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Darwin vs. Lamarck

The genetic and epigenetic contribution to phenotypic variation in a vertebrate and an invertebrate model

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Preface

In this master's thesis, I aim to assess the relative contribution of genetic and non-genetic effects on phenotypic variation in natural populations of two distinct organisms. This research is important in the light of evolution, as it might suggest we need to revisit and expand our modern view on evolution.

During the process, I was surrounded by people kind enough to help or support at all times. First, I would like to thank Professor L. Brendonck, without whom this research would not have been possible and who's enthusiasm is truly inspiring. My gratitude also goes to my mentor, Arnout Grégoir, who has been an excellent coach during my mental gymnastics and who has spent quite a considerable amount of time helping me in the lab. I would also like to thank Charlotte Philippe, who helped me with, among other things, rearing the fish and who, when time seemed to be against us, cared for our water fleas as much as Arnout and I did. Furthermore, I thank the technical staff who helped us maintaining and improving our needed infrastructure.

As research not only asks for support in practical and intellectual context but from time to time also asks for social and perhaps even emotional support, I would like to thank friends and family who took excellent care of this. Primarily, I would like to thank my second half, Sarah Princen, who crossed boundaries by, in addition to taking tremendous care of me, also supported me and my work on an intellectual level. My gratitude also goes to my close relatives for listening to my stories (which are, obviously, at all times fascinating beyond thought) and, on a more serious note, who's sympathy has always been stimulating. For all my friends I didn't mention, you can either wither about it, or acknowledge the fact that I have the time nor the inspiration to write personal notes. What I mean is, thank you.

To all readers of this thesis, thank you for struggling through this part so far. I promise the text below is more interesting and I would strongly encourage you to read through the whole of it. Good luck, and have fun along the way!

In closing this preface and opening the door to science, I would like to, again, thank all of you. Science is a work of many hands.

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1 Introduction

1.1 Darwin vs. Lamarck

"Nothing in biology makes sense, except in the light of evolution." (Dobzhansky, 1973) This famous quote, originally the title of an essay by evolutionary biologist Theodosius Dobzhansky (1900-1975), is a popular opening sentence in many works on evolutionary biology[1,2]. The concept of biological evolution and its underlying mechanisms have, however, throughout history always been subject of controversy, as illustrated by the countless views on the matter and the numerous theories of evolution that have challenged the scientific community[3,4].

The currently accepted theory of evolution, the Modern Evolutionary Synthesis, is the result of a merge between the Darwinian vision on evolutionary change and Mendelian genetics[4], and was first coined in 1940 by Julian Huxley in his book *Evolution: The Modern Synthesis*. It states that phenotypic variation results from genetic variation arising through recombination and, ultimately, mutations[5]. This variation is encrypted in the DNA sequence and transmitted to the next generation[6,7], a process often referred to as hard inheritance[8,9]. Selection (e.g. natural selection) acts on this phenotypic variation and by doing so changes the frequency of alleles from genes under selection[6,7].

This vision, however, suggests that biological information is transmitted to next generations merely through the DNA sequence, and leaves no room for heritable phenotypic variation acquired in an organism's lifetime[10]. This contrasts with the Lamarckian vision on evolutionary change. Lamarck's first law, the law of use and disuse, states that a frequent and continuous use of an organ leads to a profound development of that organ, with the strength of this development being proportional to the time in which the organ has been used. Disuse of an organ weakens and degrades the organ until it has completely faded away. Use or disuse of an organ depends on the needs of the organism, as imposed by the environment. According to Lamarck's second law, on inheritance, acquired or lost characteristics from individuals are preserved in the individuals of the next generation ('inheritance of acquired traits')[11]. Long ridiculed, the second law has gained more attention in recent years, which we here refer to as neo-Lamarckism. Associated with Lamarck's ideas, Ernst Mayr proposed the term soft inheritance, often referred to as 'non-Mendelian inheritance' by evolutionary biologists[9] and defined as the transgenerational transmission of phenotypic variation that arises during the development. This phenotypic variation is due to other effects than variation encoded in the DNA-sequence[5,6,8], and can be adaptive as well as non-adaptive. In case of the latter, however, it is not regarded as (neo-)Lamarckian[8].

1.2 Systems of inheritance

In their book *Evolution in four dimensions*[12], Eva Jablonka and Marion Lamb claim the existence of four partially overlapping inheritance systems ('dimensions'), each providing variation as a substrate for natural selection[6,13,14]. The first dimension is a DNA-sequence based inheritance system (i.e. hard inheritance), confer the Modern Synthesis. In general, this genetic information is being transmitted between generations, from ancestor to descendants (vertical transmission)[10]. The three other dimensions are behavioural, symbolic and cellular epigenetic inheritance systems (see below and Fig. 1). All three involve transmission of non-genetic information across generations and are therefore regarded as soft inheritance systems[6,12–14].

Behavioural inheritance involves transmission of animal behaviour ('animal tradition') associated with observational social learning to subsequent generations[6,12]. A widely cited case of behavioural inheritance involves naïve Great tits (*Parus major* L.)[15], who learn to open milk bottles by observing experienced individuals[16].
In a study on mice, it was found that wild-type female offspring of mutant mothers with impaired maternal care exhibited impaired maternal care as well (e.g. increased neophobia, decreased exploratory behaviour and were unable to retrieve pups to a nest). Furthermore, also the third generation exhibited increased neophobia and reduced exploration, despite having wild-type mothers[17].

The **symbolic inheritance system** is largely restricted to humans and refers to thought-based systems such as language and music that enable humans to communicate and to transmit information among each other[6,12]. Cultural information units are coined as 'memes' by Richard Dawkins in his book *The selfish gene*, and are related to cultural evolution as are genes to biological evolution[18,19]. However, being based on neo-Darwinian models of evolution, memetics has been criticised for ignoring the influence of development on memes[13].

Finally, **cellular epigenetic inheritance** is an inheritance system from mother- to daughter cell in which the DNA-sequence remains unaltered, but in which the changes occur on a higher level mainly by regulating the expression-behaviour of genes[6,20,21], by means of a wide array of mechanisms (see 1.3). These changes may ultimately lead to phenotypic variation in the next generation of cells (or organisms) without genotypic variation[20,22]. Forms of a gene that are epigenetically regulated in a different way are often termed epialleles[23].

A distinction can be made between mitotically and meiotically heritable epigenetic variation, the latter here regarded as transgenerational cellular epigenetic inheritance. In the first case, epigenetic variation arises in somatic cells and can be transmitted to daughter cells during mitosis (cell-to-cell), possibly leading to phenotypic changes within one generation[20]. In this way, for instance, phenotypic plasticity - when defined as the ability of an individual organism to undergo environmentally-induced phenotypic changes[24], and when restricted to a non-transgenerational concept - can be related to this cell-to-cell epigenetic inheritance[22,25]. Another example holds the differentiation of cells within a multicellular organism during its development (ontogeny)[14,22,26]. In the second case, epigenetic variation occurs in the germ-line and can be transferred to the offspring during meiosis, provided that the epimutations are not reset following fertilisation[8,26,27]. This process has been found in an array of organisms (see Table 1), including humans. For instance, it was found that overeating during the slow growth period (just before puberty) of a male child increases the chance for cardiovascular and diabetes mortality in his grandchildren[28].

Table 1: Cases of transgenerational cellular epigenetic inheritance

Organism	Trait	Reference
Plants		
Yellow toadflax (*Linaria vulgaris*)	- flower symmetry	164
Common flax (*Linum usitatissimum*)	- flowering age	48
Thale cress (*Arabidopsis thaliana*)	- genomic flexibility (homologous recombination)	165
	- transmission of retroposon-epialleles	166
Fungi		
Fission yeast (*Schizzosaccharomyces pombe*)	- sexual compatibility (mating-types)	167
Animals		
Caenorhabditis elegans	- transgenerational gene-silencing by RNAi	168
Drosophila melanogaster	- ectopic outgrowth	169
Mice (*Mus musculus*)	- agouty phenotype (yellow fur, obesity, diabetes, tumour-susceptibility)	170
	- male fertility	171
	- β-cell dysfunction	172
	- behavioural sensitivity to conditioned odour, neural structure	47
Rats (*Rattus norvegicus*)	- mate preference	173
Humans (*Homo sapiens*)	- health and disease conditions	174, 175, 176, 177

The latter three dimensions (behavioural -, symbolic - and cellular epigenetic inheritance) fall under the banner of epigenetics[9]. There is however no consensus on how to define epigenetics and epigenetic inheritance, and the terms are commonly used in different ways. The term epigenetics originated in the field of developmental biology, where it is defined in a broad sense as the study of the processes behind the translation of the genotype into the phenotype[9,22]. In the field of evolutionary biology, epigenetics is strongly related to soft inheritance and is referred to as the study of inheritance of phenotypic variation that is not encoded in the DNA sequence[8,22,29]. Epigenetic inheritance, in turn, can be considered in a narrow sense including only cellular processes (cellular epigenetic inheritance), whereas when considered in a broad sense also non-cellular processes like behavioural inheritance and symbolic inheritance are taken into account[22]. Some authors also consider ecological inheritance as a consequence of niche construction[10,30] to be a form of epigenetic inheritance[22]. Where DNA-sequence based inheritance occurs mainly in a vertical manner, epigenetic inheritance can occur between generations as well as within generations, i.e. vertically, horizontally and obliquely (diagonally from generation 1 to generation 2)[10].

In this work, epigenetic inheritance is considered in its broad sense including all inheritance systems based on other than in the DNA sequence encoded phenotypic variation.

Figure 1: Illustrative examples of different systems of soft inheritance. Left: a Great tit learns to open a milk bottle (behavioural inheritance system). Middle: Egyptian hieroglyphs, a writing system as an example of symbolic inheritance. Right: schematic illustration of DNA methylation, a mechanism of cellular epigenetic inheritance. From left to right, images are obtained from http://www.telegraph.co.uk - http://wallpaperest.com/ - http://esrcgenomicsforum.blogspot.be/

1.3 Mechanisms of cellular epigenetic inheritance

Cellular epigenetic inheritance systems can be categorized in different types, mainly based on the molecular mechanisms involved.

A first example involves 'self-sustaining transcriptional feedback loops', where gene products regulate gene transcription in a direct or an indirect manner. These gene products can be transmitted to daughter cells over many generations, where they induce the same effects as in the parental cells and result in a similar phenotype[6,31].

A second type is 'structural inheritance', in which self-perpetuating cellular structures are being transmitted to daughter cells. This type comprises different mechanisms of which prion-based inheritance forms a comprehensive example[6]. Prions are proteins that are able to exist in different conformations, each being structurally and functionally different, and of which at least one conformational state acts as a template for conformational conversion of other proteins of the same type, thus managing their own replication[32–34].

Chromatin marks constitute another type of cellular epigenetic inheritance system[6]. Chromatin is a complex of DNA and histone proteins forming nucleosomes as fundamental, repeating subunits in the nucleus of eukaryotic cells. Two different states are distinguished: "active" domains (euchromatic) and "silent" domains (heterochromatic), both representing another expressive – and conformational state of the chromatin[35–37]. Conformation of chromatin is determined by chromatin marks including methylation or acetylation of the DNA[38,39], the so far best-known epigenetic mechanisms[21], as well as proteins non-covalently bound to the DNA[6]. The latter includes modifiable histone proteins, e.g. by histone-acetylation[40]. Chromatin marks, and therefore the pattern of chromatin expression state, can be transmitted to daughter cells[6]. This way, cells sharing the same DNA sequences can show phenotypic differences nonetheless[41].

A fourth type of cellular epigenetic inheritance system involves RNA interference[6]. Genes that are being transcribed yield mRNA molecules, which are then being translated to corresponding proteins[42]. However, small, noncoding RNA strands may interfere by interacting with the mRNA molecules or with their associated DNA regions (potentially leading to heritable chromatin marks[37]) and thereby influence gene-expression, either by up- or downregulation[6,37,43,44]. Especially the latter two types of epigenetic inheritance systems (i.e. chromatin marks and RNA interference) seem to be important in meiotic epigenetic inheritance (i.e. through the germ-line)[14].

1.4 Environmentally induced epigenetic variation

Variations in epigenotype are brought about by genomic stress[23], including stochastic processes[14] like spontaneous mutation, developmental signals[8] or by environmental changes[22,25,45]. Environmentally induced epigenetic changes have for instance been demonstrated in inbred mice. Prior to and during pregnancy, female mice feed was supplemented with methyl, which led to an increase in DNA methylation. In turn, this altered the expression behaviour of the agouti gene, leading to offspring with significant phenotypic differences (in coat colour, health and longevity, see Fig. 2) compared to control offspring[46].

A recently discovered example involves the inheritance of parental traumatic exposure. Offspring up to at least two generations of mice that were subjected to odour fear conditioning – in which they were learned to associate a scent, acetophenone, with pain – exhibited an increased sensitivity to acetophenone despite never having been exposed to the scent before. It was found that the DNA sequence of the inherited Olfr151 gene, which encodes for the acetophenone-activated receptor, had not changed. However, the gene underwent CpG hypomethylation, leading to its increased expression[47].

Figure 2: A wild-type mouse (left) compared with the agouty-phenotype (right). (Image obtained from http://jaxmice.jax.org/)

In yet another study, flax plants were treated with 5-azacytidine, altering DNA methylation. Phenotypes, including plant height and flowering age, of up to four generations of offspring were significantly affected[48]. Also antipredator defences were found to potentially be transmitted to offspring. Agrawal *et al.*[49] found that *Daphnia cucullata* non-lethally exposed to carnivores, and wild radish plants (*Raphanus raphanistrum*) exposed to herbivores expressed an induced antipredator defence that was subsequently being transmitted to their offspring.

The growing findings and insights concerning soft inheritance are in favour of Lamarckian evolutionary theory and ask for an extension of the current Modern Synthesis[4,12,50]. However, despite the scientific consensus about the existence and commonness of non-genetic heritable variation[10,14], not all authors agree on the claim that current findings pose a challenge to the Modern Synthesis and believe there is need for a theoretical extension[51]. Moreover, little is known about the evolutionary importance of transgenerational epigenetic effects so far and there is no consensus about its role in adaptation and speciation[22,25].

1.5 Within-generation phenotypic plasticity

1.5.1 What is phenotypic plasticity?

In contrast with transgenerational epigenetic effects, the evolutionary role of phenotypic plasticity has been studied more frequently. As mentioned above, (within-generation) phenotypic plasticity can be regarded as a result of the transmission of epigenetic variation from cell-to-cell (via mitosis)[25]. It is defined as the ability of a single genotype to express different phenotypes, depending on the prevailing environmental conditions[24,52].

Different environmental stimuli can lead to phenotypic changes, e.g. in behaviour, physiology, morphology or life-history[24,53]. These novel traits may result in a higher fitness and hence be adaptive[24,54], as is often the case with so called active phenotypic plasticity[53]. Active phenotypic plasticity involves complex phenotypic changes in response to changing environmental conditions coordinated by multiple regulatory genes and modified developmental pathways, and are most often anticipatory to the changing environment. In contrast, passive phenotypic plasticity involves phenotypic changes that are merely a direct consequence of being exposed to the environmental stresses, e.g. a nutrition-poor environment might result in slim or small-sized individuals[53,55]. There is however a thin line between active and passive phenotypic plasticity as they constitute most forms of phenotypic plasticity together and interact with each other[55].

An example of phenotypic plasticity involves the exposure to predation risk, i.e. predator-induced phenotypic plasticity[57,56]. Predator-induced phenotypic plasticity of behavioural, physiological, morphological or life history traits has been demonstrated in an array of organisms[56]. Tadpoles of the Common frog (*Rana temporaria*) were found to exhibit a decreased feeding and swimming activity and an increase in hiding responses, i.e. antipredator behaviour, in presence of predators[58]. Similarly, Dionne *et al*.[59] demonstrated an increased hiding behaviour in damselfly larvae exposed to predatory Pumpkinseed sunfish (*Lepomis gibbosus*). Behavioural responses were also found in Striped killifish (*Fundulus majalis*) exposed to predation risk from Sand seatrout (*Cynoscion arenarius*). Moreover, the exposed killifish were found to exhibit increased plasma cortisol levels, increased mass-specific oxygen consumption rate and a decrease in short-term growth[60]. In another study, Leopard frog tadpoles (*Rana pipiens)* exposed to caged, predatory dragonfly larvae (*Aeshna spp.*) were found to grow larger and exhibited a taller tail fin compared to the control condition[61]. Finally, Skelly and Werner[62] demonstrated that larval American toads (*Bufo americanus*) reduced their activity, exhibited reduced growth rates and metamorphosed at smaller sizes in presence of predatory *Anax junius*.

1.5.2 Phenotypic plasticity and evolution

When the environmental conditions change, a genotype might be able to express a novel phenotype without any required genetic changes, exposing its capacity for phenotypic plasticity[52]. This is often illustrated by means of genotypic reaction norms[52,55]. If natural selection in the changed environment favours the novel phenotype and persists, the novel trait may become genetically fixed (i.e. 'genetic assimilation') in the population; this means the trait is expressed regardless of any provoking environmental stimuli. Moreover, the capacity for phenotypic plasticity may decrease (i.e. the reaction norm becomes less steep) due to genetic drift or due to costs related to the maintenance of plasticity: in an environment without any selection favouring phenotypic plasticity, it will disappear from the population[52,54].

A classic example of genetic assimilation was demonstrated by C. H. Waddington[63]. A thermal shock in the pupal stage of *Drosophila melanogaster* led to the expression of the crossveinless wings phenotype (see Fig. 3), which is considered to be an example of stress-induced non-adaptive phenotypic plasticity[52]. After 14 generations of artificial selection in favour of this phenotype, it was found to be expressed without applied heat shock, i.e. the trait was found to be genetically fixed or assimilated[52,63,64]. Although this looks like the inheritance of an acquired character (see 1.1 Darwin vs. Lamarck), Wallace Arthur states that *"genetic assimilation looks, but is not, Lamarckian. It is"*, he said, *"a special case of the evolution of phenotypic plasticity"* (Arthur, 2011, p23) and can be explained in standard Darwinian terms[52].

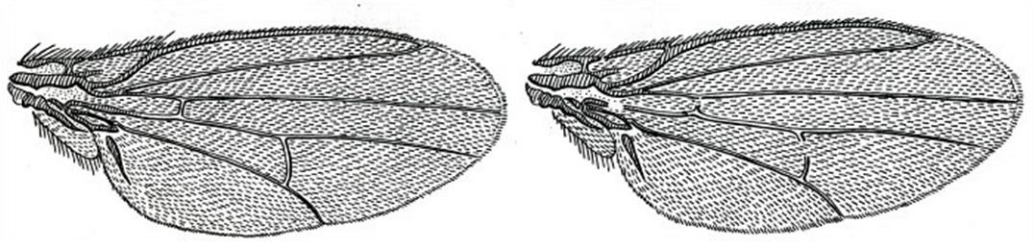

Figure 3: Right wing of the fruit fly (*Drosophila* sp.). Left: a normal *Drosophila*-wing has two cross veins. Right: crossveinless *Drosophila* have broken cross veins (different grades of severity exist). (Image obtained from http://bulbnrose.x10.mx/Heredity/Waddington/Characteristic/)

High levels of phenotypic plasticity increase the probability of population persistence in the changed environment by providing the population an open road to a new adaptive peak in the adaptive landscape. Nevertheless, higher levels of phenotypic plasticity also reduce the probability of genetic change or evolution, because a new adaptive peak has already been reached by means of the plasticity itself and subsequent directional selection towards an adaptive peak is no longer required. Hence, there is no adaptive genetic differentiation between the initial population and the population under the novel conditions. If, on the other hand, the capacity for phenotypic plasticity is too limited, the population might never reach a new adaptive peak and go extinct, or stagnate on a sub-optimal adaptive peak.

Intermediate levels of phenotypic plasticity increase the probability of population survival in the changed environment by bringing the population closer, but not all the way to, a new adaptive peak. Subsequent directional selection, favouring the extreme phenotypes, leads the population further towards the adaptive peak. This way, intermediate levels of phenotypic plasticity may facilitate genetic evolution[24].

The adaptive role of phenotypic variation is however dependent on the variability and predictability of the environment. Plastic responses can be maladaptive in unpredictable environments[25,65]. In rapidly changing environments, populations with very high levels of plasticity were found to have a significantly higher extinction probability compared to populations with less plasticity[65].

