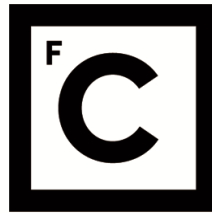UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

Evolutionary lessons from *Drosophila melanogaster* for colonization. How do history, selection and effective population size shape evolution?

Doutoramento em Biologia
Especialidade de Biologia Evolutiva

Marta Alexandra Arandas dos Santos

Tese orientada por:
Professora Doutora Margarida Matos e Professor Doutor Michael R. Rose

Documento especialmente elaborado para a obtenção do grau de doutor

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To *Inês*, and all the little girls out there, whom deserve a better tomorrow. Stay strong, be humble, and keep going. Look within, where it lies all the strength and resilience to struggle, to fight vigorously, and to strive! It really does not matter what road you take…

The Road Not Taken

Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveler, long I stood
And looked down one as far as I could
To where it bent in the undergrowth;

Then took the other, as just as fair,
And having perhaps the better claim,
Because it was grassy and wanted wear;
Though as for that the passing there
Had worn them really about the same,

And both that morning equally lay
In leaves no step had trodden black.
Oh, I kept the first for another day!
Yet knowing how way leads on to way,
I doubted if I should ever come back.

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I–
I took the one less traveled by,
And that has made all the difference.

– Robert Frost –

Acknowledgements

Abstract

Understanding the factors that constrain adaptation, namely in a colonization scenario, has been a major topic in evolutionary biology and was the chief focus of this thesis. Using a highly-replicated experimental evolution design with well-characterized *Drosophila melanogaster* populations, we aimed to respond to several evolutionary questions relevant for the colonization of a new habitat. First, we showed that reduced effective population size (1) impaired the responses to directional selection, (2) increased between-population differentiation, and (3) shaped the signatures of history and chance, which were overrun by selection in larger populations. Second, we saw that interpopulation hybridization can have strong effects on a population's subsequent evolution, especially under a sustained bottleneck. Most importantly, the outcome of hybridization is unpredictable, due to the complex genetic architecture of fitness-related traits and the multitude of interfering factors. This calls for caution on the use of hybridization in conservation management, especially in small, endangered populations. Third, we showed that evolutionary history is very important for a population's subsequent evolution and fate, namely in a reverse colonization scenario. We additionally showed that the evolutionary patterns during reverse evolution are contingent to the trait under study. Finally, we presented the first, while crude, experimental test of the *Hamiltonian wave* of adaptation. We found that (1) small changes in diet can have significant effects on age-specific mortality but could not determine whether adaptation to a novel diet was greater at earlier than later ages, and (2) the age-specific decrease in differentiation between adapted and non-adapted populations, predicted by the Hamiltonian hypothesis, was not verified in our system. Despite the high replication and complex design of our experiments, many questions remain unanswered. Other studies involving genomic analysis of our populations, other traits, and diets will shed light on how history, selection, and effective population size shape evolution during colonization.

Keywords: *adaptive evolution, colonization, Drosophila melanogaster, effective population size, experimental evolution*

Resumo

A compreensão dos factores que limitam a adaptação, nomeadamente durante a colonização, é um tema importante em evolução, sendo o objectivo principal desta tese. Utilizando populações de *Drosophila melanogaster* bem caracterizadas, num estudo de evolução experimental altamente replicado, procurámos responder a questões evolutivas relevantes para a colonização de um novo habitat. Primeiro, mostrámos que a redução do efectivo populacional (1) diminuiu as respostas à selecção direccional, (2) aumentou a diferenciação interpopulacional e (3) modelou as assinaturas da história e do acaso, rapidamente superadas pela selecção em populações grandes. Segundo, vimos que a hibridação interpopulacional pode ter fortes efeitos na evolução das populações, especialmente sob N_e reduzido. Sobretudo, verificámos que as consequências da hibridação são imprevisíveis, pela complexa arquitectura genética das características da história da vida e multiplicidade de factores que intervêm na sua evolução. Como tal, alertamos para o uso da hibridização em programas de conservação, especialmente em populações pequenas e ameaçadas. Terceiro, mostrámos que a história evolutiva é fundamental para a subsequente evolução da população, nomeadamente num cenário de colonização reversa, e que os padrões evolutivos durante a evolução reversa são contingentes às características analisadas. Finalmente, apresentámos o primeiro, apesar de rudimentar, teste experimental da *onda Hamiltoniana* da adaptação. Vimos que (1) alterações pequenas na dieta das populações podem ter efeitos significativos na mortalidade específica de cada idade, mas não pudemos determinar se a adaptação à nova dieta era maior em idades mais precoces e (2) a diminuição da idade-específica na diferenciação entre populações adaptadas e não adaptadas, previstas pela hipótese Hamiltoniana, não foi verificada. Apesar da elevada replicação e do complexo design destas experiências, muitas questões permanecem sem resposta. Outros estudos envolvendo análise genómica, outras características e dietas, dar-nos-ão uma melhor compreensão de como a história, a selecção e o efectivo populacional modelam a evolução durante a colonização.

