part this is due to a role of PC levels in regulating fat synthesis via the transcription factor SBP-1; SAM cycle mutants display increased rates of TAG synthesis (Walker et al. 2011). The bubble phenotype is likely also due to a

dearth of PC for lipid droplet membranes (Ehmke et al. 2014), exacerbated by the roughly 3-fold enrichment of PC over PE in lipid droplet membranes relative to whole-worm PC/PE ratios (Vrablik et al. 2015).

If enlarged gut granules are indeed caused by a deficiency of phospholipids, would we not also see lipid droplets enlarged in the progeny of young mothers, as in SAM cycle mutants? Not necessarily, for several reasons: 1) we expect offspring of young mothers to display a general reduction in PE as well as PC, reducing the specific impact on PC-enriched lipid droplet membranes and on SBP-1 signalling; 2) SBP-1 mediated feedback on lipid synthesis is likely not important in this context given that neutral lipids in developing embryos and newly hatched larvae will be largely maternally supplied; and 3) any effect may be difficult to detect given the dynamic nature of lipid droplets during development, growth and starvation (Ehmke et al. 2014, Palgunow et al. 2012). Conversely, similar reasoning can be applied to explain why we may not necessarily see grossly enlarged gut granules in SAM cycle mutants. However in early embryos of *rme-2* mutants, presumably representing an extreme case of phospholipid deprivation, enlarged vesicles that are likely to be lipid droplets are apparent (personal observation). Similarly, *rme-2* mutants display grotesquely enlarged gut granules at hatching (**Fig x, b**). Ultimately phospholipid deficiency would be expected to affect multiple organelles but perhaps the effects on gut granules in newly hatched larvae are simply more noticeable, given their large size (Coburn and Gems 2013) and their autofluorescent and light-scattering properties (Fouad et al. 2017).

Figure x





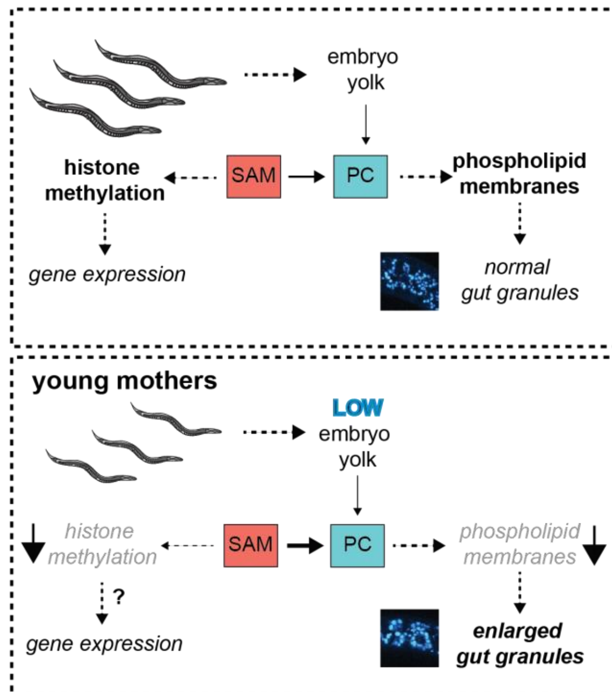


Figure x. Low phosphatidylcholine supply in the yolk of young mothers may act during embryogenesis to produce enlarged gut granules and potentially secondary metabolic effects.

(**a**) Synthesis of phosphatidylcholine (PC) uses S-adenosyl methionine (SAM) as a cofactor (after Walker et al. 2011). The three genes required by this pathway are strongly expressed or strongly induced during embryogenesis. Expression profiles were taken from Wormbase.com.

(**b**) Gut granules are grossly enlarged in *rme-2* mutants which lack any detectable embryonic yolk. Scale bar main image 10 um, inset 5 um.

(**c**) Hypothesised mechanism for secondary metabolic effects of low maternal phosphatidylcholine supply via yolk. Competition for SAM, a universal methyl donor, between histone methylation and PC synthesis for phospholipid membranes may lead to reduced levels of SAM available for histone methylation, affecting gene expression, and reduced PC levels reflected in observably larger intestinal gut granules.

Panel **a** *C. elegans* images credit, I Chin Sang.