1.6 Transgenerational phenotypic plasticity

Phenotypic plasticity can also be regarded as a transgenerational mechanism instead of being considered within just one generation, requiring non-genetic (epigenetic) inheritance. Similar to within-generation plasticity, in a novel environment transgenerational nongenetic inheritance can bring a population in the vicinity of a new adaptive peak, followed by directional selection towards the fitness optimum. Eventually, the environmental stimuli leading to the expression of the novel phenotype are no longer required, i.e. the trait is genetically assimilated[25,66]. Jablonka *et al.*[67] demonstrated that non-genetic inheritance is potentially more advantageous than within-generation plasticity. Parents can anticipate on future environmental conditions by producing offspring that is immediately adapted to its own environment, thereby avoiding the offspring's lag-phase between assessing the environmental conditions and expressing the novel phenotype that is associated with within-generation plasticity[25,67]. However, similar to within-generation plasticity, transgenerational phenotypic plasticity can be disadvantageous in rapidly changing and unpredictable environments. When the parental environment is a poor predictor of the environmental conditions of the next generation, non-genetic information transmitted to the offspring can be maladaptive in their actual environment[25,68].

In conclusion, non-genetic inheritance influences phenotypic change as well as genetic change. Evolution is thus not based solely on genetic inheritance, but on an expanded context of inheritance, covering genetic inheritance as well as non-genetic inheritance[25,69]. This raises the question of what the relative importance of transgenerational epigenetic heritability, within-generation phenotypic plasticity and genetic heritability is on the process of adaptation and adaptive radiation in natural populations. Anthropogenic impact on the environment and global change in relation to nature- and biodiversity conservation stress the importance of this question.

Excellent study systems to investigate this matter are species with (genetically) isolated populations that have a low standing genetic variation and are subject to a fluctuating but predictable environment. One such a study system is the African killifish, particularly the widespread *Nothobranchius furzeri*[70]. To date, this species has been subjected to genetic and genomic analyses[71] and a highly homozygous laboratory strain (GRZ-strain) has been maintained[71–73].

In addition to *N. furzeri*, also *Daphnia*, a well-studied freshwater zooplankton genus[74], proves to be a valuable system in investigating phenotypic plasticity and the (epi)genetic heritability of traits, especially due to their short generation-time and their ability to create clonal (i.e. genetically identical) lines[75]. In the following, both study systems (*N. furzeri* and *Daphnia* sp.) are discussed separately.

1.7 Study system – *Nothobranchius furzeri*

1.7.1 *N. furzeri* in general

Nothobranchius is a genus of annual fishes within the Nothobranchiidae family (Teleostei, Ordo Cyprinodontiformes)[76] from eastern and southern Africa[70,77,78]. All *Nothobranchius*-species show a strong sexual dimorphism (dimorph and dichromatic) with robust and colourful males and smaller pale females[70,79,80]. *Nothobranchius* inhabits isolated, ephemeral freshwater pools[81,82] that desiccate during the dry season (Fig. 4). When the habitat dries out, all adults die[77]. These fishes are however adapted to this seasonal habitat disappearance by producing desiccation-resistant eggs which are deposited in alluvial vertisol soils[70,78,83,84], allowing the resident population to bridge the dry period[77,85,86].

The eggs are dormant, with three successive facultative stadia that are easily distinguishable[86,87]. After the onset of the new rainy season, the pools fill and the eggs hatch[86]. Within only a few weeks of rapid growth, the fish reach sexual maturity and start to produce new desiccation-resistant eggs that will survive the next dry season[70,77,84].

Figure 4: An isolated, ephemeral freshwater pool as habitat of *N. furzeri*. (Image obtained from http://www.practicalfishkeeping.co.uk/)

Due to the seasonal desiccation, the post-hatching natural lifespan of the fish is limited to only a couple of months[72,77]. *N. furzeri* (Turquoise killifish) is the vertebrate with the shortest recorded captive lifespan; the current laboratory strain, GRZ, was found to have a maximal post-hatching lifespan of only three months[77,82,88], comparable to that of wild *Drosophila*[84], and a median lifespan of nine weeks[77,86]. Wild strains of *N. furzeri* were found to exhibit a maximal lifespan of 25 to 32 weeks and a median lifespan of 20 to 23 weeks in laboratory conditions[77], reaching sexual maturity within three to four weeks[79,84,86,88]. The short lifespan of *N. furzeri* makes this species a convenient model system for drug screening and ageing research[70,82,84]. Moreover, lifespan can be modulated by means of water temperature and resveratrol, a natural phytoalexin produced by several plants including grapevines[82,84,89,90]. Valenzano *et al*.[89] demonstrated that lowering the water temperature from 25°C to 22°C increased the median (from nine to ten weeks) and maximum (from eleven to twelve and a half weeks) lifespan of *N. furzeri* and reduced the accumulation of lipofuscin in the liver, a commonly used histological age marker. Moreover, locomotor and learning deficits related to age were delayed in the low-temperature condition compared to the high-temperature condition. In another study, Valenzano *et al*.[90] demonstrated that resveratrol increased both the median and maximum lifespan of *N. furzeri* in a dose-dependent manner, with respectively a 56% and 59% increase in the condition that received the highest amounts of resveratrol (600 µg/g food). Similar to the effects of lowered temperature, age-related locomotor and learning deficits were delayed in fish treated with resveratrol. Furthermore, resveratrol-treatment reduced neurofibrillary degeneration in the brain compared to control fishes.

N. furzeri inhabits pools (often shallow) with high turbidity and soft substratum[70], separated from rivers[83]. Few other teleost fish species have been recorded to occur in sympatry with *N. furzeri*: *N. orthonotus*, *N. rachovii*, small members of the Cyprinidae, North African catfish (*Clarias gariepinus*) and West African lungfish (*Protopterus annectens*)[70,83]. Most populations of *N. furzeri* appear to be female biased[70]. This is possibly caused by a higher extrinsic mortality risk for males compared to females, since no differences in mortality rate between males and females have been reported under laboratory conditions, nor any biases in sex ratio[70,84,88]. The higher mortality risk for males might be due to costs associated with male colouration for sexual selection[70,91,92], cf. Zahavi's Handicap Principle[93], or due to male-male competition for spawning grounds and associated aggression[70,85].

The natural distribution of *N. furzeri* covers parts of Zimbabwe and Mozambique[70]. Dispersion capacity of *N. furzeri* is limited to occasional flooding events due to high rainfall[83]. Also, large mud-wallowing mammals or waterbirds might possibly serve as a transport vector of dormant eggs in mud[83,94]. In 1968, the species was collected in (and described from) the Gona Re Zhou National Park in south-eastern Zimbabwe along the border of Mozambique[70,77]. The current inbred laboratory strain GRZ originated from this collection, and has been maintained (and kept as a pure line) by hobbyists for approximately 90 generations so far, if the generation-time is estimated as 6 months[77]. Three new *N. furzeri* populations were discovered in 1999 in the Limpopo River basin in southern Mozambique, and were at first considered to be a new, yet undescribed species[70]. Males from this populations all exhibited a red caudal fin, while previously collected *N. furzeri* from Gona Re Zhou all exhibited a yellow vertical stripe at the caudal fin[70,95]. Subsequent captive generations produced red as well as yellow phenotype males, and were hence considered to be different colour morphs of *N. furzeri* (Fig. 5)[70,79]. Ever since, new populations (with yellow morphs as well as red morphs) throughout southern Mozambique have been discovered[70].

Figure 5: Yellow (left) and red (right) colour morph of male *N. furzeri*.

In Mozambique, *N. furzeri* populations occur along an aridity gradient, mainly determined by the annual amount of precipitation and evaporation. Combined with basin properties like size and shape, these factors determine the inundation length of the ponds[72,77]. In southern and central Mozambique, the inland region receives less annual precipitation (400 mm/year) than more coastal regions (1200 mm/year) (Fig. 6). The inland, arid region is characterised by pools with a short inundation length while more humid regions are characterised by pools with a longer inundation length[72]. The maximum lifespan of the resident fish population is determined by the inundation length, and thus the duration of the ephemeral pool[77].

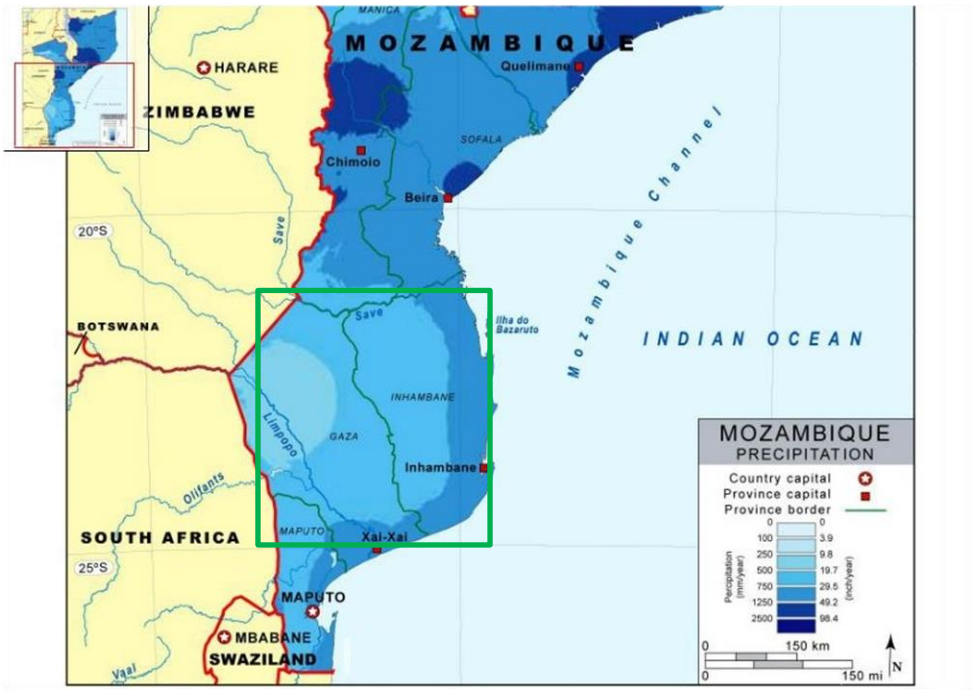

Figure 6: Precipitation map of southern and central Mozambique. Green box: our study area. (Obtained from Stock Map Agency; http://www.stockmapagency.com).

1.7.2 *N. furzeri* and the evolutionary theories of ageing

Evolutionary theories of ageing state that the evolution of senescence is a consequence of a lower selection pressure later in life[88,96,97], often referred to as the selection shadow[98]. Senescence is defined as a decrease in an individual's fertility with increasing age, along with an increase in the intrinsic mortality[99–101]. Two non-mutually exclusive main models of the evolution of senescence have been proposed: the 'Mutation Accumulation Hypothesis' by Medawar[102] and the 'Antagonistic Pleiotropy Hypothesis' by Williams[96,98,103]. Both hypotheses are based on individual fitness and selection[98] and differ from previous theories, e.g. the Theory of Programmed Death by Weissmann[104], in that the latter regards the evolution of senescence as an adaptation to life since senescence (and eventually death) allows older individuals to be replaced by younger, fecund individuals (i.e. group selection)[96,98].

Both main models predict that populations experiencing lower extrinsic mortality (i.e. ecological mortality due to for instance predators or diseases) will evolve less senescence, i.e. the onset of senescence will be delayed[77,105,106]. In support of the evolutionary theories of ageing, Blanco and Sherman[107] found that poisonous and venomous species of fish, amphibians and reptiles (experiencing lower extrinsic mortality) in general had longer maximum lifespans than non-poisonous or –venomous species (experiencing higher extrinsic mortality). Despite many empirical studies in favour of the theories, their generality has been questioned[77,105,106,108]. When more factors are being accounted for, for instance the fact that susceptibility to extrinsic mortality might differ between age classes[72,106], higher or lower extrinsic mortality can be predicted to lead either to the evolution of an earlier onset of senescence, a delayed onset of senescence or have no influence on the evolution of

senescence[105,106,108]. While Reznick *et al.*[109] found that, in accordance to the classic evolutionary theories of ageing, populations of guppies (*Poecilia reticulata*) originating from habitats with higher predation- or mortality rates reached maturity earlier in life and allocated a higher proportion of their energy resources to reproduction, a subsequent study found no effects on the lifespan, nor on the span of the reproductive period of these populations[77,105].

In natural populations of *N. furzeri*, the intensity of extrinsic mortality pressure caused by seasonal desiccation of the ponds abruptly reaches 100% and does not differ between populations[72]. However, the timing of the desiccation (and thus the occurrence of the extrinsic mortality event) differs. As described above, the timing of this event is earlier in populations inhabiting arid regions, while it occurs only later in life in populations inhabiting more humid regions[72,77]. In accordance to the classic evolutionary theories of ageing, populations from arid regions are expected to evolve an earlier onset of senescence and thus a shorter life span, while populations from more humid regions are expected to evolve a longer life span. The short longevity of *N. furzeri* was found to be a natural characteristic of the species, i.e. their short lifespan was conserved in laboratory conditions[77,84]. Moreover, it was found that populations from arid regions exhibited a shorter lifespan than populations from more humid regions, confirming the predictions of the classic evolutionary theories of ageing[72,77].

1.8 Study system – *Daphnia* sp.

Daphnia is a cosmopolitan genus of more than 100 freshwater zooplankton species within the Daphniidae family (Subphylum Crustacea, Ordo Cladocera)[74,110,111], commonly referred to as water fleas[112]. Some well-known representatives of the genus include *D. magna* (Fig. 7) and *D. pulex*[111]. *Daphnia spp.* inhabit a range of freshwater bodies, such as rivers, lakes and small temporary ponds[111,113] and are generally found in the pelagic zone[111]. Most *Daphnia* species reproduce both sexually and asexually through cyclic parthenogenesis[75,111,114]. During the growth period, as long as the environmental conditions remain favourable, females produce diploid eggs asexually; this mode of reproduction is termed ameiotic parthenogenesis and gives rise to genetically identical offspring (clones)[74,75,111]. The eggs hatch in the brood chamber of the mother, where they develop further before being released, which is generally after three days. Most daphnids reach maturity (i.e. are able to produce a clutch of eggs) within four to six juvenile instar stadia, generally around an age of five to ten days in favourable conditions[111]. Clutch sizes vary between species, from only a couple of eggs in *D. cucullata* up to more than 100 eggs in *D. magna*, and can be produced every three to four days[111,115].

At the onset of unfavourable conditions associated with environmental change, daphnids will reproduce sexually in order to produce resting eggs that are able to survive the harsh period and hatch as soon as the conditions turn favourable again. In this mode of reproduction, males are produced asexually through ameiotic parthenogenesis and fertilise sexually produced haploid eggs[75,111]. Once fertilised, the resting eggs become encapsulated in a so-called ephippium, a hard shell which protects the eggs during their diapause[74,75,111]. Being viable for over 100 years[116], the eggs constitute an egg bank (similar to seed banks in plants) in lacustrine sediments[117–119]. This characteristic provides a record of the evolutionary history of local natural populations and allows for reconstruction of the past environmental conditions by retrieving ancestors or ancestral DNA (resurrection ecology)[118,120,121].

Figure 7: Adult female of *D. magna*. (Photograph taken by Hajime Watanabe, obtained from http://journals.plos.org/plosgenetics/)

Due to its short generation-time, easy cultivation, the formation of a persistent egg bank and the ability to create clonal (i.e. genetically identical) lines, *Daphnia* sp. is an attractive study organism in many research fields including ecology, ecotoxicology, evolutionary biology and evolutionary developmental biology[75,110]. For instance, *Daphnia* sp. have often been used as study system to investigate phenotypic plasticity such as antipredator responses. A series of antipredator responses has been recorded in multiple species of *Daphnia*[112,122], from morphological modifications such as the formation of neck spines[111,123] or cranial extensions known as 'helmets'[49], to changes in behaviour such as phototactic behaviour[124,125], and life-history traits such as age at maturity and fecundity[74,126,127]. Moreover, *Daphnia* sp. is an emerging model organism in epigenetic research, more specifically in the study of transgenerational epigenetic effects[128].

It is worth noting that, while maternal effects are in essence transgenerational epigenetic phenomena (given that epigenetics is defined in a broad sense, see 1.2 Systems of inheritance), maternal effects have been well documented in *Daphnia*[114,129] but have only recently gained more attention in the light of epigenetics[130]. For instance, Agrawal *et al.*[49] found that the formation of helmets as an induced antipredator response in kairomone-exposed (exposed to chemical cues from fish) *D. cucculata* was being transmitted to subsequent, non-exposed generations. Similar results have been found in studies focussing on life-history traits such as growth and fecundity[112,131]. Also, chemicals like 5-azacytidine proved to decrease DNA methylation in exposed daphnids, an effect that was found to be transferred to subsequent, untreated generations[131].

1.9 Aims and hypotheses

In this thesis, we aim to assess the relative contribution of within-generation phenotypic plasticity, genetic inheritance and epigenetic inheritance to natural variation in life-history traits in two distinct study organisms, the vertebrate *Nothobranchius furzeri* (but see Materials and Methods) and the invertebrate *Daphnia magna*, by comparing covariances between relatives (see Materials and Methods for details). For *N. furzeri*, we use four natural populations originating from well-documented pools with different desiccation regimes, while for *D. magna*, we use four clones originating from sediment from a period with relatively low fish predation pressure and four clones originating from the same pond but from a period with high fish predation pressure. In addition, we investigate possible predator-induced phenotypic plasticity on life-history traits by non-lethal exposure to predatory Pumpkinseed sunfish (*Lepomis gibbosus*), both within and across generations.

We expect that, in *N. furzeri* as well as in *D. magna*, the observed variation in life-history traits will be partly due to epigenetic phenomena given their commonness in nature[14]. In addition, the setup of the *Daphnia* experiment enables us to assess the relative contribution of genetic inheritance and epigenetic phenomena to the observed phenotypic variation along an 'epigenetic gradient' (parent vs. offspring, among offspring, niece vs. niece, grandniece vs. great-aunt, grandniece vs. grandniece) while keeping the genetic factor constant since they all are genetically identical. Given there is one epigenetic reset generation between parent and offspring, two reset generations between full siblings, four reset generations between nieces, five reset generations between grandniece and great-aunt and six reset generations between grandniece-grandniece, we expect the covariance to decrease from parent-offspring to grandniece-grandniece (same order).

We hypothesise that both *N. furzeri* and *D. magna* will respond to the perceived predation risk with adaptive shifts in life-history traits. More specifically, *N. furzeri* might reach maturity earlier and at smaller sizes, and produce more offspring, a response documented in many species including species of fish[109,132]. Similarly, *D. magna* might respond with a higher fecundity, earlier maturation and smaller size at maturation, consistent with results from previous studies[112,122].

In addition, we expect populations of *N. furzeri* and clones of *D. magna* that have a history of (intense) predation exposure to exhibit on average a stronger antipredator response due to a higher degree of exposure than do populations or clones with a history of no or low predation pressure. This hypothesis is supported by results of previous studies[74,124].

Moreover, as there is increasingly more evidence of predator-induced transgenerational phenotypic plasticity, we might observe a shift in life-history traits in naive offspring of kairomone-exposed individuals.

Aside from our hypotheses and expectations about phenotypic plasticity and epigenetic inheritance, we expect for *N. furzeri* to observe a shorter maturation time and lifespan for populations originating from pools with a strong desiccation regime (i.e. short-lived pools) relative to populations originating from longer-lived pools. This expectation is based on the hypothesis that populations from short-lived pools have evolved an earlier onset of senescence due to earlier desiccation of the pool (an event of 100% extrinsic mortality) in accordance with the classic evolutionary theories of ageing. These predictions have been confirmed by previous studies on *N. furzeri*[72,77].
Similarly, clones of *D. magna* from a high-predation habitat (high extrinsic mortality) might exhibit a shorter maturation time (and lifespan, although not assessed in this study) than clones from a low-predation habitat (lower extrinsic mortality), in accordance with the classic evolutionary theories of ageing.

2 Materials and methods

2.1 *N. furzeri* experiment

2.1.1 General setup of the experiment

Natural populations of *N. furzeri* occur along an aridity gradient in Mozambique. In this experiment, fish originating from four different, well-documented pools were used (Fig. 8). Two of these populations (NF222 and NF414) originate from two different shorter-lived pools in a more arid region of central Mozambique while the other two populations (NF2 and NF121) originate from two different longer-lived pools in a more humid region of southern Mozambique. Due to flooding events, three of these pools (NF414, NF2 and NF121) are frequently in contact with adjacent rivers while NF222 inhabits a more isolated pool that most probably never makes contact with rivers.