Palavras-chave: *evolução adaptativa, colonização, Drosophila melanogaster, efectivo populacional, evolução experimental*

Resumo alargado

A compreensão dos factores que influenciam e limitam o processo adaptativo, em geral, e cenários de colonização de novos habitats, em particular, tem sido alvo de elevado interesse tanto da biologia evolutiva, como da biologia da conservação. Esta tese de doutoramento foi desenvolvida com o intuito de melhor compreender estes factores, recorrendo a populações de *Drosophila melanogaster* do laboratório do Professor Michael R. Rose, evolutivamente bem caracterizadas num estudo de evolução experimental muito rigoroso e altamente replicado.

O objectivo desta dissertação foi responder, em cinco capítulos experimentais, a dez questões evolutivas relevantes num cenário de colonização: *(i)* como é que as populações respondem a novas condições demográficas? *(ii)* como é que as características da história da vida evoluem em resposta a um ambiente novo e inóspito? *(iii)* qual o efeito do tamanho efectivo da população (N_e) na resposta evolutiva à selecção direccional? *(iv)* populações com a mesma origem evoluem da mesma maneira? *(v)* quais os papéis relativos da história, do acaso e da selecção na evolução das populações? *(vi)* como é que a hibridização a diferentes N_e afecta a dinâmica evolutiva das populações sob selecção direccional? *(vii)* manter as populações em N_e reduzido influencia e/ou dificulta a colonização reversa? *(viii)* qual o impacto de novos desafios ambientais, como a mudança de dieta, durante um evento de colonização? *(ix)* a adaptação a uma nova dieta é específica da idade? E, finalmente, *(x)* a adaptação a longo prazo a condições novas leva à perda de adaptação no ambiente ancestral?

A tese está estruturada em sete capítulos. O primeiro apresenta uma revisão da literatura relevante para os assuntos abordados nos capítulos dois a seis, que reportam a contribuição empírica deste trabalho. Finalmente, no capítulo sete são discutidos de uma forma integrada os resultados obtidos, sumarizadas as conclusões gerais da tese e apresentadas algumas questões pertinentes a desenvolver no futuro.

As experiências do segundo capítulo, o estudo central da tese, foram realizadas com o intuito de responder às cinco primeiras questões. Primeiro, as idiossincrasias do protocolo de selecção para a resistência à inanição causaram alterações demográficas nas populações experimentais, aumentando a duração do ciclo de vida em relação às populações ancestrais B. Essas alterações, por si só, levaram a um aumento temporal da resistência à inanição e a uma diminuição da fecundidade jovem, padrão anteriormente observado no nosso laboratório. Segundo, a dinâmica evolutiva das características estudadas corroborou tanto previsões