High demand for PC during embryogenesis may cause secondary metabolic effects. As argued above, PC is likely to be actively synthesised during embryogenesis in a pathway requiring SAM. Limited SAM availability caused by dietary deficiency of the essential SAM cycle co-factor cobalamin (vitamin B12) has been shown to limit post-embryonic developmental rate in *C. elegans* by unknown mechanisms (Watson et al. 2014). SAM is also an essential methyl donor for the methylation of DNA in other species (Sharma and Rando 2017) and of histone tails (Shyh-Chang et al. 2013). Global changes in the availability of essential enzymatic substrates such as SAM have the potential to affect chromatin modifications (Sharma and Rando 2017). It may be that reduced maternal PC supply leads to competition for SAM between PC synthesis and histone methylation, which also undergoes extensive dynamic changes during embryogenesis (Cui and Han 2007). If that seems far-fetched, it was found that SAM availability had a dramatic influence on the global levels of the H3K4me3 histone mark required for high growth rate and maintenance of pluripotency in cultures of mammalian induced pluripotent stem cells (Shyh-Chang et al. 2013). Intriguingly, the alteration of gene expression in *C. elegans* caused by an ancestral

mutation in *wdr-5*, responsible for deposition of H3K4me3, differs substantially in progeny from the first or second day of egg-laying (Greer et al. 2011). I did observe higher expression in starved L1 larvae from day 2 mothers of the *pmyo-2:mcherry* transgenic marker relative to day 1 progeny; as transgenes in worms are very sensitive to alterations in chromatin this may reflect differences in chromatin modifications or architecture. It is therefore possible that some effects of maternal age on progeny phenotypes could act via reduced phospholipid provisioning and consequent alteration of metabolism, affecting chromatin state and perhaps ultimately gene expression (**Fig x, c**).

Cholesterol is another specific yolk component that may underlie some phenotypic consequences of maternal age. Cholesterol deprivation can produce germline defects, including the production of endomitotic oocytes, that resemble the germline defects produced by extended L1 starvation that are exacerbated in the progeny of young mothers (Shim et al. 2002). Cholesterol deprivation can also produce defects in growth and development (Shim et al. 2002). It may be that cholesterol levels become depleted during starvation and thus worms become sensitised to differential maternal supply of cholesterol after a long larval starvation. It would be interesting to see if supplementation of cholesterol during starvation ameliorates any impacts of extended L1 starvation and whether this reduces the effect of maternal age.

As it has been shown that components of the pseudocoelomic milieu, including dsRNA, can enter embryos along with yolk (Marré et al. 2016), it is also possible that an increased uptake of yolk in the progeny of older mothers may be associated with concomitant increase in uptake of extracellular molecules, such as RNAs, that could impact progeny phenotypes. Indeed, as speculated in section **1.4.4**, it may be that yolk complexes directly transport intestinal RNA molecules to the oocyte, which could play a regulatory role in developing progeny.

3.6 The difficulty of understanding vitellogenin regulation

Although limited by biosynthetic capacity, the increase in yolk production can still be subject to regulation, insofar as that the increased capacity of the older worm can be directed towards or away from vitellogenin synthesis. Here I have shown that the increase in provisioning to progeny with maternal age can be suppressed by loss-of-function of the insulin receptor orthologue *daf-2*, which acts independently of the canonical downstream master stress regulator *daf-16*, an orthologue of FOXO. Further I have shown that the Sma/Mab TGF-β pathway also plays a role in the increase in progeny provisioning.

As the regulation of vitellogenin increase by *daf-2* appears to be *daf-16* independent, it will be interesting to identify the downstream effectors that directly regulate the *vit* genes. A pair of excellent candidates are *sgk-1* and *pqm-1*. These genes have been demonstrated to regulate vitellogenins (Dowen et al. 2016, Shi et al. 2017, Wang et al. 2016) and *sgk-1* lies downstream of *daf-2* (Murphy and Hu 2005), in addition to its role in TOR signalling. Additionally the localisation of *pqm-1* undergoes a progressive shift from the nucleus to the cytoplasm at maturity (Dowen et al. 2016) and during ageing (Tepper et al. 2013), which provides an obvious mechanism for how it might regulate the age-related increase in vitellogenins.

Although the total level of yolk provisioning to individual embryos remains the same across the reproductive period in *daf-2* mutants, this does not imply that the level of vitellogenin production remains constant. The rate of progeny production itself is highly dynamic. If the rate of progeny production at the height of the reproductive period, approximately coinciding with the maternal age time point referred to as ‘day 2’ in this study, is roughly 2-3 times higher than progeny production in early adulthood (‘day 1’) as in wildtype, then a constant level of provisioning to individual embryos in *daf-2* mutants must still reflect an increase

in yolk production that is proportional to the increase in ovulation rate. Likewise, this logic implies that in wildtype hermaphrodites, where progeny provisioning increases substantially from day 1 mothers to day 2 mothers despite the latter's rapid rate of ovulation, the increase in total yolk production substantially exceeds the fold difference observed in provisioning to individual embryos. Indeed, we can use the same reasoning to argue that the increased yolk provisioning to progeny from day 2 to day 3 is not inconsistent with the observation of no increase in vitellogenin transcription over the same period. The rate of progeny production at the day 3 time point is already in decline and as such, a similar or even reduced available yolk pool will be divided between fewer progeny, resulting in elevated provisioning to individual embryos.