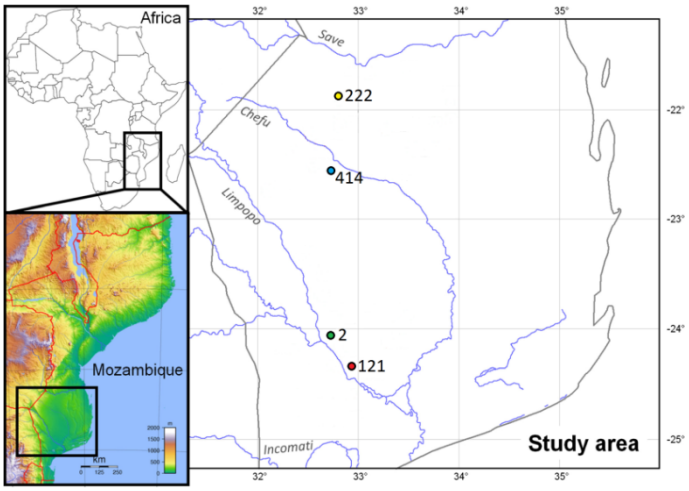

Figure 8: The four different original populations of *N. furzeri* used in this experiment (NF2, NF121, NF222, NF414). (Modified from Bartáková *et al.*[83])

The experiment comprised two conditions, a control condition and a predator condition (see Table 2). In the control condition, killifish were housed in 20L aquaria without exposure to predatory Pumkinseed sunfish, while in the predator condition, killifish were housed in aquaria in which they were exposed to visual and olfactory cues of sunfish for four hours/day. (Note that below, for practical matters, 'kairomone-exposed individuals' refers to individuals that were exposed to olfactory as well as visual cues in case of *N. furzeri*.)

Each aquarium had one big compartment (approximately 8L); neighbouring to this compartment were three smaller compartments (each approximately 4L) with an individual killifish in each of the three compartments. By using non-transparent plastic partitions, the killifish were not able to see each other. In the predator treatment, the big compartment housed one Pumpkinseed sunfish (for four hours a day), separated from the killifish-compartments by means of a transparent and perforated, plastic partition.

This way, the killifish were exposed to visual and olfactory cues of the predatory sunfish, without being exposed to actual predation. In the control condition there were no predators present in the aquaria, but the arrangement of each aquarium was the same as in the predator condition.
Pumkinseed sunfish were fed frozen *Chironomus* sp. larvae *ad libitum* prior to being transferred to killifish-aquaria.

Table 2: Sample sizes of the first generation of *N. furzeri*. Fish that died before all fish had reached maturity, and hence could not participate in breeding (see below) are excluded from the sample. Due to abnormally high juvenile mortality in NF121, this population is excluded in analyses. Also NF2 is excluded in analyses due to abnormally high mortality and extremely low fecundity.

	Predator treatment		Control	
	Number of ♂	Number of ♀	Number of ♂	Number of ♀
NF222	12	10	13	9
NF414	3	5	7	5
NF2	4	6	13	8
NF121	5	1	0	2

2.1.2 Fish maintenance

Fish were kept in a climate-controlled room at a constant air temperature of 24°C and a 14 h: 10 h light:dark regime.

At the onset of the experiment, eggs deposited by adults of the second generation kept in captivity were hatched by submerging the eggs, together with the peat they were diapauzing in, in slightly-aerated hatching medium (50% demineralised water and 50% dechlorinated tap water) at a temperature of 14°C. Eggs that were ready to hatch were recognized by their clearly-visible, well defined eyes.

Newly-hatched fry were housed individually in 250mL containers and were fed *ad libitum* three times a day with *Artemia fransiscana* nauplii. In order to prevent growth of fungus, one apple snail (*Pomacea* sp.) was added to each jar to feed on the excess of food. In addition, every jar received a little piece of filter material as a substrate for bacteria that are able to converse harmful ammonia into nitrite and nitrate. Levamisole (in order to expel intestinal worms, 1mL of 5% solution per 3.8L), Flubendazole (in order to prevent parasitic worms and fungus, 2mg/L) and a FMC solution (as an antiseptic and an aid in respiration, see 2.3) were added to the water, along with a pinch of natriumchloride (NaCl) in order to prevent infection by *Oodinium* sp. At day five, fish were moved to individual 1L containers. At an age of fourteen days, the fish were transferred to compartmentalized 20L aquaria filled with dechlorinated tap water, supplemented with one tablespoon of salt/5L and equipped with an air-driven sponge filter.

Starting from an age of 3 weeks, fish were weaned with finely chopped *Chironomus* sp. larvae twice a day and *Artemia* nauplii once a day. Onwards from week four, the fish were fed *ad libitum* frozen *Chironomus* larvae two times per day. All fish were housed together with two apple snails to clear away any excess food.

From day five onwards, each jar/aquarium was cleaned three times a week by suctioning away all dirt from the bottom. 25% of the original water was retained and supplemented with dechlonarinated water. NaCL concentrations were kept constant.

2.1.3 Assessing life-history traits and breeding the fish – first generation

Several key life history traits were quantified. To quantify growth, fish were photographed at different ages (5, 7, 9, 12, 14, 16, 19, 21, 23, 26, 29, 34, 37, 41, 44, 47, 51, 54, 58, 62, 69, 76, 79, 107, 120, 134 & 145 days). For this, fish were placed in a small amount of water to prevent movement in the vertical plane and photographed from above over mm paper. Pictures were analysed using the open source *Analyzing digital images* software. For each fish, this was done over the whole course of its life, yielding individual growth curves.

Secondly, lifespan of each individual fish was recorded. Next, for maturity, different criteria were used for males and females. For males, we scored the age at which colouration of the fins appeared. For females, we recorded the age at which the first egg was produced. To that end, different (complementary) methods were applied. First, females were provided with a spawning substrate (fine white sand), allowing them to spawn eggs even in the absence of males. Until maturation was ensured in females, this sand was sieved and checked for eggs daily. In general, however, gravid females only deposit eggs when stimulated by an adult male. Therefore, in addition, starting from week six until maturity was confirmed, every fish - except for mature males - was placed in a 1L jar with a bottom layer of sand together with an older, not experimental adult male individual for 30 minutes in order to stimulate potential egg deposition. This was done three times a week, and after every spawning session the sand was sieved and checked for eggs.

Starting from when a female had matured, females were allowed to spawn with a male of the same population and treatment for three times per week and 21 times in total. This was done by putting each female fish in individual 1L jars (with sand substrate) together with one male for one hour. Individual males and females were coupled in a semi-random fashion so that at the end of the experiment every possible male-female combination had been made. After every spawning session, the sand was sieved and the eggs were retrieved and counted.

Per couple, eggs were then transferred to damp peat in a petri dish. Next, the petri dish was sealed with parafilm in order to prevent desiccation during storage, and incubated at 27°C until the eggs were fully developed and ready to hatch. During the first weeks of incubation, all eggs were inspected daily so to remove dead, mouldy eggs and restrain them from infecting other eggs.

2.1.4 Assessing life-history traits – second generation

Due to a technical defect during incubation, the eggs produced by the first generation were unable to hatch and hence the experiment lacks the necessary second generation to assess (epi)genetic heritability's. However, possible antipredator responses (within-generation phenotypic plasticity) of life-history traits could still be assessed.

2.2 *D. magna* experiment

This experiment allowed for the assessment of the relative contribution of genetic inheritance and epigenetic phenomena to the observed variation in life-history traits as well as for the assessment of possible antipredator responses. First, a basic theoretical background - based on Tal *et al*.[133] - with regard to the method of the first aim is presented.

2.2.1 Theoretical background – covariance between relatives

Heritable phenotypic variation can be assigned to the inheritance of genetic variation and the inheritance of epigenetic variation. Comparison of covariances between relatives can be used as a tool to assess the relative contribution of genetic and epigenetic effects, a method presented in Tal *et al*.[133]. When assuming asexual, asymmetric reproduction, the genetic relatedness between parent and offspring as well as among offspring equals one (or 100%, see Fig. 9), i.e. they are genetically identical. During cell division and the early development of the offspring, however, part of the chromosomal parental epigenetic information is randomly reset. This means that although the genetic relatedness between relatives equals one, relatives are at least partly epigenetically different from each other. In this respect, Tal *et al*.[133] introduced 'the reset coefficient (v)' which refers to the probability in which the epigenetic state is altered between generations, or analogue, to the portion of epigenetic information that is restrained from transmission to the next generation, and made the simplifying assumption the reset coefficient to be a constant in the population. If v equals one, then the complete epigenetic state is altered and no parental chromosomal epigenetic information is transmitted to the next generation. If v equals zero, then the epigenetic state has not been altered and all of the epigenetic information is transmitted to the next generation. Complementary, Tal *et al*.[133] introduced 'the coefficient of epigenetic transmissibility ($1 - v$)', which refers to the probability in which the epigenetic state is transmitted to the next generation, or analogue, to the portion of epigenetic information that is transmitted to the next generation.

In the case of asexual reproduction, the covariance between parents and offspring should equal the covariance among offspring when assumed the transmission of variation is of a purely genetic nature. However, when transmission of epigenetic variation is taken into account, the covariance between parents and offspring should exceed the covariance among offspring since the number of random epigenetic reset generations between parent and offspring and between full siblings (i.e. among offspring) is one and two respectively (see Fig. 10). In other words, the epigenetic dissimilarity among offspring exceeds the epigenetic dissimilarity between parent and offspring.

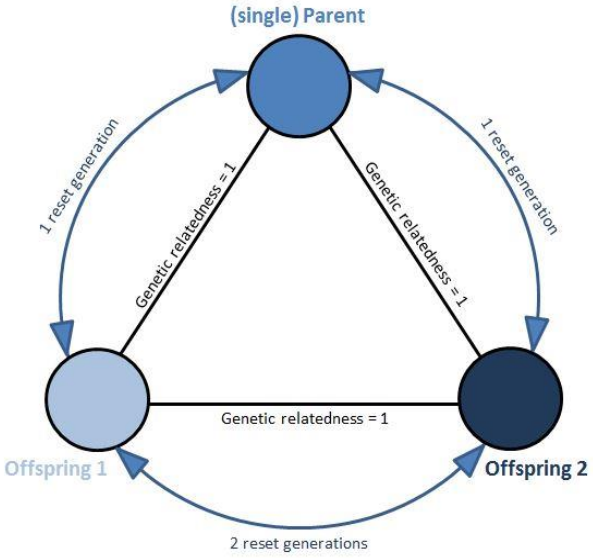

Figure 9: Parent-offspring relationship in asymmetric, asexual reproduction. Note that the circular shape of each individual is the same, representing their 100% genetic relatedness. The colour of each circle represents their epigenetic state; note a bigger (epigenetic) dissimilarity among offspring than between parent-offspring. Figure based on Tal *et al.*[133].

Not discussed in Tal *et al.*[133] is the case in which the covariance among offspring exceeds the covariance between parents and offspring, suggesting the epigenetic dissimilarity among offspring to be smaller than the epigenetic dissimilarity between parent and offspring. We postulate this phenomenon would be due to a plastic response in the offspring generation (i.e. within-generation phenotypic plasticity).

2.2.2 General setup of the experiment

In this experiment, clones of *D. magna* established by Cousyn *et al.*[118] were used. Clones originate from the resident population in Oud Heverlee Pond, Belgium. The history of the fish stock in this pond has been well documented, see Cousyn *et al.*[118] for details. Four clones were used originating from a period (early 1970's) of relatively low fish predation pressure, and four clones originating from a period (1973-1982) of relatively high fish predation pressure.

Clones were originally hatched from diapausing ephippia from Oud Heverlee Pond sediment and cultured under common garden laboratory conditions for several generations prior to being used in this experiment. The experiment comprised two conditions, a control condition and a predator condition. In the control condition, *Daphnia* were house in individual 100mL jars of dechlorinated tap water. In the predator condition, *Daphnia* were housed in individual 100mL jars of kairomone water.
The kairomone water was prepared by isolating a Pumkinseed sunfish in 8L fresh, dechlorinated tap water for 24 hours. The sunfish was fed *ad libitum* frozen *Chironomus* larvae and, in addition, was fed approximately 50 live adult *D. magna* to include alarm cues from conspecifics. Prior to use, the water was removed from the aquarium and filtered down to 45 µm using a membrane filter in order to remove debris, and diluted with dechlorinated tap water to a concentration of one fish/20L. (Note that below, for practical matters, 'kairomone-exposed individuals' refers to individuals that were exposed to kairomones as well as alarm cues from conspecifics in case of *D. magna*.)

In the second generation of *Daphnia* (see below), daphnids born from mothers in predator treatment were either assigned to a predator treatment or to the control condition, referred to as the K-K condition and K-C condition respectively (see table 3).

2.2.3 Maintenance, culturing and assessing life-history traits

The starting generation of *Daphnia* (here referred to as the 'great parental generation') in the experiment consisted of four replicate individuals per clone (i.e. four times eight, or 32 individuals). Until an age of five days, all four replicate individuals were housed, per clone, in a common jar of 100 mL dechlorinated tap water and received daily 100µL algae (concentration of 2.5 10^5 *Scenedesmus* sp. cells ml^{-1}) per jar. On day five, each daphnid was transferred and housed individually in a jar of 100mL dechlorinated tap water and received daily 100µL algae until day 7, from when all daphnids daily received 200µL algae. All daphnids, including subsequent generations, were kept in a climate-controlled room at a constant temperature of 20°C and a 14 h: 10 h light:dark regime. Starting from day three, every daphnid was transferred to a clean jar with fresh water every two days; this protocol applies for all generations of *Daphnia* in this experiment.

Every time a daphnid from the great parental generation produced a second clutch of offspring, eight neonates from that clutch were isolated in order to initiate the 'parental generation' (here also referred to as the 'first generation') of the experiment. Four of these neonates were housed in a common jar of 100 mL dechlorinated tap water (control condition) and the other four were housed in a common jar of 100 mL kairomone-water (predator condition).

On from day five, one random daphnid per jar was isolated and housed individually in a 100mL jar with dechlorinated tap water or kairomone-water for the control and predator condition respectively. The remaining three daphnids per jar were discarded. Thus, the first generation consisted of 64 individuals; 32 in the predator treatment and 32 as a control. *Daphnia* from the first generation were fed in a similar fashion as were *Daphnia* from the great parental generation, i.e. every jar received daily 100µL algae from day one until day seven, and on from day seven every jar received 200µL algae.

Every time a daphnid from the first generation produced a second clutch of offspring, 16 neonates in case of control condition and 24 neonates in case of predator condition were isolated in order to initiate the 'offspring generation' (here also referred to as the 'second generation'). In case of offspring from a mother in control condition, neonates were housed per four in a common jar of 100 mL dechlorinated tap water. On from day five, one random daphnid per jar was isolated and housed individually in a 100mL jar with dechlorinated tap water. The remaining three daphnids per jar were excluded from the experiment, thus preserving four neonates (replicates) per mother. In case of neonates born from a mother in kairomone treatment, neonates were housed per four in three jars of 100mL kairomone-water (kairomone-kairomone condition or 'K-K') and three jars of 100mL dechlorinated tap-water (kairomone-control condition or 'K-C'). On from day five, one random daphnid per jar was isolated and housed individually in a 100mL jar with kairomone-water or dechlorinated tap water, depending on the condition. The remaining three daphnids per jar were again excluded from the experiment, thus preserving 6 (two times three) neonates per mother. Thus, the second generation consisted of 320 individuals (32 times four control condition + 32 times three K-K + 32 times three K-C). Feeding protocol was the same as in the previous generations.

Table 3: Sample sizes of *D. magna* over clones.

	Control condition	**Predator condition**	**K-K condition**	**K-C condition**
Great parental generation	32	0	/	/
First generation	32	32	/	/
Second generation	128	/	96	96

As all Daphnia were housed per four until an age of five days, size at birth was assessed by measuring the length of all four daphnids by means of a stereo microscope and calculating their average length. Length was measured from the top of the head to the base of the tail spine as well as from the top of the head to the orthogonal projection of the end of the tail spine on the body axis. The individual size was measured again at day five, when daphnids were transferred to individual jars. Also, when the *Daphnia* produced their first clutch of offspring, their size was measured together with the number of produced offspring and the average size of the neonates by measuring a random sample of five neonates. Maturation time was recorded as the number of days until the first clutch was produced. For subsequent clutches only the number of produced offspring and the average size of the neonates was recorded; fecundity was assessed as the total number of produced offspring. The size of each daphnid was measured again at day 21 prior to being killed by means flash freezing. Life-history traits were only recorded in the first and second generation, life-history traits in the great parent generation were not assessed.

2.2.4 The epigenetic gradient

The setup of the experiment allows not only for comparison – within treatment – between parent-offspring covariance and among-offspring covariance, but also for comparison with niece-niece covariance, grandniece-great aunt covariance and grandniece-grandniece covariance. As there is one epigenetic reset generation between parent and offspring and two reset generations between full siblings, there are four, five and six reset generations between niece-niece, grandniece-great aunt and grandniece-grandniece respectively (Fig. 10). The relative contribution of genetic and epigenetic effects to variation in life-history traits can thus be assessed along an epigenetic gradient with a constant genetic background.

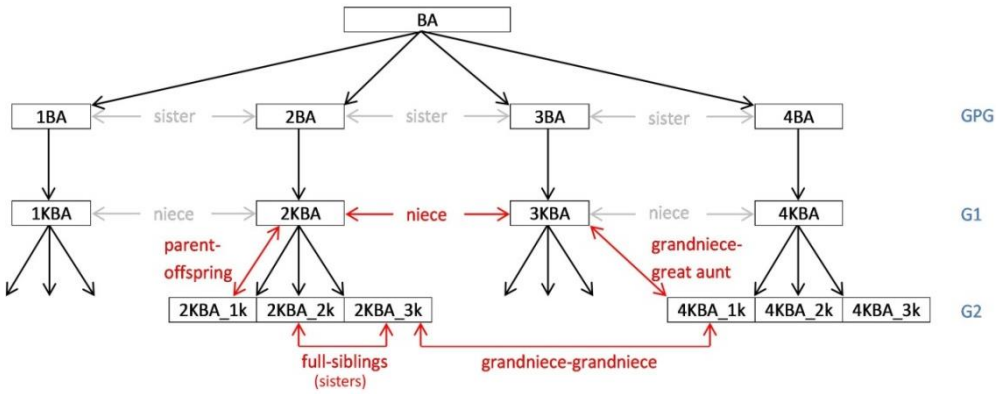

Figure 10: Family tree of an exemplary clone in kairomone treatment (i.e. both generation 1 and generation 2 in predator condition) depicting the assessed relationships (in red) that constitute our epigenetic gradient. 'A' stands for a particular clone, 'B' stands for the origin of the clone (in this case habitat with low fish predation pressure), 'K' stands for predator condition in the first generation, 'k' stands for predator condition in the second generation. (GPG = great parental generation; G1 = generation 1; G2 = generation 2)

2.3 Data analysis

All analyses were performed in R-3.1.3 (R Development Core Team, 2015).

2.3.1 *N. furzeri* experiment

Both maturation time and mortality were normally distributed and hence were analysed using a full-factorial ANOVA with population, sex and treatment (control - and predator condition) added to the model as fixed factors. Since weekly and total egg production (fecundity) are Poisson distributed, these variables were analysed using a full-factorial generalised linear mixed model with population, sex and treatment added as fixed factors, and time as a random factor.

The Von Bertalanffy curvature parameter (K) was calculated per individual fish and used as a proxy for growth. Growth was analysed using a second degree full-factorial ANOVA with the Von Bertalanffy curvature parameter as dependent variable and population, sex and treatment as fixed factors.

2.3.2 *D. magna* experiment

In total, 20 dependent variables have been analysed by means of either full-factorial ANOVA, ANODEV (Type II Wald chi-square tests) or Generalised Least Squares (GLS) for both generations of *D. magna* separately (see Table 4 and 5). Layer of origin (bottom and middle layer) and treatment (control and predator condition in the first generation; C-C, K-K and K-C in the second generation) were added as fixed factor in each test, while 'clone' was added as random factor in every test except for all GLS-tests and ANOVA's from the second generation. ANOVA's were further analysed by means of post-hoc Tukey HSD tests. In addition to these 20 variables, an additional 12 variables – taking in to account the size of the spine – have been analysed by means of similar methods (see Table S2a and S2b, Addendum 2).

Covariances were calculated on bootstrapped data. Each bootstrap was performed on the lowest level possible (i.e. the level of the individual). For parent-offspring and great aunt-grandniece, covariances were calculated on these data. In case of between sisters, between nieces and between grandnieces, the covariance was calculated as the variance on the means of sister-groups, niece-groups and grandniece-groups respectively.

Covariances were calculated within control - and within predator conditions in case of among nieces, and within CC, KK and KC in case of parent-offspring, among sisters, great aunt-grandniece and among grandnieces. This was done for 15 variables and for an additional 7 variables taking into account the size of the spine.