teóricas como experiências anteriores no laboratório: o aumento temporal da resistência à inanição como resposta à selecção direccional forte, acompanhado pelo declínio da fecundidade precoce. Terceiro, a redução no N_e resultou (1) no declínio significativo da fecundidade, revelando a perda de função causada pelo tamanho populacional reduzido e (2) na redução significativa da resposta directa à selecção. Estes resultados corroboram as expectativas de menor desempenho associado ao N_e reduzido, devido ao aumento da consanguinidade, maior perda de heterozigosidade por deriva e consequente menor efectividade da selecção. Quarto, em experiências de evolução experimental em que as populações são mantidas separadas por várias gerações, espera-se que ocorra diferenciação interpopulacional por deriva, mais acentuada quanto menor o tamanho populacional. Os resultados confirmaram estas expectativas, com uma variância significativamente maior entre as populações pequenas em comparação com as populações maiores, na ausência de selecção direccional. Quando sob selecção forte, os padrões não foram tão claros. Quinto e por fim, é esperado que o papel relativo da história, do acaso e da selecção na evolução das populações dependa do tamanho populacional e com o tipo de características estudadas. Os nossos resultados, para além de corroborarem experiências anteriores, mostraram que a história e o acaso desempenham papéis preponderantes em populações mais pequenas, mas que são rapidamente ultrapassados pela selecção, especialmente em populações maiores.

A hibridização entre populações tem sido sugerida como estratégia para atenuar os efeitos da depressão consanguínea, aumentando o desempenho das populações pequenas (e em declínio) e diminuindo o risco de extinção – resgate genético. Porém, a complexa interacção do fluxo génico, mutação, deriva e selecção dificulta a previsão da consequência evolutiva da hibridação. As experiências do terceiro capítulo foram feitas com o objectivo de melhor compreender a evolução de populações sob diferentes combinações de selecção direccional, tamanho efectivo e fluxo génico. Durante as quinze gerações que precederam a hibridação, seria esperado que as populações sofressem um aumento de homozigotia e diferenciação interpopulacional devido aos efeitos combinados da deriva e selecção, especialmente em populações mais pequenas. Seria também expectável que a hibridação recuperasse alguma da heterozigotia, levando ao aumento dos valores médios das características relevantes para a *fitness* (*heterose*). Os resultados mostraram, como esperado, que os efeitos da heterose são generalizados e geralmente diluídos com o passar das gerações. Os resultados sobre as consequências evolutivas da hibridação revelaram padrões complexos, sugerindo que a hibridação pode ter efeitos muito fortes na subsequente evolução da população, especialmente

sob N_e reduzido, e que alguns caracteres (como a fecundidade) são mais susceptíveis à hibridação do que outros (como a resistência à inanição). Em conclusão, a complexidade da arquitectura genética das características da história da vida e a multiplicidade de factores que podem interferir com a hibridação, tornam os seus efeitos muito imprevisíveis. As expectativas teóricas clássicas podem, assim, não ser atingidas, pelo menos no intervalo de gerações e de efectivos populacionais que abordámos. Portanto, o uso da hibridação em programas de conservação deve ser feito com muito cuidado, especialmente em populações pequenas em risco de extinção.

As experiências do quarto capítulo pretenderam reproduzir um cenário de colonização reversa, onde populações com diferentes histórias evolutivas migraram de volta ao ambiente ancestral. Estudos anteriores no nosso laboratório com populações de N_e moderadamente elevado mostraram que a imposição de diferentes regimes selectivos leva à rápida divergência fenotípica e que, mais tarde, sob o mesmo regime, convergem rapidamente para um fenótipo semelhante. Com este projecto pretendíamos analisar as consequências de, após selecção direccional, manter o N_e reduzido durante as primeiras 21 gerações de selecção reversa. Os dados obtidos sugerem que a evolução reversa não é impossibilitada pelo efeito de gargalo continuado, reproduzindo os possíveis padrões teóricos de reversão ao estado ancestral: convergência completa, reversão parcial e reversão abrupta ultrapassando o ancestral. Estes padrões de reversão revelaram-se contingentes às características e/ou população em questão. Em particular, verificou-se que a assinatura da história definida pelos regimes selectivos contrastantes é muito marcada, podendo ser atenuada – mas não apagada – pela selecção reversa, pelo menos durante o período de tempo ensaiado. É, contudo, possível que estudos mais prolongados venham a revelar completa reversão.