daf-2 mutations have numerous, highly pleiotropic effects. As such, the dynamics of progeny production - the total reproductive span and the temporal distribution of reproductive effort across this period - will be affected by the mutation, just as will yolk production itself. Consequently the data available from the experiments conducted is incomplete and does not afford an easy interpretation of at which point the *daf-2* mutation regulates provisioning to progeny.

To fully comprehend the effect of various mutations on regulation of yolk synthesis and provisioning to progeny, it would be necessary to collect data on: 1) the dynamics of egg-laying over the reproductive period; 2) the dynamics of vitellogenin transcription, as quantifiable by qPCR; 3) the per-embryo level of yolk, as quantifiable with the endogenous *vit-2:gfp* reporter. Ideally a simple model would be constructed where the total yolk synthesis could be inferred from the per-embryo yolk level and the rate of embryo production at any particular time point (**Fig xi**). Comparison of this inferred profile of yolk production with the directly measured dynamics of vitellogenin transcription could also illuminate cases where particular genes act on yolk synthesis at the post-transcriptional level, as has been claimed for the IIS pathway (DePina et al. 2011).

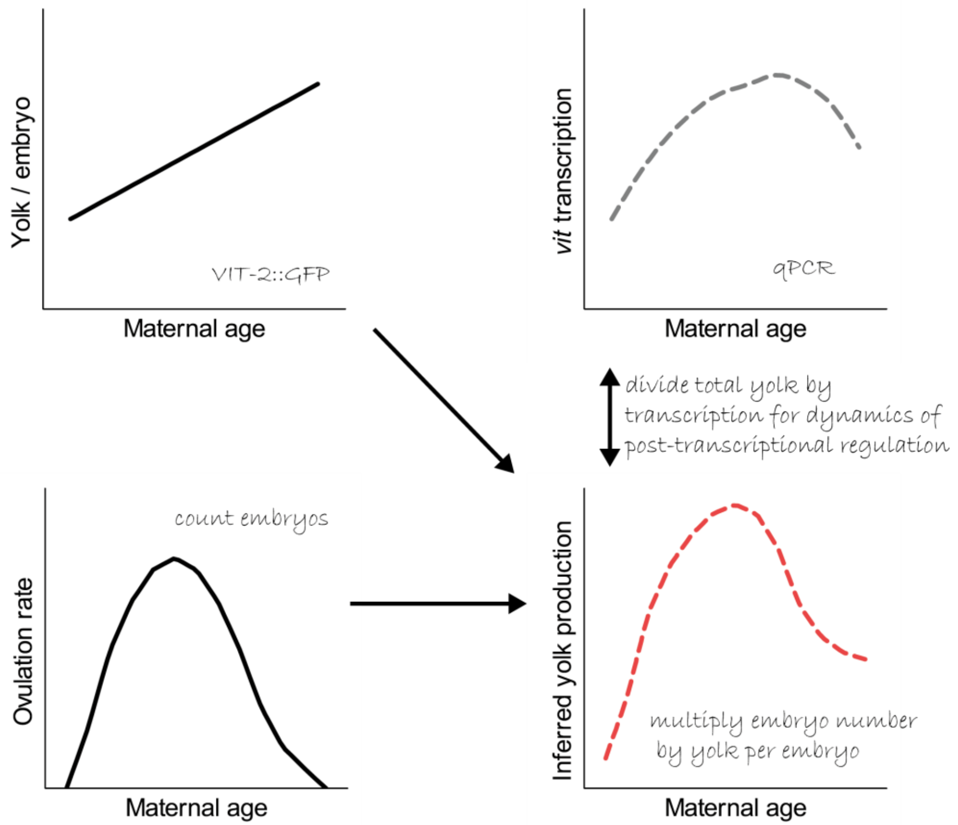


Fig xi. Inter-relation of dynamic processes complicate interpretation of genetic regulators of embryonic vitellogenin content.

A sketch suggesting how total yolk production may be inferred from ovulation rate and embryonic yolk content, with comparison of transcription and total yolk protein production illuminating genetic regulation of post-transcriptional dynamics.