3 Results

3.1 *N. furzeri* experiment

An ANOVA for female maturation time yielded no significant effect for neither population ($F_{1,25}$ = 0.304, p = .587) nor treatment ($F_{1,25}$ = 0.778, p = .386) nor the interaction of population with treatment ($F_{1,25}$ = 0.340, p = .565). Likewise, an ANOVA for male maturation time yielded no significant effect for population ($F_{1,31}$ = 0.467, p = .499), treatment ($F_{1,31}$ = 0.002, p = .969) or the interaction of population with treatment ($F_{1,31}$ = 0.019, p = .891).

A full factorial ANOVA (see Fig. 11) for lifespan with population, treatment and sex as factors yielded a significant effect for sex with males exhibiting a longer lifespan then females ($F_{1,55}$ = 6.565, p < .05). Also the interaction of population with sex was significant ($F_{1,55}$ = 4.641, p = .0356, with (control) NF222 males exhibiting a longer lifespan than (control) NF222 females, and NF414 males and females exhibiting no significant difference in lifespan.

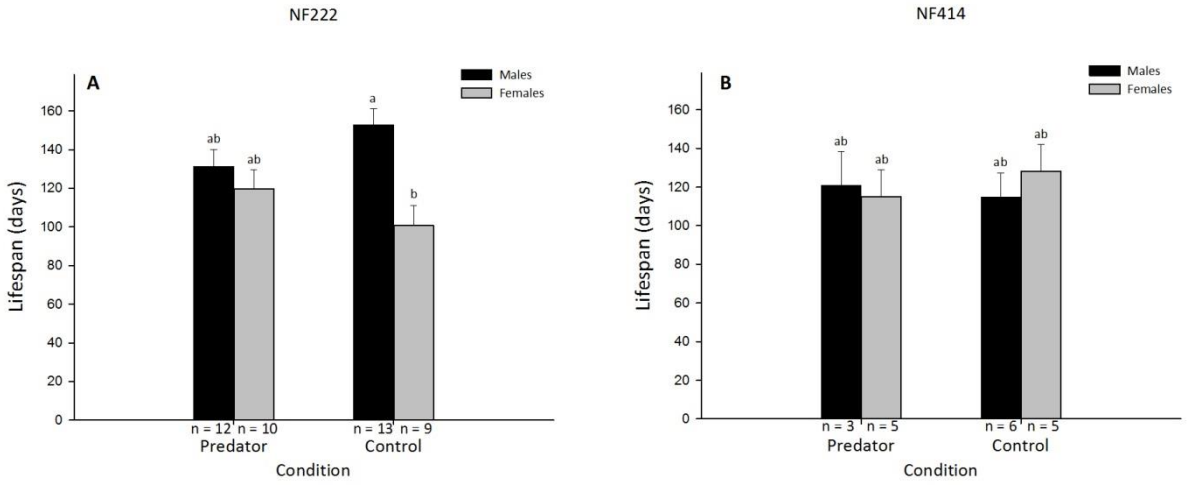

Figure 11: Mean lifespan (in days) of males and females for population (A) NF222 and (B) NF414. Whiskers delineate the standard error of mean. Letters indicate significant differences (based on post-hoc Tukey HSD test, p < .01).

A generalised linear model for total female fecundity with population and treatment as fixed factors yielded a highly significant effect for population, with NF222 females exhibiting a higher total fecundity than do NF414 females (z = 17.82, p < .0001). Females overall from the predator condition exhibited a significantly higher total fecundity than females from control condition (z = 27.57, p < .0001). The interaction of population with treatment was highly significant (z = -28.96, p < .0001), with NF222 kairomone-exposed females exhibiting a significantly higher total fecundity than NF222 control females, while NF414 kairomone-exposed females exhibited a significantly lower total fecundity than NF414 control females (see Fig. 12).

A generalised linear mixed model for female fecundity with population and treatment as fixed factors and time as random factor (see Fig. S2.1, Addendum 2) yielded similar results; population (z = 18.33, $p < .0001$), treatment (z = 8.121, $p < .0001$) and the interaction of population with treatment (z = -21.04, $p < .0001$) were highly significant.

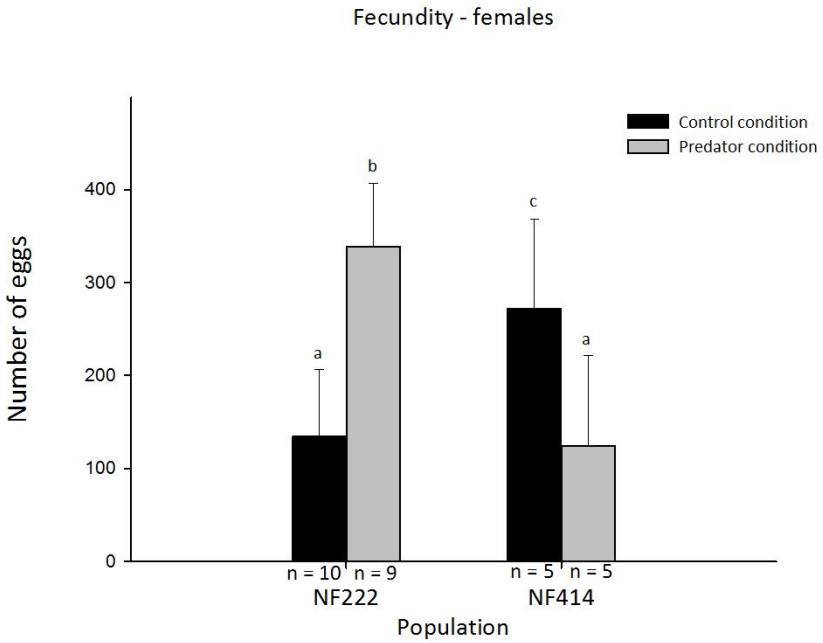

Figure 12: Mean of total number produced eggs (fecundity between week six and week 20) between predator and control condition for females. Whiskers delineate the standard error of mean. Letters indicate significant differences (based on post-hoc Tukey HSD test). All significant differences have a *p*-value < .0001.

A full-factorial (2nd degree) ANOVA for the Von Bertalanffy curvature parameter (growth parameter) only yielded a significant effect for population ($F_{1,57} = 5.22$, $p = .0261$) with a higher mean growth parameter for NF222 compared to NF414. All other factors were not significant.

3.2 *D. magna* experiment

3.2.1 General results

Results of the tests on the 20 dependent variables are displayed in Table 4 for the first generation and Table 5 for the second generation.

Figures showing the comparison between groups for variables related with size of parental daphnids and number and size of offspring are presented Figure 13, 14 and 15 for the first generation and Figure. 16, 17 and 18 for the second generation. In addition for the second generation, Figure. 19 displays the comparison between groups for variables related with age.

In the first generation, the kairomone treatment significantly increased the number of offspring produced in the first two broods (see Fig. 14), as well as their entire reproductive output in those two broods (taking into account the number of offspring and the size of the neonates). On from the third brood, however, kairomone-exposed daphnids produced significantly smaller offspring in terms of size than daphnids from control condition (see Fig. 15). The sediment layer where the clones used in the experiment originated from significantly impacted the size of the parental individuals, both at birth and at an age of five days (see Fig. 13). At birth, individuals originating from the middle sediment layer were significantly larger than individuals originating from the bottom sediment layer. In contrast, at an age of five days, individuals originating from the bottom sediment layer were significantly larger than individuals originating from the middle sediment layer. At maturation, the effect of sediment layer on size of the individuals was close to significance, with bottom-layer-originating individuals being larger than middle-layer-originating individuals, while at 21 days (final size) the effect of sediment layer seems to be absent. No significant effects on reproductive age could be observed.

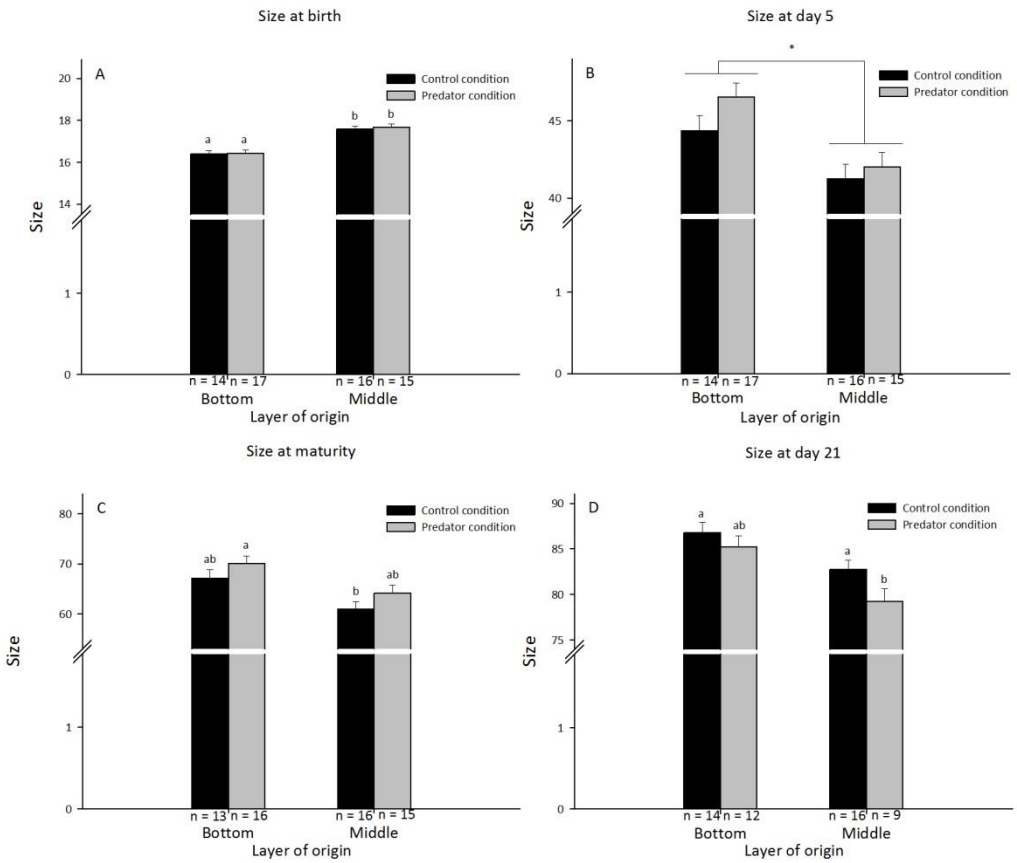

Figure 13: Mean size of first-generation parental daphnids at (A) birth, (B) day 5, (C) maturity and (D) day 21. Note that size has not been resized and therefore lacks a unit of length. Whiskers delineate the standard error of mean. Letters indicate significant differences (based on post-hoc Tukey HSD test) in case of ANOVA. An asterisk (*) indicates a significant difference in case of ANODEV.

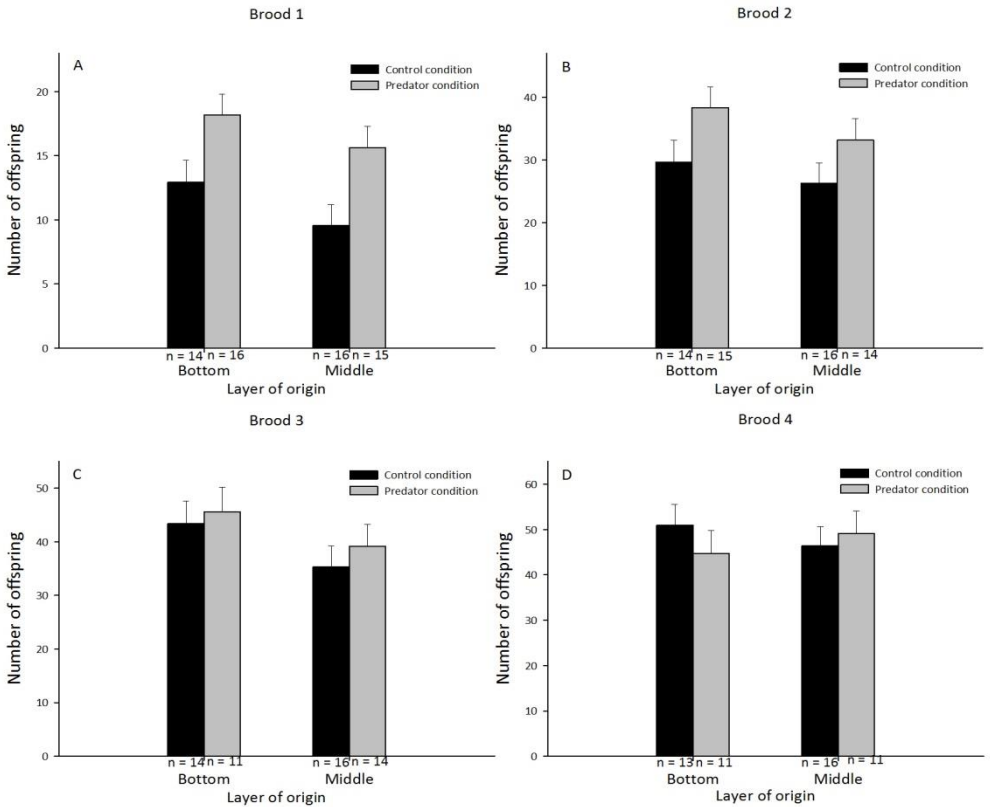

Figure 14: Mean number of offspring of first-generation parental daphnids at the (A) first , (B) second, (C) third and (D) fourth brood. Whiskers delineate the standard error of mean. Formal post-hoc tests for ANODEVs have not been performed and therefore significant effects are not depicted.

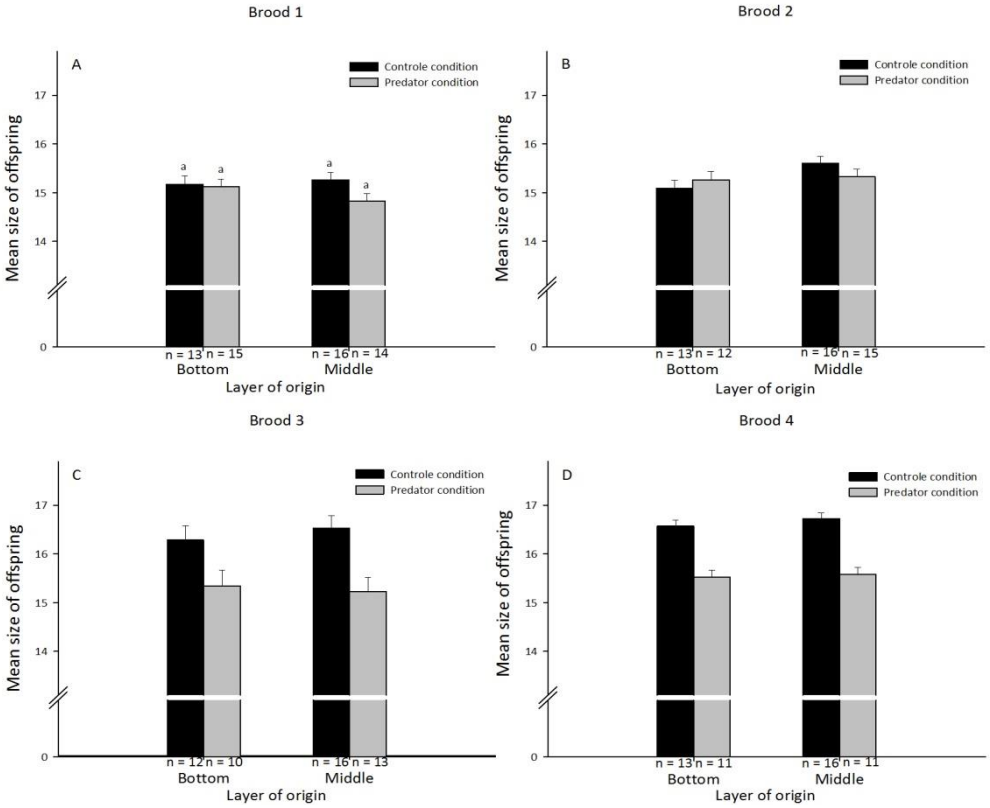

Figure 15: Mean sizes of offspring (neonate-size) of first-generation parental daphnids at the (A) first, (B) second, (C) third and (D) fourth brood. Whiskers delineate the standard error of mean. Letters indicate significant differences (based on post-hoc Tukey HSD test) in case of ANOVA. Formal post-hoc tests for ANODEVs and GLS-tests have not been performed.

Table 4: Results of separate tests on 20 variables of the first generation of *D. magna* in the experiment. Significant effects are depicted in bold and underlined. (Total reproductive output = number of offspring times the mean size of offspring)

			Fixed effects						
			Layer		Treatment		Layer*Treatment		
Category	**variable**	**Test**							**Random effects**
Size	Size at birth	ANOVA	$t = 4.16$	**$p < .005$**	$t = 0.402$	$p = .689$	$t = 0.131$	$p = .897$	'Clone'
	Size at day 5	ANODEV	$X^2 = 6.70$	**$p < .01$**	$X^2 = 2.29$	$p = .130$	$X^2 = 0.224$	$p = .636$	'Clone'
	Size at maturity	ANOVA	$t = -2.17$	$p = .0534$	$t = 1.26$	$p = .212$	$t = 0.207$	$p = .837$	'Clone'
	Size at day 21	ANOVA	$t = -1.80$	$p = .108$	$t = -0.794$	$p = .432$	$t = -1.42$	$p = .163$	'Clone'
Reproductive age	Age at maturity	GLS	$t = -1.71$	$p = .0932$	$t = 1.50$	$p = .140$	$t = -1.91$	$p = .0609$	None
	Age at brood 2	GLS	$t = -1.46$	$p = .150$	$t = 1.11$	$p = .274$	$t = -1.62$	$p = .112$	None
	Age at brood 3	GLS	$t = -1.35$	$p = .183$	$t = -1.21$	$p = .231$	$t = 0.002$	$p = .998$	None
	Age at brood 4	GLS	$t = -1.49$	$p = .142$	$t = -1.84$	$p = .0724$	$t = 1.07$	$p = .288$	None
Number of offspring	# at brood 1	ANODEV	$X^2 = 0{,}609$	$p = .435$	$X^2 = 21.5$	**$p < .0001$**	$X^2 = 0.006$	$p = .936$	'Clone'
	# at brood 2	ANODEV	$X^2 = 0{,}225$	$p = .635$	$X^2 = 17.6$	**$p < .0001$**	$X^2 = 0.562$	$p = .453$	'Clone'
	# at brood 3	ANODEV	$X^2 = 0{,}199$	$p = .656$	$X^2 = 0.0079$	$p = .929$	$X^2 = 1.06$	$p = .304$	'Clone'
	# at brood 4	ANODEV	$X^2 = 0{,}066$	$p = .798$	$X^2 = 1.05$	$p = .307$	$X^2 = 2.51$	$p = .113$	'Clone'
Size of offspring at birth	Size of brood 1	ANOVA	$t = 0.341$	$p = .737$	$t = -0.252$	$p = .802$	$t = -1.19$	$p = .240$	'Clone'
	Size of brood 2	GLS	$t = -0.911$	$p = .367$	$t = -0.162$	$p = .872$	$t = -0.573$	$p = .569$	None
	Size of brood 3	GLS	$t = 1.67$	$p = .101$	$t = -2.07$	**$p < .05$**	$t = -0.546$	$p = .588$	None
	Size of brood 4	ANODEV	$X^2 = 0.370$	$p = .543$	$X^2 = 81.4$	**$p < .0001$**	$X^2 = 0.246$	$p = .620$	'Clone'
Total reproductive output	Brood 1	ANODEV	$X^2 = 0.583$	$p = .445$	$X^2 = 19.1$	**$p < .0001$**	$X^2 = 0.0346$	$p = .853$	'Clone'
	Brood 2	ANOVA	$t = -0.200$	$p = .848$	$t = 3.80$	**$p < .0005$**	$t = -1.30$	$p = .202$	'Clone'
	Brood 3	ANOVA	$t = -1.64$	$p = .155$	$t = -1.11$	$p = .272$	$t = 0.867$	$p = .391$	'Clone'
	Brood 4	ANOVA	$t = -0.177$	$p = .865$	$t = -2.22$	**$p < .05$**	$t = 1.39$	$p = .171$	'Clone'

Table 5: Results of separate tests on 20 variables of the second generation of *D. magna* in the experiment. The mean size of offspring from brood 2, 3 and 4 were not analysed. Significant effects are depicted in bold and underlined. (Total reproductive output = number of offspring times the mean size of offspring)

			Fixed effects						
Category	**variable**	**Test**	**Layer**		**Treatment**		**Layer*Treatment**		**Random effects**
Size	Size at birth	ANOVA	$F_{1,252} = 0.225$	$p = .635$	$F_{2,252} = 47.5$	**$p < .0001$**	$F_{2,252} = 0.493$	$p = .612$	None
	Size at day 5	ANOVA	$F_{1,269} = 1.81$	$p = .179$	$F_{2,269} = 13.1$	**$p < .0001$**	$F_{2,269} = 0.982$	$p = .376$	None
	Size at maturity	ANOVA	$F_{1,258} = 77.4$	**$p < .0001$**	$F_{2,258} = 13.3$	**$p < .0001$**	$F_{2,258} = 2.85$	$p = .0599$	None
	Size at day 21	ANOVA	$F_{1,230} = 112$	**$p < .0001$**	$F_{2,230} = 1.39$	$p = .251$	$F_{2,230} = 4.04$	**$p < .05$**	None
Reproductive age	Age at maturity (3x log-transformed)	ANOVA	$F_{1,264} = 56.7$	**$p < .0001$**	$F_{2,264} = 2.69$	$p = .0698$	$F_{2,264} = 0.092$	$p = .912$	None
	Age at brood 2 (3x log-transformed)	ANOVA	$F_{1,259} = 18.1$	**$p < .0005$**	$F_{2,259} = 3.13$	**$p < .05$**	$F_{2,259} = 0.619$	$p = .539$	None
	Age at brood 3 (3x log-transformed)	ANOVA	$F_{1,238} = 0.188$	$p = .665$	$F_{2,238} = 4.83$	**$p < .01$**	$F_{2,238} = 2.51$	$p = .0836$	None
	Age at brood 4	ANOVA	$F_{1,190} = 1.60$	$p = .207$	$F_{2,190} = 7.13$	**$p < .005$**	$F_{2,190} = 1.76$	$p = .175$	None
Number of offspring	# at brood 1	ANOVA	$F_{1,264} = 15.6$	**$p < .0001$**	$F_{2,264} = 33.2$	**$p < .0001$**	$F_{2,264} = 1.13$	$p = .324$	None
	# at brood 2	ANODEV	$X^2 = 1.17$	$p = 279$	$X^2 = 45.1$	**$p < .0001$**	$X^2 = 6.87$	**$p < .05$**	'Clone'
	# at brood 3	ANOVA	$F_{1,237} = 31.3$	**$p < .0001$**	$F_{2,237} = 2.84$	$p = .0607$	$F_{2,237} = 0.039$	$p = .962$	None
	# at brood 4	ANOVA	$F_{1,190} = 29.4$	**$p < .0001$**	$F_{2,190} = 1.53$	$p = .220$	$F_{2,190} = 0.583$	$p = .559$	None
Size of offspring at birth	Size of brood 1	ANOVA	$F_{1,261} = 0.169$	$p = .681$	$F_{2,261} = 4.28$	**$p < .05$**	$F_{2,261} = 0.044$	$p = .957$	None
Total reproductive output	Brood 1	ANODEV	$X^2 = 0.697$	$p = .404$	$X^2 = 63.0$	**$p < .0001$**	$X^2 = 2.88$	$p = .237$	'Clone'
	Brood 2	ANODEV	$X^2 = 0.859$	$p = .354$	$X^2 = 46.9$	**$p < .0001$**	$X^2 = 7.47$	**$p < .05$**	'Clone'
	Brood 3	ANOVA	$F_{1,233} = 27.1$	**$p < .0001$**	$F_{2,233} = 2.55$	$p = .0805$	$F_{2,233} = 0.0260$	$p = .974$	None
	Brood 4	ANOVA	$F_{1,188} = 31.4$	**$p < .0001$**	$F_{2,188} = 1.06$	$p = .348$	$F_{2,188} = 0.807$	$p = .448$	None

In the second generation, treatment had a significant effect on the number of produced offspring, with individuals from K-K or K-C condition generally producing larger numbers of offspring in the first two broods than do individuals from the C-C condition (see Fig. 17). The general trend observed is that of the lowest number of offspring in C-C condition and the highest number of offspring in K-K condition. Treatment also had a significant effect on the total reproductive output in the first two broods, with individuals from K-K or K-C condition generally exhibiting a higher reproductive output than individuals from the C-C condition. The same general trend was observed as for the number of offspring. The number of offspring in brood one, three and four was significantly higher for individuals originating from the bottom sediment layer than for individuals from the middle sediment layer (see Fig. 17), and the total reproductive output was significantly higher in brood three and four for bottom-layer individuals than for middle-layer individuals. Treatment had a significant effect on the mean size of offspring (spine excl.) in brood one, but post-hoc Tukey HSD test found no significant differences between groups. The observed trend displayed the biggest neonate size in K-K condition and the lowest neonate size in C-C condition. Figure 18 shows the comparison between groups for size of offspring including the size of the spine. Size of the individuals at birth was significantly decreased by kairomone treatment, with individuals from K-K and K-C condition being smaller than individuals from C-C condition. However, kairomone treatment significantly increased size at day five and at maturity, with individuals from K-K or K-C condition in general being larger than individuals from C-C condition. Size at maturity and at day 21 was significantly higher for bottom-layer individuals than for middle-layer individuals (see Fig. 16). Age at maturity and at brood two was significantly higher for bottom-layer than for middle-layer individuals. Age at brood three and four was not dependent on the sediment layer of origin, but was however significantly higher for C-C individuals compared to K-K individuals (see Fig. 19).

Results regarding variables that account for the size of the spine are displayed in Table S2a for the first generation and Table S2b for the second generation (see Addendum 2) and are generally in compliance with the results displayed in Table 4 and Table 5 respectively.

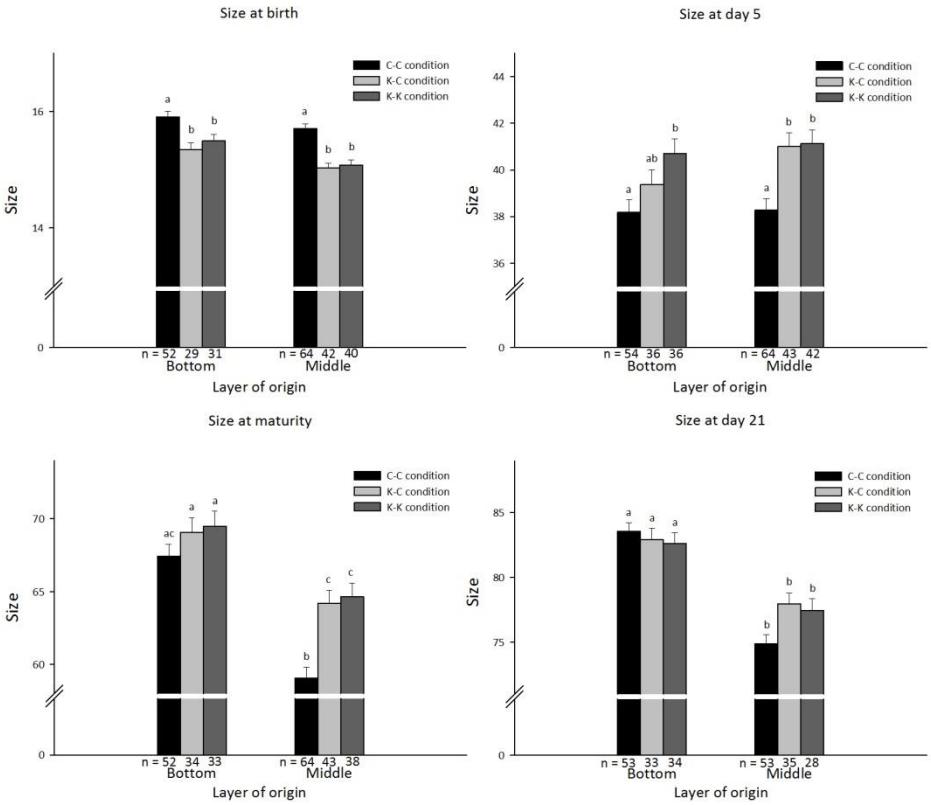

Figure 16: Mean size of second-generation parental daphnids at (A) birth, (B) day 5, (C) maturity and (D) day 21. Note that size has not been resized and therefore lacks a unit of length. Whiskers delineate the standard error of mean. Letters indicate significant differences (based on post-hoc Tukey HSD test).

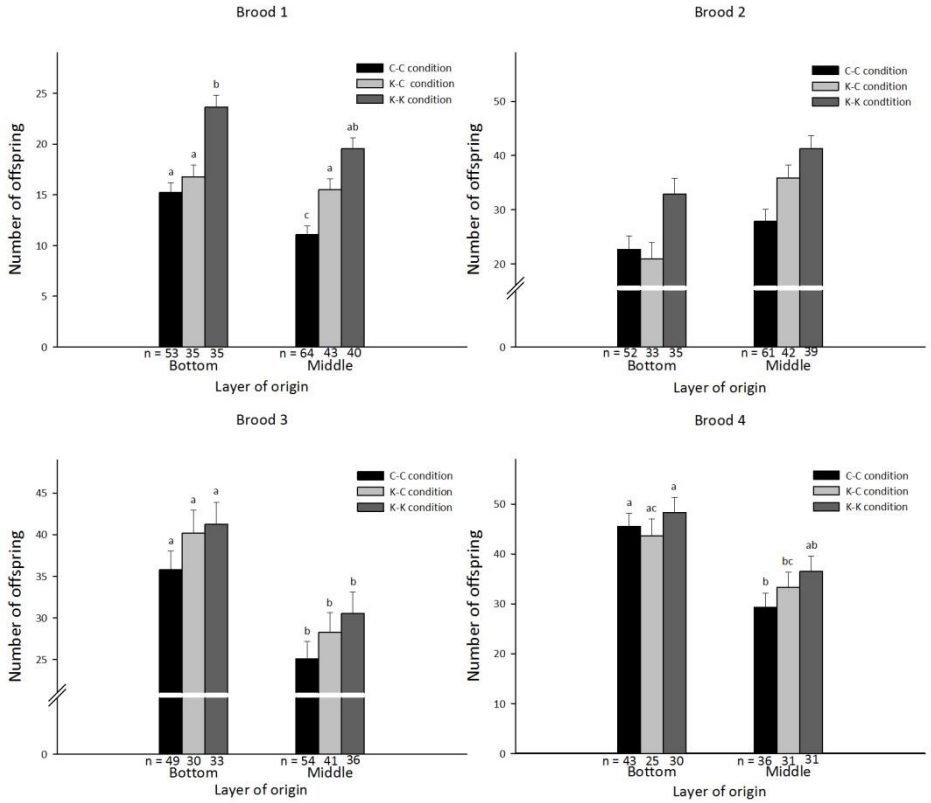

Figure 17: Mean number of offspring of second-generation parental daphnids at the (A) first, (B) second, (C) third and (D) fourth brood. Whiskers delineate the standard error of mean. Letters indicate significant differences (based on post-hoc Tukey HSD test).

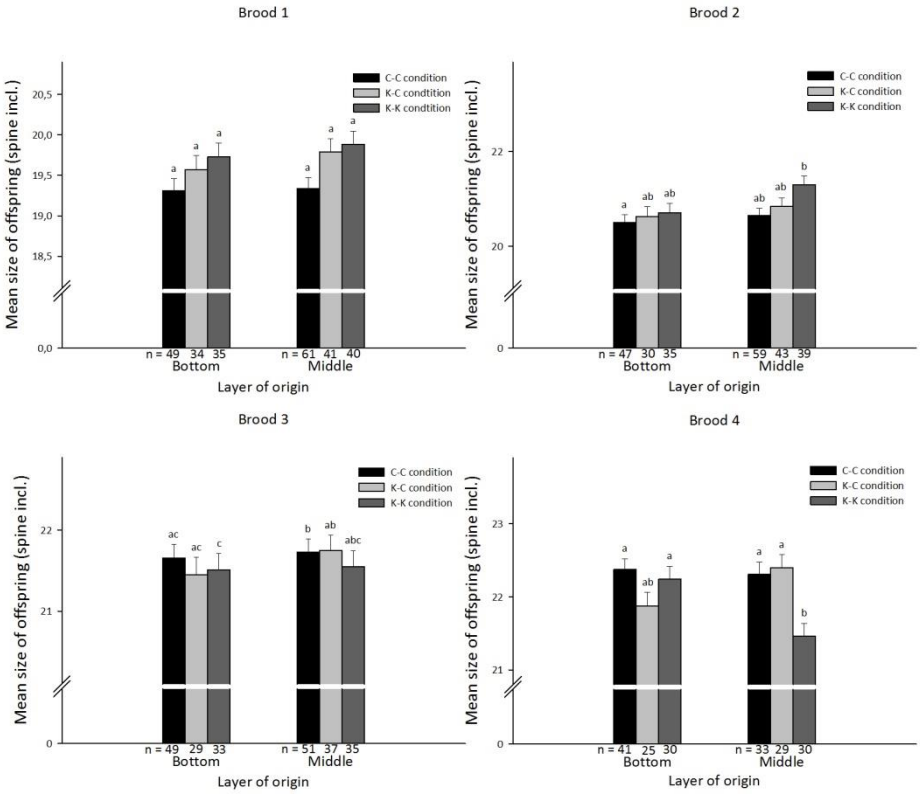

Figure 18: Mean sizes (spine incl.) of offspring (neonate-size) of second-generation parental daphnids at the (A) first, (B) second, (C) third and (D) fourth brood. Whiskers delineate the standard error of mean. Letters indicate significant differences (based on post-hoc Tukey HSD test).

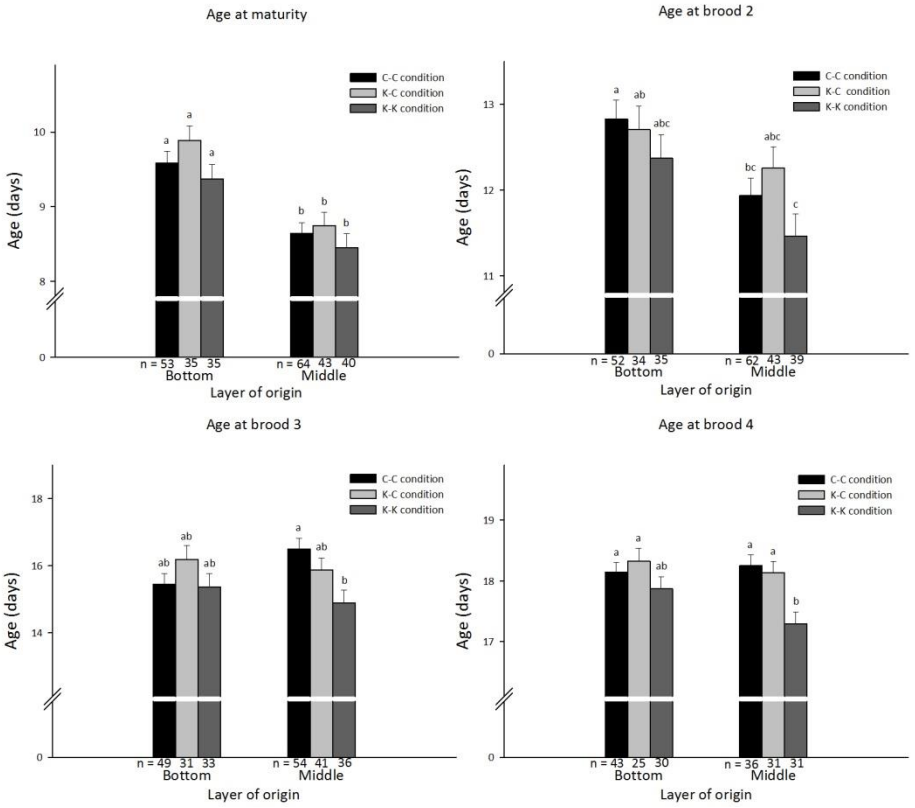

Figure 19: Mean age of second-generation parental daphnids at (A) brood 1 (maturity), (B) brood 2, (C) brood 3 and (D) brood 4. Whiskers delineate the standard error of mean. Letters indicate significant differences (based on post-hoc Tukey HSD test).

3.2.2 Covariance results

Covariances calculated on variables related with size of parental daphnids are displayed in Figure 20. For size at day five and size at maturity, the mean covariances were in general highest between sisters and lowest between great aunt - grandniece. The mean covariances between nieces were lower than between sisters, followed by the mean covariances between grandnieces and between parent – offspring. Covariances also differ between conditions; for sisters and nieces, covariances were generally lowest for C-C and control conditions. In case of grandnieces, parent – offspring and great aunt – grandniece, the pattern was less clear.
For size at day 21, covariances were in general higher between sisters than between grandnieces, followed by the covariances between parent – offspring and great aunt – grandniece. However, the covariance between nieces in predator condition exceeds all other covariances. *Confer* size at day five and size at maturity, covariances between sisters and nieces were generally lowest for C-C and control conditions and a less clear pattern exists for grandnieces, parent – offspring and great aunt – grandniece.

Covariances calculated on variables related with age of parental daphnids are displayed in Figure 21. In general, covariances between grandnieces, between parent - offspring and between great aunt - grandniece were smaller than between sisters and nieces. For all variables except age at brood four, covariances were generally lowest for C-C and control conditions between sisters and between nieces. The between-condition pattern for grandnieces, parent - offspring and great aunt - grandniece was less clear. Except for age at brood three, the covariance between nieces in predator condition exceeds all other covariances. For age at brood three, the highest covariance was the covariance between sisters in K-C condition.

Covariances calculated on variables related with number of offspring are displayed in Figure 22. In general, covariances between sisters were highest, followed by the covariances between nieces, between grandnieces, between parent - offspring and between great aunt - grandniece. For the number of offspring at the first two broods, covariances are generally lowest for C-C and control conditions, except for brood two between grandnieces where the C-C covariance was highest. For these two variables, covariances between sisters and between nieces were highest in K-K conditions.
For the number of offspring at brood three and four, the covariances between sisters and between nieces were in general lowest in C-C and control conditions, and highest in K-K and predator conditions. For grandnieces, parent - offspring and great aunt - grandniece however, the covariance in C-C conditions is higher than in K-C and K-K conditions.

Covariances calculated on variables related with size of offspring are displayed in Figure 23. For the mean size of offspring of all four broods, covariances were generally highest between sisters, followed by the covariances between grandnieces, between parent - offspring and between great aunt - grandniece. However, the covariance between nieces exceeds all other covariances for all broods except brood 1. Moreover, the highest covariance for brood two and three was the covariance between nieces in control condition, while the highest covariance for brood four was between nieces in predator condition. For the mean size of offspring of brood one, all covariances except for parent-offspring were relatively much higher in C-C and control conditions than in K-C and K-K conditions.

Covariances on variables that take into account the size of the spine are presented in Figure S2.2 (size of parental daphnids, see Addendum 2) and Figure S2.3 (size of offspring, see Addendum 2), and are generally in compliance with the results displayed in Figure 20 and Figure 23 respectively. However, for the mean size of offspring (spine incl., Fig. S2.3 in Addendum 2) of brood one, the between-condition pattern is not clear and the highest covariance is the covariance between sisters in K-C condition.