3.7 Potential confounding effects of maternal age

I have shown here that maternal age affects progeny phenotypes in *C. elegans* under standard laboratory culture conditions. However, a number of correlated maternal experiences are potential confounders that may produce some effects rather than maternal age *per se*. These potential confounders have been accepted in the present study due to the difficulty of controlling for them explicitly, but merit discussion. Maternal size and its likely impact on yolk via biosynthetic capacity has already been discussed in section **3.3**.

Population density is a factor that is undergoing constant change during the reproductive period. If the population density of the maternal generation is controlled, there is still the constant production and growth of progeny, which will lead to a constant increase in worm density on the plate. Although among young mothers the maternal density does not appear to influence embryonic vitellogenin content (data not shown), the maternal response to density could also be age-dependent. Likewise, larval worms are known to sense population density via secreted pheromones (Butcher 2017). Perceived population density can influence rates of larval development (Ludewig et al. 2017) and the rate of hermaphrodite reproductive maturation (Wharam et al. 2017). The production of these pheromones is strongly dependent on developmental stage (Kaplan et al. 2011), so an evolving population structure may affect perception of density even if absolute numbers of worms could be controlled for. Secreted pheromones could also accumulate in the environment and have an intergenerational influence that is proportional to the dose and duration of maternal exposure. Maternal exposure to plates that have previously harboured worms can influence the relative developmental rates of somatic and germline tissues in progeny (Mirko Francesconi, unpublished data), although it does not appear to affect embryonic vitellogenin levels (data not shown). The level of food on plates is in constant flux, at a rate that varies with density and population structure. The decision to

enter the dauer stage is based on integration of density and food signals (Hu 2007); likewise the influence of age, density perception and food availability could interact in a complex fashion to shape maternal influence on progeny.

3.8 Embryo size does not reflect maternal provisioning in *C. elegans*

Maternal age affects embryonic provisioning with yolk, which is the predominant protein component of *C. elegans* embryos. A strong vitellogenin knockdown reduces embryo size (data not shown). Surprisingly, maternal age does not affect embryo size. Embryo size and embryo energy content often correlate, although this is not necessarily the case (McIntyre and Gooding 2000, Uusi-Heikkilä et al. 2012). However, a pair of studies conducted in *C. elegans* have shown that dietary restriction increases the size of embryos, explicitly assumed in both papers to reflect maternal provisioning (Harvey and Orbidans 2011, Hibshman et al. 2016). Assaying lipid and vitellogenin content is a direct measure of the extent of provisioning of embryos. The discordance between yolk and lipid content and embryo size in the present work suggests that embryo size likely does not reflect direct maternal provisioning in *C. elegans*, and that the reported effect of parental dietary restriction influences embryo size via a different mechanism.

4. CONCLUSION

To conclude, I have shown in this work that maternal age is a major source of phenotypic variation in isogenic populations of *C. elegans* reared in an identical laboratory environment. Maternal age affects size at hatch, growth, development, fecundity and starvation resistance. I have shown that maternal provisioning to offspring increases as a function of maternal age, and that this increase is likely regulated by the Sma/Mab TGF-β signalling pathway and non-canonical signalling via the insulin receptor orthologue *daf-2*. Lastly I have shown that this differential provisioning underlies the phenotypic effects observed in the offspring of young mothers relative to mothers at the peak of the reproductive period.

Given the importance of *C. elegans* as a model for studies of development and other processes, my findings are certainly relevant to future research in this species. I have discovered an important source of phenotypic variation among individuals under standard laboratory conditions that may have additional, as yet uncharacterised effects on various traits beyond those reported here. It is to be assumed that maternal age has often not been controlled for in the literature, potentially giving rise to spurious reported differences between experimental groups that owe little or nothing to the interventions performed. In particular, any of the countless mutations or environmental influences that slow development may appear to produce spurious phenotypic effects that are in fact due to differential physiological age of mothers, even when chronological age has been carefully controlled. It is my hope that the work presented here will inform and facilitate the careful interpretation of many other laboratory studies of *C. elegans*, both past and future.

Further investigation is merited into other potential effects of maternal age in *C. elegans*, for example on chromatin landscape, gene expression, stress resistance

and mutation penetrance, and whether maternal provisioning or other as-yet-undiscovered mechanisms are responsible. As reported in section **1.3.1.6**, associations of maternal age or size in other species are widely reported in ecological literature, but existing reports largely lack the carefully controlled environmental conditions and powerful genetic and molecular techniques available to researchers studying such effects in model organisms under laboratory conditions. Indeed it will be illustrative to search for similar effects of maternal age in model insects, amphibians and fish, and to establish whether such effects in oviparous taxa may also be mediated by maternal provisioning.

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