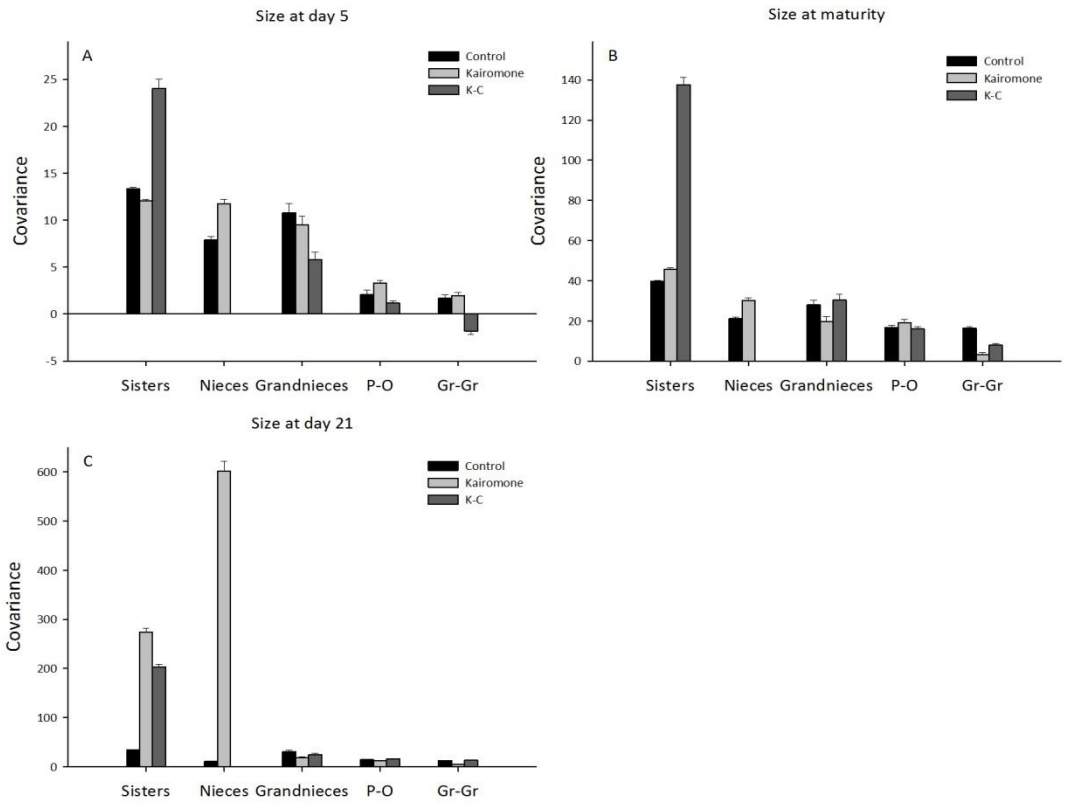

Figure 20: Mean covariance between sisters, nieces, grandnieces, parent–offspring and great aunt–grandniece in C-C (or control), K-K (or predator) and K-C condition for (A) size at day 5, (B) size at maturity and (C) size at day 21. Whiskers delineate 95% confidence intervals. (P-O = parent-offspring ; Gr-Gr = great aunt-grandniece)

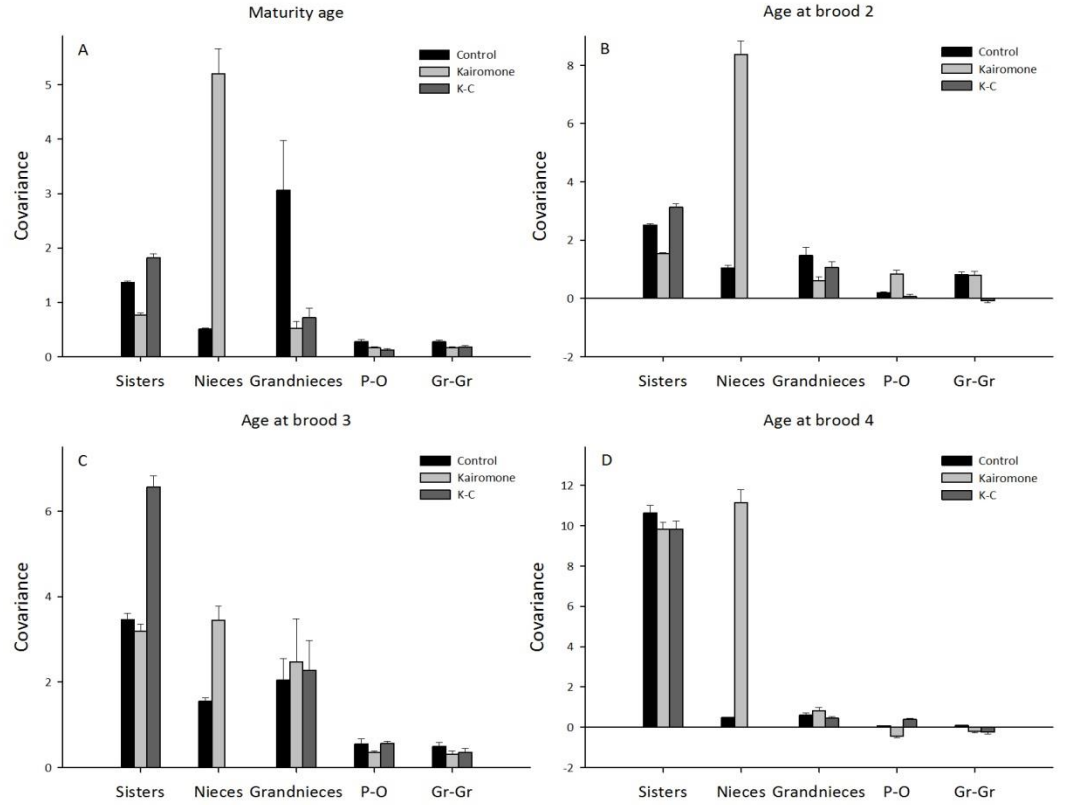

Figure 21: Mean covariance between sisters, nieces, grandnieces, parent–offspring and great aunt–grandniece in C-C (or control), K-K (or predator) and K-C condition for (A) age at brood 1 (maturity), (B) at brood 2, (C) at brood 3 and (D) at brood 4. Whiskers delineate 95% confidence intervals. (P-O = parent-offspring ; Gr-Gr = great aunt-grandniece)

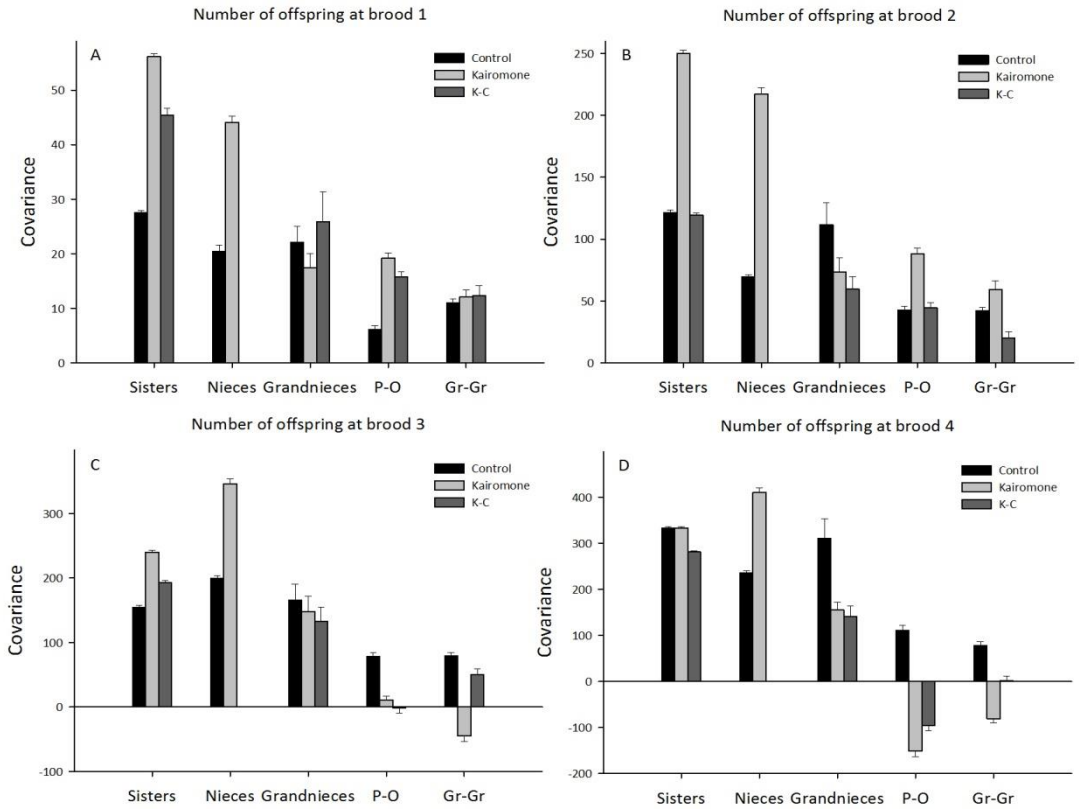

Figure 22: Mean covariance between sisters, nieces, grandnieces, parent–offspring and great aunt–grandniece in C-C (or control), K-K (or predator) and K-C condition for number of offspring at (A) brood 1, (B) brood 2, (C) brood 3 and (D) brood 4. Whiskers delineate 95% confidence intervals. (P-O = parent-offspring ; Gr-Gr = great aunt-grandniece)

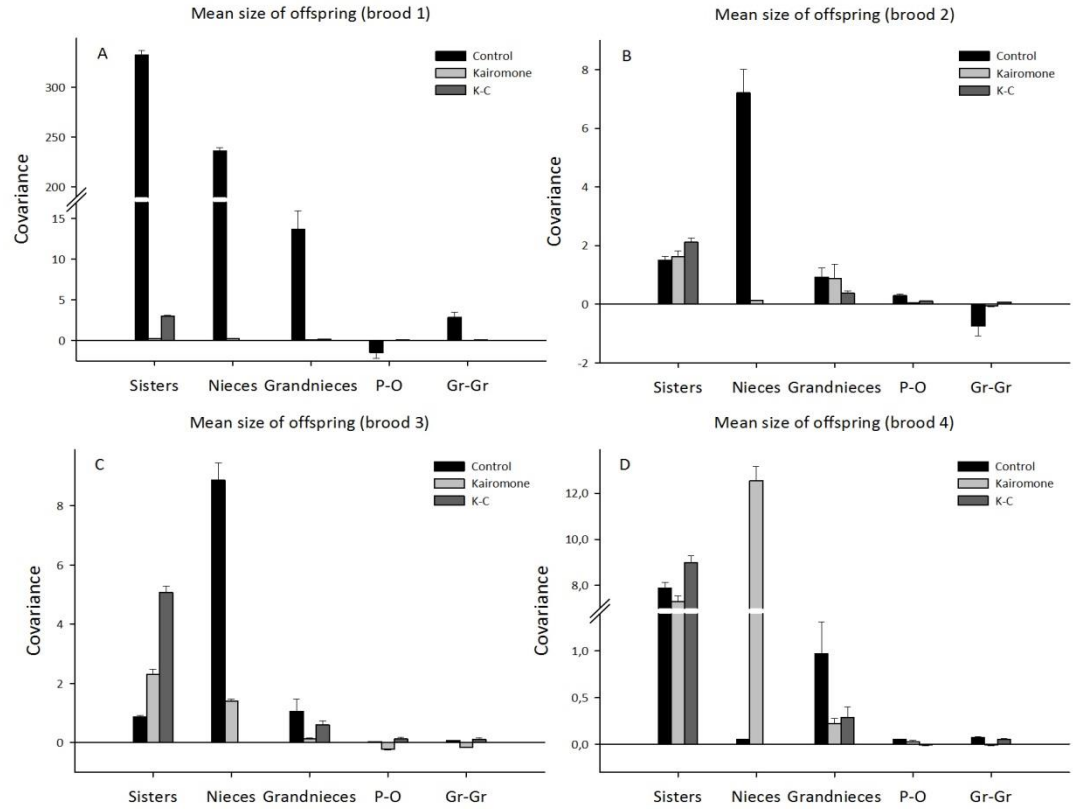

Figure 23: Mean covariance between sisters, nieces, grandnieces, parent–offspring and great aunt–grandniece in C-C (or control), K-K (or predator) and K-C condition for size of offspring (neonate-size) at (A) brood 1, (B) brood 2, (C) brood 3 and (D) brood 4. Whiskers delineate 95% confidence intervals. (P-O = parent-offspring ; Gr-Gr = great aunt-grandniece)

4 Discussion

Both genetic and epigenetic phenomena contribute to the phenotypic variation observed in a population of organisms. In this study, we examined this in a vertebrate model, *N. furzeri*, as well as in an invertebrate model, *D. magna*.

4.1 *N. furzeri* experiment

Although we were unable to assess transgenerational epigenetic phenomena for *N. furzeri* since only one generation could be observed, within-generation non-genetic effects in the form of predator-induced phenotypic plasticity have been recorded. Overall, female killifish exposed to olfactory and visual cues of a predatory pumpkinseed sunfish exhibited a higher fecundity (i.e. number of produced eggs between week six and week 20) compared to control females. Similar results have been reported in previous studies on fish[105,109,132].

Surprisingly, this effect was strongly pronounced in the NF222 population. This population originates from a pool that most probably never makes contact with rivers during flooding events and was hence assumed to harbour no predatory fish. Therefore, the NF222 population was expected to have no history of predation exposure and hence lack the ability for an antipredator response given the absence of a selective force favouring the response and due to costs associated with the maintenance of phenotypic plasticity[52,54]. The presence of an antipredator response might imply that he premise of a predatory fish-free pool is not as applicable as expected as we cannot completely rule out the possibility, as small as it may be, that predatory fish (for instance lungfish, *Protopterus annectens*[70]) occasionally invade the pool. This might require, however, for the predatory fish to be present for several generations in order to allow for the establishment of the adaptive plastic response. Moreover, predatory fish should have been present in the recent past since the response is expected to disappear from the population in the absence of selection favouring the response due to costs associated with the maintenance of plasticity. An alternative and possibly reinforcing explanation is that of gene flow with populations of *N. furzeri* that have a history of predation exposure and hence might possess the capacity to exhibit an antipredator response. These gene flow events might occur during flooding events or, in this case more likely, might be mediated by transport vectors of dormant fish eggs such as mud-wallowing mammals or waterbirds[83,94]. In the same line of thought, it is plausible that the NF222 population was founded by fish with a history of predation exposure and there has not yet been sufficient time for the plastic response to disappear from the population.

In contrast with the findings for the NF222 population, killifish from the NF414 population exposed to predation risk exhibit a smaller fecundity than control fish. Although this response is unexpected and might at first seem maladaptive, it might also be the result of a functional trade-off between reproduction and competing traits, cf. constrained adaptive evolution. It has, for instance, been found that an increased reproductive effort implies an increased locomotor cost in the Trinidadian guppy (*Poecilia reticulata*), hence increasing the risk of predation[134]. In such a case, expectations concerning the direction of the plastic response might not be straighforward.

Both populations appear to exhibit alternative antipredator strategies; differences in fecundity between populations might be attributable to differences in selective forces between their respective pools (e.g. due to different predator species). It is, however, worth noting the results should be relativized due to the small sample size (especially in NF414, see Fig. 12).

Although a negative correlation between reproductive effort and lifespan has been demonstrated in an array of organisms[135–137], lifespan was, unlike fecundity, found to be unaffected by the simulated predation risk. As such, no tradeoff between reproductive effort and lifespan was found in this experimental setting. This is consistent with the results of a previous study on *N. furzeri* in which female fish were either separated from or housed together with male fish. The study demonstrated that separated female fish, compared to female fish that were housed together with males, had a lower reproductive effort but no prolonged lifespan[136].

In the NF222 population however, lifespan was significantly longer in control males compared to control females. This might be an artifact of the setup of the experiment. There was an excess of males in the control condition of NF222 (13 males over nine females, see Table 1), hence each time females were allowed to spawn with a male, approximately 44% of the males were not transferred to individual 'spawning jars' and were less frequently exposed to handling stress. Alternatively, as approximately only 66% of the males were allowed to reproduce each spawning session, males overall might have allocated relatively more energy to somatic maintenance (lifespan) rather than to reproduction, or might have experienced less somatic damage as a direct consequence of reproduction. Both hypotheses are supported by the fact that in NF222 fish exposed to kairomones, where only 20% of the males were excluded each spawning session, the same yet non-significant trend (males longer-lived than females) is observed. The latter hypothesis (relatively less energy allocated to reproduction) however contradicts the findings of Graf *et al*.[136] (see above) who found no correlation between lifespan and reproductive effort.

The studied populations show no difference in lifespan. This is as expected given that both populations originate from pools in central Mozambique with a similar desiccation regime. However, given the expectation that NF414 is subjected to higher predation pressure (by predatory fish) compared to NF222, NF414 was expected to experience a higher extrinsic mortality pressure and hence to exhibit an earlier onset of senescence conform the classic evolutionary theories of aging. The absence of this result might imply that NF222 does have a history of predation exposure (see above), or that the extrinsic mortality pressure due to predation by fish in the NF414 pool did not effectively select for shorter lifespan. Many studies have pointed to the hiatus in the predictions of the classic evolutionary theories of aging (See 1.7.2). For instance, predation might translate into a higher extrinsic mortality pressure, which in turn might translate into a decreased population density accompanied by a decrease in intraspecific competition and a higher resource availability[105,138]. Thus, the classic evolutionary theories of aging constitute a basic framework that needs to take into account many additional factors so to effectively predict the evolution of senescence in various systems. Another plausible explanation as to why no difference in lifespan between populations was recorded is that populations of *N. furzeri* already are at their physiological limits for rate of development. The latter explanation is supported by the finding that maturity time is unaffected by treatment and does not differ between populations.

Fish from NF222 grew significantly faster compared to fish from NF414, regardless of treatment. Consequently, as no differences in maturity time between the two localities was recorded, this implies that fish from the NF414 population attain maturity at smaller sizes than do fish from the NF222 population, which is in accordance with our expectations. Although no (predator-induced) plasticity for growth was found, this finding could possibly imply genetic adaptation to predation. This idea is supported by a previous study reporting that livebearing fish (*Brachyrhaphis rhabdophora*) from habitat with predatory fish reached maturity at a smaller size than did fish from predator-free habitat[139].

4.2 *D. magna* experiment

4.2.1 Clutch size, offspring size and total reproductive effort

Also in *D. magna*, predator-induced phenotypic plasticity was recorded. In accordance with our hypothesis and previous studies (e.g. Walsh *et al*.[112]), daphnids exposed to olfactory cues of predatory pumpkinseed sunfish (and alarm cues of conspecifics) exhibited a higher reproductive effort in the form of an increased clutch size and a higher reproductive effort overall (taking into account clutch- and neonate size). This effect was observed for the first two clutches in both generations, but deteriorates in subsequent clutches. Subsequent clutches of kairomone-exposed mothers consist of offspring that is significantly smaller than offspring from non-exposed mothers. Although the latter effect could only be assessed in the first generation of *Daphnia* (when the spine is excluded), it is also visible in the fourth brood of second-generation daphnids when the size of the spine of the neonates is taken into account.

Possibly, the observed effect implies a switch-over in time from one antipredator strategy (increased clutch size[112,122]) to another (decreased neonate size[74]). Alternatively and not necessarily exclusive, decreased neonate size might also be a consequence of a higher allocation of a limited amount of energy to reproduction during the first clutches, decreasing the energy available to allocate to the production of subsequent clutches. The latter hypothesis appears to be more likely given that decreased neonate size is an antipredator response more often associated with predation threat by *Chaoborus spp.* (phantom midges) rather than by fish[140]. Moreover, Hanazato *et al*.[127] reported increased neonate size to be an antipredator response in case of fish predation.

As such, kairomone-exposed individuals seem to allocate more energy to reproduction early in life than do non-exposed individuals. This is consistent with the results of previous studies[112,141,142]. Given the commonness of a tradeoff between reproductive effort and lifespan[135–137], the lifespan of kairomone-exposed individuals is expected to be shorter than the lifespan of non-exposed individuals. As all daphnids were euthanized/flash frozen at an age of 21 days, this tradeoff was not assessed. However, although speculative, the fact that 31 percent of kairomone-exposed daphnids in the first generation died before reaching an age of 21 days compared to zero percent in the case of non-exposed daphnids might imply a negative correlation between reproductive effort and lifespan to be present. The same trend could also be observed in the second generation, where 23 percent of K-K daphnids died before an age of 21 days compared to eleven percent in the case of C-C daphnids. The mortality of K-C daphnids was intermediate (15 percent), in conformity (see below) with a possible negative correlation between reproductive effort and lifespan.

In addition, the observed trend might also be a consequence of increased stress associated with predation-perception[143] possibly resulting in a decreased immune functioning[144]. This hypothesis is supported by the observation that K-C daphnids (who experienced predation-perception only during their embryonic development) seemed to have a lower mortality than did K-K individuals (who were exposed for a longer time and hence might have experienced more stress). Worth noting is that immune functioning has been shown to trade-off with reproduction[145,146]. Thus, immune functioning and reproduction with regard to lifespan might in this light be regarded as two sides of the same coin.

In compliance with the implied tradeoff between reproduction and lifespan, individuals from the K-C condition (second generation) exhibit a clutch size that is in general intermediate between clutch sizes from C-C and K-K daphnids for all four broods. Moreover, this trend could also be observed for the size of the neonates (spine incl.) for the first two broods (and for neonate size of the first brood, spine excl.) as well as for the total reproductive output in the first two broods. Offspring from kairomone-exposed mothers seem, as expected, to exhibit an anti-predator response even when these are not subjected to the simulated predation risk themselves. This effect can be interpreted as a transgenerational epigenetic effect in the form of transgenerational phenotypic plasticity (or a maternal effect, as often called in *Daphnia*-literature[147]), in conformity with previous studies reporting transgenerational epigenetic effects in *Daphnia*[49,112,131].

It is worth noting, however, that the observed transgenerational effect may result from an embryonic exposure while residing in the mothers' brood chamber, rather than from an inherited epigenetic effect. Embryonic exposure has been reported to be important in the establishment of the epigenome in the regulation of the lifecycle of *Daphnia*, whereas postembryonic exposure largely maintains the established epigenome[128]. Under the premise that this is not only the case for the epigenetic regulation of the lifecycle but also applies for the epigenetic regulation of anti-predator responses, this might be an explanation as to why the observed response in K-C individuals is not as pronounced as in K-K individuals.
Both hypotheses (transgenerational epigenetic effect vs. embryonic exposure) however are not mutually exclusive.

Furthermore, differences between bottom- and middle layer individuals were observed in the second generation. Bottom-layer daphnids produced a larger number of offspring in three out of four quantified broods. Moreover, also the total reproductive investment appears to be higher in brood three and four in the bottom-layer daphnids. These results imply that bottom-layer individuals allocate more energy to reproduction than do middle-layer individuals, contradicting our expectations.

While we observed that kairomone-exposed daphnids in general allocated more energy towards reproduction than did non-exposed daphnids (i.e. phenotypic plasticity) during the first two broods, we find the opposite effect in terms of genetic adaptation for this trait: individuals with a history of relatively high predation pressure (middle layer) allocate less energy towards reproduction than do individuals without a predation-intense history, at least in second-generation daphnids. This counterintuitive pattern could be explained if assumed that upon creation of the pool in 1970[118], the pool was colonised by daphnids originating from a pool with high predation pressure. As such, these founder daphnids might have been genetically adapted to their prior, high-predation environment mediated by a strong selective force (as possibly imposed by fish-predation) in favour of larger clutch sizes. After colonisation of the new pond, the following three years of relatively low benthivorous fish-predation pressure[118] might not have been sufficient to possibly select again for lower clutch sizes. Aside from the short period of time, this idea is based on the fact that benthivorous fish have been demonstrated to effectively reduce the biomass of large zooplankton such as *Daphnia*. This was done directly through increased predation pressure and/or indirectly via increased levels of suspended sediment[148], the latter because suspended sediment hinders the grazing of *Daphnia*[149]. On from 1973 and for approximately ten years (corresponding with the middle sediment layer), the pool was stocked with a high number of planktivorous fish, resulting in a high predation pressure. However not observed, this was expected to effectively select for daphnids producing larger clutch sizes. We postulate the lack of this observation to possibly be due to an indirect consequence of the presence of planktivorous fish, rather than a direct effect of predation (e.g. through changes in competition and resource availability).

The layer-specific difference observed in the second generation (for clutch- and offspring size and total reproductive output, see above) was not observed in the parental generation. This is possibly due to a larger sample size in the second generation (n_{bottom} = 126 and n_{middle} = 149) compared to the first generation (n_{bottom} = 31 and n_{middle} = 31).

4.2.2 Reproductive age

In contrast to our hypothesis, simulated predation risk did not alter maturation time. Yet, age at brood two, three and four of the second generation was lower for K-K individuals compared to C-C individuals. Thus, from the second brood onwards, K-K individuals produced clutches of offspring at a faster rate. This effect becomes more pronounced at later broods, possibly because the effect sums up in time and is therefore more prominent later in life. Unlike clutch size, offspring size and total reproductive effort, reproductive age (at brood two, three and four) of K-C individuals is not intermediate between reproductive age of C-C and K-K individuals.

This implies that, while kairomone-exposed mothers from the first generation did not exhibit a shift in reproductive age, they neither transmitted non-genetic information (related with reproductive age linked with perceived predation risk) to their offspring. The fact that K-C individuals did not differ significantly from C-C individuals (nor showed a consistent trend) might suggest that, in this case, post-embryonic exposure is the determining factor to elicit a detectable antipredator response in reproductive age.

Although the interaction of treatment with sediment layer of origin was never significant, individuals originating from the bottom layer seem not to exhibit a shift in reproductive age in response to perceived predation risk, while individuals originating from the middle layer did exhibit a shift, as indicated by post-hoc Tukey HSD tests (Fig. 19). Individuals originating from the bottom sediment layer have a history of relatively low fish predation pressure, hence we expected the selective force favouring an antipredator response to be relatively weak. Conversely, individuals originating from the middle sediment layer have a history of relatively high fish predation pressure, hence the selective force favouring an antipredator response was expected to be strong. Although a formal conclusion about this genotype-dependent response cannot be made from our results, our above observation is in accordance with our expectations. Moreover, previous studies reported similar results. One such a study found that the effect of simulated predation threat on phototactic behaviour and neonate size was more strongly pronounced in *Daphnia* originating from fish-habitat compared to *Daphnia* originating from fishless habitat[74], confirming a similar result found in another study on phototactic behaviour in *Daphnia*[150].

Age at maturity and at brood two was significantly higher for bottom-layer than for middle-layer individuals. Daphnids originating from the middle layer thus reproduce significantly faster compared to daphnids originating from the bottom layer; this is an example of genetic adaptation to predation threat and is conform our expectations. Note that the same response (although in that case the response was plastic) was observed when daphnids were exposed to kairomone-conditioned water (see above), but could only be observed at later broods. Layer-specific differences in reproductive age could not be observed in first-generation daphnids, possibly due to the smaller sample size in the first generation compared to the second generation.

4.2.3 Size of parental daphnids

The simulated predation risk only had a significant effect on size at birth, size at day five and size at maturity for the second generation, but had no effect on the size of the first-generation individuals. (Note that, although not explicitly stated, this was also the case for reproductive age of first-generation daphnids.) This difference might be due to the fact that K-C and K-K individuals from the second generation all have been exposed to simulated predation risk during their embryonic development, while first-generation individuals from the predator condition were only exposed during their postembryonic stages. Embryonic exposure might thus be important for the establishment of the epigenome in the regulation of body size (and reproductive age, see above) in response to predation risk (confer the regulation of the life cycle[128]). Alternatively, the observed difference between both generations might also be explained by a higher sample size in the second generation compared to the first generation.

Size at birth was decreased when the mother was exposed to kairomones. Many predators of *Daphnia* have been shown to be size-selective[147]. Specifically, fish – being visually oriented predators – may preferentially predate on larger prey[151,152]. In this light, the observed response is adaptive as it decreased the likelihood of being predated upon. The response observed in K-C individuals might be either due to a transgenerational epigenetic effect or be a consequence of embryonic exposure to predation risk (or both), as was also the case for clutch size, offspring size and total reproductive effort (see above).
Contrary to the response for size at birth, and contrary to our expectations, the size at five days and at maturity was increased by the simulated predation. This suggests kairomone-exposed daphnids grow significantly faster than C-C individuals. This response appears to be maladaptive as an increased size also increases the vulnerability to fish predation (see above). However, size might be linked with other traits that are stronger selected for in predatory environments. For instance, swimming behaviour was found to be linked with size[153,154], with larger daphnids exhibiting an increased swimming velocity. As locomotion allows organisms to avoid predators, faster (and larger) individuals might have an advantage over slower (and smaller) individuals. This hypothesis is however highly unlikely since such an escape behaviour seems very ineffective in case of fish predation. In fact, it has been demonstrated that fish predate more heavily on faster-swimming daphnids[155]. The increased size under simulated predation risk at maturity could also have been explained if age at maturity would have been significantly increased by perceived predation risk. However, this was not the case. The observed response is also contrary to the response observed in the size of offspring (see above), suggesting this difference in antipredator response between generations should be investigated more thoroughly.

Sizes for K-C individuals were in general intermediate between sizes for C-C and K-K, suggesting a transgenerational epigenetic effect or an effect of embryonic exposure to predation risk (or both), as was also the case for size at birth, clutch size, offspring size and total reproductive effort (see above).

Size at maturity was not only dependent on treatment but was also significantly affected by the sediment layer of origin, with individuals originating from the bottom layer being significantly larger compared to individuals originating from the middle layer. The same was observed for size at day 21. Moreover, for size at day 21, the interaction of layer and treatment was significant, with no trend for treatment observed in bottom-individuals while a clear trend (C-C individuals smaller than K-C individuals smaller than K-K individuals) could be observed in middle-individuals. This observation is not unexpected since daphnids originating from the middle sediment layer have a history of higher predation pressure compared to bottom-individuals and were therefore expected to have experienced a stronger selective force favouring smaller sizes. The fact that a trend for treatment could be observed for middle-individuals but not for bottom-individuals is in compliance with the hypothesis that there was no need for an strong antipredator response to evolve in daphnids from the bottom sediment layer.

For first-generation daphnids, layer of origin had a significant effect on size at birth and size at day five. At birth, individuals originating from the bottom sediment layer were significantly smaller than individuals from the middle layer. This observation is unexpected since, in the light of predation pressure, middle layer individuals were expected to be smaller compared to bottom layer individuals since they most probably have experienced a higher selection pressure towards smaller sizes (i.e. genetic adaptation). However, at day five, bottom layer individuals were significantly larger than middle layer individuals, implying daphnids from the bottom layer grow faster early in life compared to daphnids from the middle layer. This effect seems to gradually wear off later in life, implying middle layer daphnids eventually catch up in size.

4.2.4 Covariances between relatives

Despite all daphnids within the same clone were genetically identical, mean covariances between relatives were not. This finding suggests that although there is no genetic variation within clones, there is phenotypic variation. Moreover, since the experiment was conducted in standardised conditions, this phenotypic variation can be attributed to epigenetic effects. If these epigenetic effects would be of a transgenerational nature, one would expect the covariance between parent-offspring to exceed the covariance between sisters, which in turn should exceed the covariance between nieces followed by the covariance between great aunt-grandniece and the covariance between grandnieces (in descending order).

This expectation is based on the fact that there is one epigenetic reset generation between parent-offspring, two between sisters, four between nieces, five between great aunt-grandniece and six between grandnieces (see 2.2.4).

This general pattern, however, could not be observed. Based on the experimental setup presented in Tal *et al*.[133], transgenerational epigenetic effects were not observed as the covariance between parent-offspring never exceeds the covariance between sisters. Even more so, the inversed pattern could be observed for all variables (covariance between sisters exceeds covariance between parent-offspring), regardless of the applied treatment. The phenotypic similarity between sisters hence appears to be bigger than between parents and offspring. We believe this can be attributed to within-generation phenotypic plasticity in the offspring. Although the experiment was standardised as fully as possible, environmental differences may have arisen between generations, for instance due to a difference in the conditions of the food source. All daphnids consistently received an equal amount of algae, but since these algae needed to be cultured, second-generation daphnids may have received for instance algae that were cultured on a different day (and moreover may not have been of the same age or health condition) than did first-generation algae.

Following the same rationale, the general observation is made that covariances between relatives within the same generation (sisters, nieces and grandnieces) always greatly exceed the covariances between relatives over generations (parent-offspring and great aunt-grandniece), suggestive of within-generation phenotypic plasticity. It is worth noting, however, that the covariances between grandnieces are generally larger than the covariances between parent-offspring and between great aunt-grandniece, yet generally smaller than the covariances between sisters and between nieces. Given there are more epigenetic reset generations between grandnieces than between sisters and between nieces, this is in favour of the view that a certain degree of transgenerational epigenetic effects might be present.

Interestingly, more observations imply transgenerational epigenetic effects to be present in our experimental setup. For instance, the covariance between parent-offspring in general exceeds the covariance between great aunt-grandniece. Also in favour of the theory presented in Tal *et al*.[133] is the observation that the covariance between sisters in general exceeds the covariance between nieces. In addition, the covariance between nieces in general exceeds the covariance between grandnieces and always exceeds the covariance between great aunt-grandniece.

These findings, and the findings of an increasing number of other studies, are in favour of a Neo-Lamarckian vision on biological evolution (see 1.1). One study, for instance, created recombinant inbred lines of two genetically-alike Thale cress plants (*Arabidopsis thaliana*) that differed to a great extend in their pattern of DNA-methylation. It was found that the epigenetic recombinant lines exhibited variation in quantitative traits with up to 30 percent heritability, demonstrating the high contribution of epigenetic effects on heritable phenotypic variation[156]. Additionally, a study demonstrates how genetic incompatibility between different strains of *A. thaliana* is established by epigenetic control mechanisms[157]. Yet another study demonstrated a high correlation between microhabitat differences and epigenetic differentiation within species of the invasive Japanese knotweed (*Fallopia* species complex) while genetic variation was relatively low[158].

The setup of the experiment in this study not only allowed for the comparison of covariances along a relatedness gradient, but also to compare covariances between treatments. In general, covariances calculated between sisters and nieces were higher in kairomone conditions (K-K) compared to the control (C-C). For grandnieces, parent-offspring and great aunt-grandniece, such patterns were not clear. In addition, in case of neonate-size, this pattern was irregular for all relatives. A higher covariance in K-K conditions compared to C-C conditions implies that individuals that are exposed to a simulated predation threat (K-K) are phenotypically more alike than are non-exposed individuals (C-C). This suggests predator-induced phenotypic plasticity. This observed pattern was more or less compatible with the results obtained from separate tests that examined the effect of origin and treatment (see 4.2.1-4.2.3). In general, whenever covariance-results were not compatible with results from separate tests, this was due to the fact that covariances implied the presence of predator-induced phenotypic plasticity while this was not detected with separate tests. One possible explanation for this discrepancy is the following. In case of phenotypic plasticity, the epigenomes of different individuals are more equally shaped compared to those of non-exposed individuals. In other words, a stressor adjusts the epigenomes of exposed individuals in the same fashion. Consequently, the phenotypic variation (for a certain trait) decreases in case of a plastic response. If under control conditions the variance of a certain trait exceeds the variance of this trait under kairomone conditions (i.e. a plastic response), while the mean trait-value remains more or less the same, the test will not detect a statistical difference between the two conditions. However, the covariance between kairomone-exposed individuals will exceed the covariance between non-exposed individuals.

When covariances between sisters (second generation) are regarded, covariances for the K-C condition are generally larger than covariances for the C-C condition with regard to number of offspring and parental size. This implies daphnids from the K-C condition are phenotypically more alike than daphnids from the C-C condition, which suggests predator-induced phenotypic plasticity. More specifically, if the higher covariance in K-C individuals is due to embryonic exposure to simulated predation threat, the observation suggests the presence of within-generation phenotypic plasticity. Alternatively, if embryonic exposure is not enough to invoke a clear plastic response, the observation suggests the presence of transgenerational phenotypic plasticity. Note that the results obtained from these covariances are compatible with the effects of separate tests on these traits. Moreover, covariances (between sisters) for reproductive age did not suggest predator-induced phenotypic plasticity, while the same negative result was inferred from the separate tests.

Note that Tal *et al*.[133] suggested to investigate possible transgenerational epigenetic effects based on the comparison of covariance between parent-offspring on the one hand and covariance between their offspring on the other hand. With merely this setup, transgenerational epigenetic effects could not be observed in our study system. Our expanded setup however (the 'epigenetic gradient') allowed us to investigate the presented theory more thoroughly and allowed us to make more nuanced conclusions. To our knowledge, this is the first study in the light of epigenetics to apply this expanded setup.

4.3 Conclusion - Phenotypic variation in natural populations

In biological evolution, phenotypic variation in natural populations acts as a substrate for natural selection[6,13,14] (see 1.1). As illustrated in this study and by numerous other studies, (heritable) phenotypic differences between individuals are caused by genetic differences (i.e. encoded in the DNA sequence) which is a fundamental idea upon which the Modern Evolutionary Synthesis is based[5]. When selection acts on this phenotypic variation, it indirectly acts on the (heritable) genetic variation in the population[6,7]. By doing so, it may push a population towards a phenotype that is tuned for the environment it finds itself in, a process called genetic adaptation[159]. Indeed, this study shows genetic adaptation both in *N. furzeri* as in *D. magna*. Even after several generations of breeding under standardised laboratory conditions, phenotypic differences between populations could be observed. For *N. furzeri* this is for populations separated in space while for *D. magna* this is for populations separated in time.

Phenotypic variation, however, is not merely determined by genetic variation, but is mediated by non-genetic effects imposed by the environment[22,25,45]. For instance, in response to environmental changes, individuals may alter their phenotypic appearance so to maximise their fitness in the new conditions. Such phenotypic plasticity has been demonstrated in an array of studies and has been confirmed in this study. Both *N. furzeri* and *D. magna* exhibited predator-induced responses (active phenotypic plasticity) with regard to life-history traits. In *D. magna*, also passive phenotypic plasticity could be recorded, as probably invoked by the quality-conditions of the food.

The capacity for phenotypic plasticity is encoded in the DNA sequence (hence, is heritable) and might be subjected to biological evolution[54]. In the present study, this genotype-dependent phenotypic plasticity could for instance be illustrated by the fact that *Daphnia* originating from the bottom sediment layer exhibited no shift in reproductive age in response to predation threat whereas *Daphnia* originating from the middle sediment layer did. Genotype-dependent phenotypic plasticity can also be expressed in terms of a different direction of the response. Here, for instance, *N. furzeri* from the NF222 population exhibited an increased fecundity when exposed to simulated predation risk, whereas individuals from the NF414 population exhibited a decreased fecundity under such conditions.

In the framework of the Modern Evolutionary Synthesis, phenotypic variation brought about by environmental changes are traditionally not regarded as heritable[10]. Thus in case of phenotypic plasticity, although the response itself might be evolvable, the 'acquired' phenotype *in se* is not heritable (until it potentially becomes genetically assimilated[52]). However, recent studies on the heritability of 'acquired traits' yield increasing evidence in favour the inheritance of non-genetic information[10,14], in support of the Neo-Lamarckian vision on biological evolution (see 1.4 and 4.2.4). Such transmissions of non-genetic information are collectively termed as transgenerational epigenetic effects[22]. Based on comparison of covariances as a proxy for the phenotypic similarity between genetically identical relatives (in *D. magna*), we demonstrate in the present study that even in the absence of genetic variation, heritable phenotypic variation can arise. And secondly, also in support of transgenerational epigenetics, we observed naïve offspring from kairomone-exposed daphnids exhibit to a certain degree a phenotypic plastic response despite not being exposed to simulated predation risk. Although generally termed as a maternal effect, we include such effects in the collective of transgenerational phenotypic plasticity.

In 1.5.2 and 1.6 we reviewed that a certain degree of within-generation- and transgenerational phenotypic plasticity respectively may facilitate genetic evolution (and allow for quicker genetic adaptation) in a changing environment by bringing a population in the vicinity of a new optimal adaptive peak. Transgenerational phenotypic plasticity might even be more advantageous than within-generation plasticity since it circumvents the lag-phase in the offspring between assessing the environment and responding accordingly with a shift in phenotype[25,160]. The presence and advantage of both processes is however highly dependent on the variability and predictability of the environment. In very stable environments, 'acquired' traits can become genetically fixed and, subsequently, the capacity for phenotypic plasticity might be selected against due to costs related with the maintenance of plasticity or might disappear from the population due to genetic drift[25,160] (see 1.5.2). In rapidly changing or unpredictable environments, aqcuired phenotypes might not match the changing environmental conditions. As current conditions might be a bad predictor for future conditions in such unstable environments, non-genetic information might not offer an advantage for the offspring in terms of fitness and might even be disadvantageous[25,68]. According to Lachmann & Jablonka[161], transgenerational phenotypic plasticity is advantageous when, in a fluctuating environment, each environmental state lasts longer than the organism's generation time, yet not long enough for genetic assimilation to be established.

The observation that phenotypic variation is much more than a direct reflection of genetic variation points to the hiatus in the Modern Evolutionary Synthesis and advocates for an extension of the theory. In addition, it points to the counterproductive gap between the field of evolutionary biology and the field of ecology. Despite the upcoming field of evo-eco-devo[162,163], there is still a lack of integration between evolutionary biology and ecology, as illustrated by, for instance, the use of the rather dubious distinction between 'evolutionary-' and 'ecological timescales'[4]. The current study demonstrates that the genome and ecology interact with each other and are both reflected in the heritable phenotypic variation of natural populations. Therefore, although the very notion of ecology is embedded in the concept of natural selection, also the origin of the substrate which natural selection acts upon (i.e. phenotypic variation) should be regarded as subjected to an ecological context.

4.4 Future research perspectives

For future research, it would be interesting to fine-tune the experimental setup of *D. magna* presented in this study. For instance, in order to detect transgenerational phenotypic plasticity as a pure epigenetic effect, an improved experimental setup should allow for exclusion of the possibility of embryonic exposure. This could be achieved by, for instance, assessing the persistence of a possible plastic response over more generations.

As individuals of the F3 generation would still have been exposed to the simulated stressor as germ cells of their embryonic parents, assessments should at least include the F4 generation.
Given the recent technological advances in molecular biology, such analyses at the phenotypic level should be complemented with analyses at the molecular level. Throughout the entire study suggested above, the maintenance of, for instance, epigenetic marks can be tracked and used as additional information. Genomic analyses can reveal which loci are involved in, for instance, the phenotypic response. Genetic and non-genetic analyses at those loci may lead to a much more detailed picture of eco-evolutionary processes in natural populations. Given the wide spectrum of molecular mechanisms involved in epigenetic inheritance (see 1.3), this might prove to be quite a big challenge.

Additionally, in order to gain more insight in phenotypic plasticity, the effectiveness of the antipredator responses could be investigated by subjecting the organisms to actual predation and assessing differences in fitness between plastically responding and non-responding organisms.

Also theoretical biologists can share in the interesting challenges for future research. While the theory presented in Tal *et al*.[133] is based on epigenetic reset between generations, and is thus based on the inheritance of chromatin marks, other mechanisms of cellular epigenetic inheritance (see 1.3) should be accounted for. Moreover, and even more challenging, next to different mechanisms of cellular epigenetic inheritance, also behavioural (and symbolic) inheritance systems (see 1.2) could be included in a unifying theory. All this theoretical work implies a lot of additional integrated experimental research to be done over various fields of study (ecology, evolutionary biology, developmental biology, molecular biology, genetics).

Many questions remain to be answered. For instance, if transgenerational phenotypic plasticity of a certain trait operates through the inheritance of chromatin marks, then what mechanism is responsible for the fact that these marks are not randomly reset during meiosis and development? Also, what is the relative importance of different mechanisms accountable for phenotypic variation on the process of evolution, and how do these mechanisms interact with each other?

The expanded context of inheritance gives rise to a manifold of new questions. Nonetheless, as Dobzhansky pointed out for us in 1973[1], it all makes sense in the light of evolution.

Summary

Phenotypic variation is the substrate of natural selection, one of the pillars of the Modern Evolutionary Synthesis. Despite the traditional view that heritable phenotypic variation is a mere translation of genetic variation, there is increasing evidence for heritable phenotypic variation that is acquired during the organism's lifetime. Transmissions of non-genetic information to the offspring are collectively termed as transgenerational epigenetic effects and are in favour of the Neo-Lamarckian vision on evolution. In this study, we assessed the relative contribution of genetic and non-genetic effects on phenotypic variation in distinct populations of the Turquoise killifish *Nothobranchius furzeri* and the water flea *Daphnia magna*. This was done so by investigating plastic shifts in life-history traits in response to perceived predation risk. For *D. magna*, multiple generations were assessed. In addition, we compared covariances, a proxy for the phenotypic similarity, between genetically identical relatives along an epigenetic gradient. For both study organisms, we found evidence of genetic adaptation and within-generation phenotypic plasticity responsible for the observed phenotypic variation. Moreover, we demonstrate that even in the absence of genetic variation, heritable phenotypic variation can arise. These results advocate for an extension of the current Modern Synthesis as it suggests that evolution might not be based solely on genetic inheritance, but on an expanded context of inheritance, covering genetic as well as non-genetic mechanisms.

Samenvatting

Fenotypische variatie is het substraat waarop natuurlijke selectie, één van de pijlers van de Moderne Evolutionaire Synthese, inwerkt. Ondanks de traditionele opvatting dat overerfbare fenotypische variatie louter een uiting van genetische variatie is, is er alsmaar meer bewijs voor overerfbare fenotypische variatie die verworven wordt tijdens de ontwikkeling van een organisme. De overerving van niet-genetische informatie wordt een trans-generationeel epigenetisch effect genoemd en staat ten gunste van de Neo-Lamarckiaanse kijk op evolutie. In dit onderzoek werd onderzocht wat de relatieve bijdrage is van genetische en niet-genetische effecten op fenotypische variatie in afzonderlijke populaties van de Turquoise killivis *Nothobranchius furzeri* en de watervlo *Daphnia magna*. Hiertoe werden plastische responsen in levensgeschiedeniskenmerken als reactie op predatiedreiging onderzocht. Voor *D. magna* werden meerdere generaties opgevolgd. Bijkomend werden covarianties, een proxy voor fenotypische gelijkenis, tussen genetisch identieke verwanten onderzocht overheen een epigenetische gradiënt. Voor beide studieorganismen werd bewijs gevonden van genetische adaptatie en binnen-generatie fenotypische plasticiteit verantwoordelijk voor de geobserveerde fenotypische variatie. Bovendien werd aangetoond dat zelfs in de afwezigheid van genetische variatie er toch overerfbare fenotypische variatie kan ontstaan. Deze resultaten pleiten voor een uitbreiding van de huidige Moderne Synthese daar ze suggereren dat evolutie niet enkel gebaseerd is op genetische overerving, maar op een bredere context van overerving die zowel genetische als niet-genetische overerving omvat.

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Addendum 1 – Risk analysis

A first risk that acquires attention is the potential exposure to hazardous chemicals. In terms of safety, a lab coat, safety goggles and clean nitrile gloves should be worn at all times when working with such chemicals. Chemicals used in this study are ethanol (80%) and FMC (3.7g malachite green and 3.7g methylene blue in 1L of 37% formalin) (see Table S1 for H- and P-phrases). When the laboratory facility was left, hands were washed and disinfected and the lab coat was left in the lab.

Drugs applied to the fish were Levamisole (5% solution) and Flubendazole (2mg/L) and were managed conform the safety instructions provided in the information leaflet.

As the animals were housed in water, the floor of the facility tended to get wet when handling the animals. Additionally, crutches were used in order to reach the upper aquaria. In this respect, it is important to wear fitting, non-slippery footwear. Spilling water was avoided and wet floors were cleaned frequently.

In order not to infect the animals, nor ourselves, the workstation was kept neat and clean, hands were frequently washed and food nor drinks were consumed in the workspace. Long hair was tied back so not to impair the vision nor to get stuck on lab equipment, nor contaminate the animal containers.

Predatory fish were handled with small fishing nets in order to avoid injure for the researcher as well as for the fish.

Table S1: H- and P- phrases of the used hazardous chemicals in the laboratory.

Chemical	H- and P- phrases
Ethanol	- H225: Highly flammable liquid and vapour - H302: Harmful if swallowed - H371: May cause damage to organs - P210: Keep away from heat/sparks/open flames/hot surfaces – No smoking - P250: Do not subject to grinding/shock/…/friction
Malachite green	- H302: Harmful if swallowed - H318: Causes serious eye damage - H361: Suspected of damaging fertility or the unborn child - H410: Very toxic to aquatic life with long lasting effects - P273: Avoid release to the environment - P280: Wear protective gloves/protective clothing/eye protection/face protection - P305+351+338: IF IN EYES: Rinse continuously with water for several minutes. Remove contact lenses if present and easy to do – continue rinsing - P501: Dispose of contents to a convenient waste disposal container
Methylene blue	- H302: Harmful if swallowed - H315: Causes skin irritation - H319: Causes serious eye irritation - H335: May cause respiratory irritation - P261: Avoid breathing dust/fume/gas/mist/vapours/spray - P305+351+338: IF IN EYES: Rinse continuously with water for several minutes. Remove contact lenses if present and easy to do – continue rinsing
Formalin	- H301: Toxic if swallowed - H311: Toxic in contact with skin - H314: Causes severe skin burns and eye damage - H317: May cause an allergic skin reaction - H331: Toxic if inhaled - H335: May cause respiratory irritation - H351: Suspected of causing cancer - H370: Causes damage to organs - P260: Do not breathe dust/fume/gas/mist/vapours/spray - P280: Wear protective gloves/protective clothing/eye protection/face protection - P301+310: IF SWALLOWED: Immediately call a POISON CENTER or doctor/physician - P305+351+338: IF IN EYES: Rinse continuously with water for several minutes. Remove contact lenses if present and easy to do – continue rinsing - P310: Immediately call a POISON CENTER or doctor/physician

Addendum 2

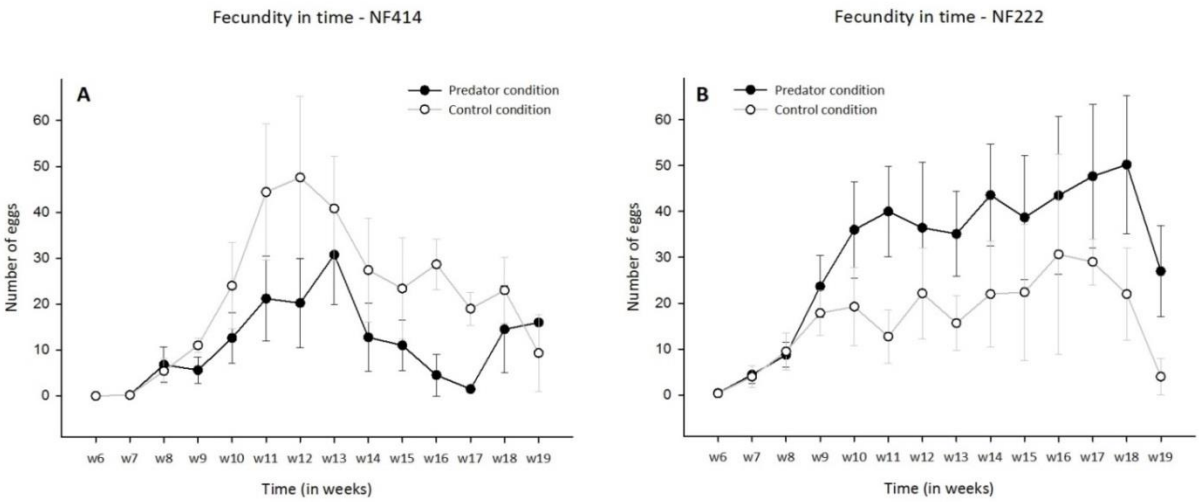

Figure S2.1: Mean number of produced eggs (fecundity) between week six and week 20 for females of population (A) NF414 and (B) NF222. Whiskers delineate standard error of mean.

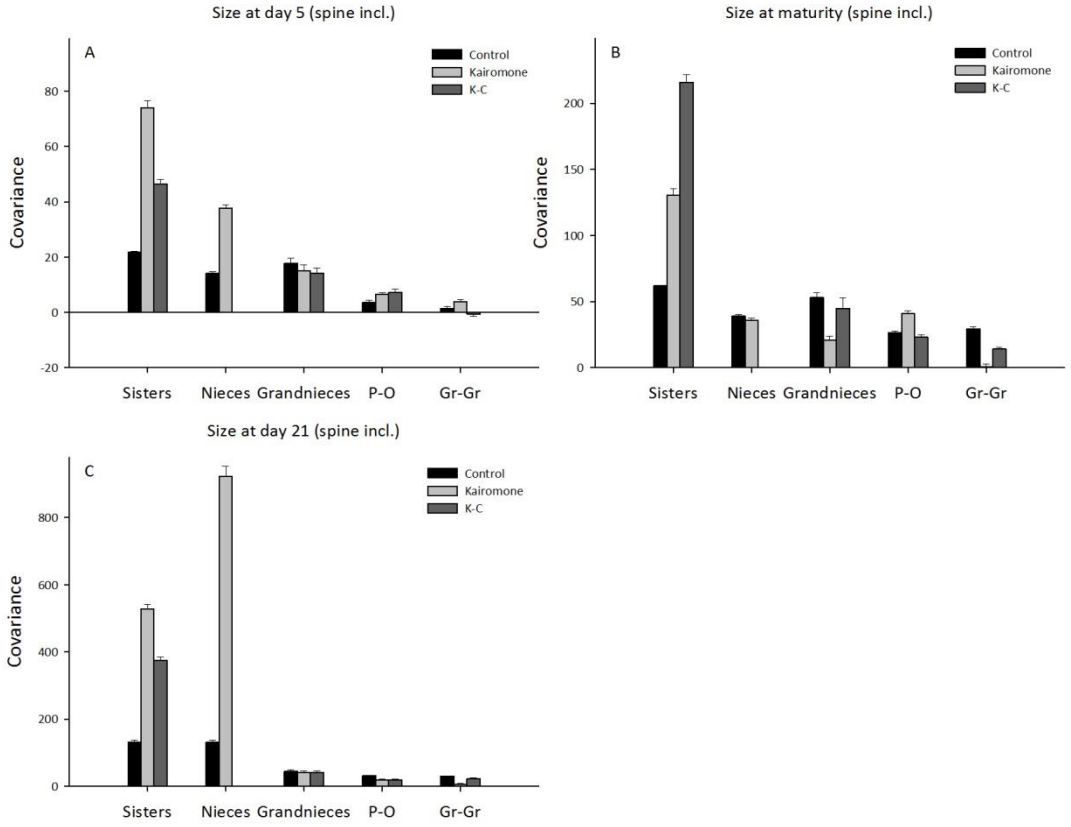

Figure S2.2: Covariances for (A) size at day 5, (B) size at maturity and (C) size at day 21. Sizes include the spine of the daphnids. Whiskers delineate 95% confidence intervals. (P-O = parent-offspring ; Gr-Gr = great aunt-grandniece)

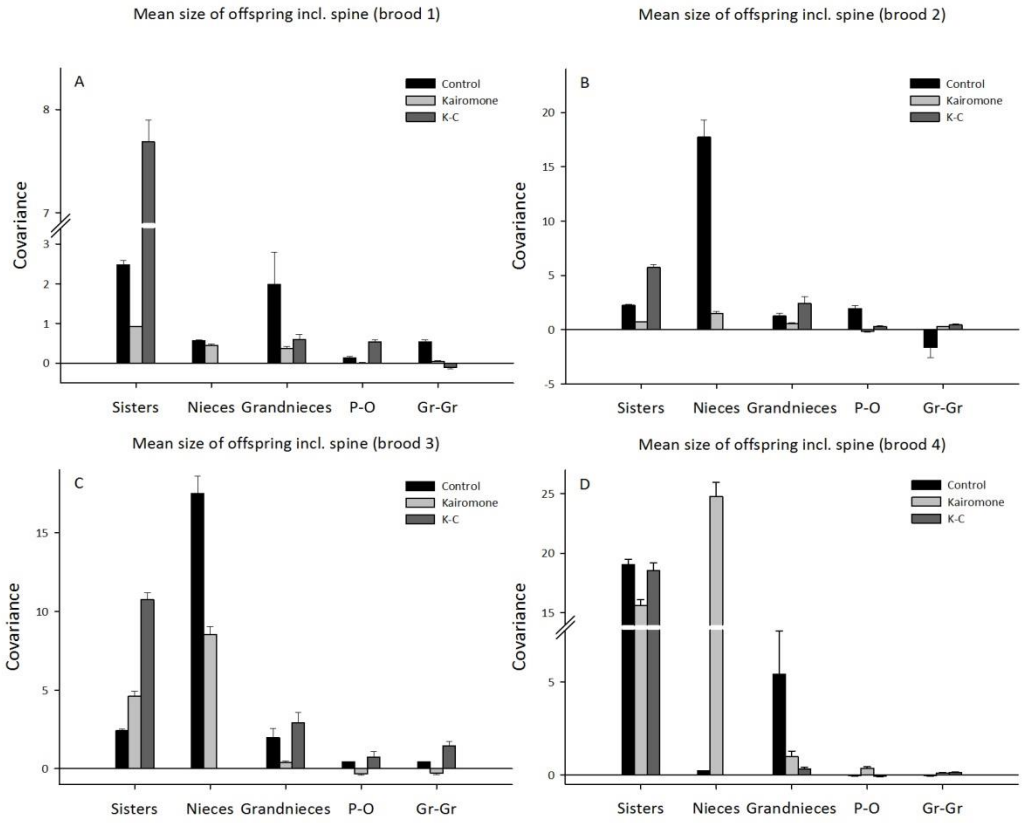

Figure S2.3: Covariances for size of offspring (neonate-size) (A) at brood 1, (B) at brood 2, (C) at brood 3 and (D) at brood 4. Sizes include the spine of the daphnids. Whiskers delineate 95% confidence intervals. (P-O = parent-offspring ; Gr-Gr = great aunt-grandniece)

Table S2a: Results of separate tests on variables that take into account the size of the spine of first-generation daphnids. Significant effects are depicted in bold and underlined. (Total reproductive output = number of offspring times mean size of offspring)

			Fixed effects						
Category	**variable**	**Test**	**Layer**		**Treatment**		**Layer*Treatment**		**Random effects**
Size	Size at birth	ANOVA	t = 5.39	**<u>$p < .0005$</u>**	t = -0.109	p = .914	t = 0.501	p = .619	'Clone'
	Size at day 5	ANODEV	$X^2 = 12.6$	**<u>$p < .0005$</u>**	$X^2 = 0.812$	p = .367	$X^2 = 0.790$	p = .374	'Clone'
	Size at maturity	ANOVA	t = -2.91	**<u>$p < .05$</u>**	t = 1.62	p = .111	t = 0.453	p = .652	'Clone'
	Size at day 21	ANOVA	t = -3.49	**<u>$p < .01$</u>**	t = -0.377	p = .708	t = -1.42	p = .166	'Clone'
Size of offspring at birth	Size of brood 1	ANOVA	t = 0.097	p = .924	t = 0.660	p = .512	t = -1.39	p = .171	'Clone'
	Size of brood 2	GLS	t = 1.20	p = .237	t = -0.944	p = .349	t = 0.0919	p = .927	None
	Size of brood 3	GLS	t = 0.218	p = .828	t = -0.553	p = .583	t = -2.12	**<u>$p < .05$</u>**	None
	Size of brood 4	ANODEV	$X^2 = 0.177$	p = .674	$X^2 = 61.3$	**<u>$p < .0001$</u>**	$X^2 = 0.958$	p = .328	'Clone'
Total reproductive output	Brood 1	ANODEV	$X^2 = 0.813$	p = .367	$X^2 = 21.6$	**<u>$p < .0001$</u>**	$X^2 = 0.0009$	p = .976	'Clone'
	Brood 2	ANOVA	t = -0.284	p = .787	t = 3.34	**<u>$p < .005$</u>**	t = -1.001	p = .323	'Clone'
	Brood 3	ANODEV	$X^2 = 4.78$	**<u>$p < .05$</u>**	$X^2 = 0.261$	p = .609	t = 0.00250	p = .960	'Clone'
	Brood 4	ANOVA	t = -0.148	p = .887	t = -2.21	**<u>$p < .05$</u>**	t = 1.32	p = .196	'Clone'

Table S2b: Results of separate tests on variables that take into account the size of the spine of second-generation daphnids. Significant effects are depicted in bold and underlined. (Total reproductive output = number of offspring times mean size of offspring)

			Fixed effects						
Category	**variable**	**Test**	**Layer**		**Treatment**		**Layer*Treatment**		**Random effects**
Size	Size at birth	ANOVA	$F_{1,252} = 0.740$	$p = .390$	$F_{2,252} = 35.6$	**$p < .0001$**	$F_{2,252} = 0.192$	$p = .826$	None
	Size at day 5	ANOVA	$F_{1,263} = 0.097$	$p = .755$	$F_{2,263} = 15.4$	**$p < .0001$**	$F_{2,263} = 1.24$	$p = .291$	None
	Size at maturity	ANOVA	$F_{1,247} = 141$	**$p < .0001$**	$F_{2,274} = 19.4$	**$p < .0001$**	$F_{2,274} = 3.30$	**$p < .05$**	None
	Size at day 21	ANOVA	$F_{1,207} = 104$	**$p < .0001$**	$F_{2,207} = .79$	$p = .0636$	$F_{2,207} = 2.43$	$p = 0.0908$	None
Size of offspring at birth	Size of brood 1	ANOVA	$F_{1,254} = 0.777$	$p = .379$	$F_{2,254} = 5.67$	**$p < .005$**	$F_{2,254} = 0.203$	$p = .817$	None
	Size of brood 2	ANOVA	$F_{1,247} = 3.88$	**$p = .05$**	$F_{2,247} = 3.22$	**$p < .05$**	$F_{2,247} = 0.850$	$p = .429$	None
	Size of brood 3	ANOVA	$F_{1,228} = 0.671$	$p = .414$	$F_{2,228} = 0.404$	$p = .668$	$F_{2,228} = 0.258$	$p = .773$	None
	Size of brood 4	ANOVA	$F_{1,182} = 1.02$	$p = .313$	$F_{2,182} = 4.44$	**$p < .05$**	$F_{2,282} = 6.85$	**$p < .005$**	None
Total reproductive output	Brood 1	ANODEV	$X^2 = 0.669$	$p = .413$	$X^2 = 61.0$	**$p < .0001$**	$X^2 = 2.34$	$p = .311$	'Clone'
	Brood 2	ANODEV	$X^2 = 0.825$	$p = .363$	$X^2 = 44.5$	**$p < .0001$**	$X^2 = 8.37$	**$p < .05$**	'Clone'
	Brood 3	ANOVA	$F_{1,227} = 22.7$	**$p < .0001$**	$F_{2,227} = 2.59$	$p = .0776$	$F_{2,227} = 0.008$	$p = .992$	None
	Brood 4	ANOVA	$F_{1,182} = 26.8$	**$p < .0001$**	$F_{2,182} = 0.913$	$p = .403$	$F_{2,182} = 0.843$	$p = .432$	None

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