De acordo com a *onda Hamiltoniana* da selecção natural, o impacto da selecção é maior nas idades mais jovens e diminui progressivamente com a idade cronológica, porque a força da selecção natural também diminui com a idade. Espera-se, então, que a adaptação a um novo ambiente – como uma dieta nova durante a colonização – seja muito eficaz em idades jovens, mas progressivamente mais reduzida em idades mais avançadas. As experiências dos quinto e sexto capítulos visavam testar o efeito evolutivo da alteração da dieta na adaptação dependente da idade. Em primeiro lugar, os resultados mostram que pequenas alterações na dieta têm efeitos significativos na longevidade média e nas taxas de mortalidade específicas da idade das populações, o que é expectável por não estarem adaptadas a estes ambientes. Em segundo lugar, uma mudança de dieta, na perspectiva Hamiltoniana, deveria ter um efeito mais severo quanto

mais cedo a mudança ocorresse. Os nossos dados não corroboraram essa expectativa, indicando apenas que a duração de exposição à nova dieta terá sido o principal factor de aumento das taxas de mortalidade. No entanto, pelo facto desta experiência ter sofrido alguns problemas de delineamento, as considerações sobre este assunto são tecidas com reserva. Terceiro, recorrendo ao sistema UX-AUC desenvolvido por Mueller e colaboradores, procurámos aprofundar a hipótese Hamiltoniana, testando a diminuição idade-específica na diferenciação entre populações adaptadas e não adaptadas a um ambiente relativamente tóxico (a ureia ambiental). Os resultados mostraram que as populações expostas a ureia em idade jovem têm, em geral, melhor desempenho em ureia que os controlos não adaptados, mas sem diminuição ao longo da vida da diferenciação das taxas de mortalidade. Finalmente, fomos testar o desempenho das populações adaptadas a ureia no ambiente ancestral (banana). Os dados de mortalidade mostraram que, independentemente do ambiente (ureia ou banana) e da idade dos indivíduos, as populações adaptadas à ureia tiveram taxas de mortalidade reduzidas, divergindo das expectativas de Hamilton.

As experiências realizadas no âmbito desta tese visaram responder a questões evolutivas fundamentais, particularmente sobre o impacto do efectivo populacional e relevantes num cenário de colonização. Devido à complexidade da base poligénica das características da história da vida, as expectativas teóricas clássicas podem não ser atingidas, pelo menos no nosso intervalo de gerações e de efectivos populacionais. Apesar da elevada replicação e da complexidade do delineamento experimental, muitas questões permanecem por responder. Outros estudos envolvendo a análise genómica das populações utilizadas, bem como outras características e diferentes dietas, permitir-nos-ão uma melhor compreensão de como a história, a selecção e o efectivo populacional influenciam a evolução durante um evento de colonização.

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Chapter One.

General Introduction: Evolutionary Constraints on Colonization

Evolutionary challenges facing colonization and reverse colonization

Consider the following scenario. A population migrates to a new environment where it encounters qualitatively novel nutrition, possibly including periods in which it starves. How will the population evolve in response to such a novel environment? Furthermore, its effective population size may be reduced in the course of migration, or as a result of an initial lack of adaptation to the new environment and a consequent population size crash. How will its effective size affect the evolutionary response to this new environment? What will happen if the population survives and its descendants migrate back to their ancestral environment? This will be from here on referred to as *reverse colonization*. How will the initial period of adaptation to a new environment affect the population's life history, and its subsequent evolution after returning to the ancestral environment? As we will discuss, this scenario is relevant for both evolutionary biology and conservation management.

Natural selection is able to produce rapid adaptive responses to sustained environmental change under propitious conditions: intense selection, abundant genetic variation, and large population sizes. A classic example of directional selection and adaptation in nature is the melanism of *Biston betularia* during the industrial revolution, where the light-colored morph became a great predation target, after the darkening of the tree trunks due to pollution (Tutt 1896; Kettlewell 1955, 1956). There is abundant evidence for directional selection on morphology and life history in many study systems, such as terrestrial plants (mainly angiosperms), invertebrates (mainly insects), and vertebrates, mainly birds and lizards (*e.g.* Endler 1986; Kingsolver *et al.* 2001; Kingsolver & Diamond 2011). Ever since the establishment of the evolutionary genetic paradigm, evolutionary geneticists have been interested in factors which constrain adaptation. Such constraints are the chief focus of this thesis.

General constraints on colonization success

When a population colonizes a novel environment and expands to new ranges, it faces several challenges that will limit and, perhaps, ultimately thwart the success of the colonization event. So, what are the evolutionary constraints facing colonization itself? The most obvious limiting factors affecting successful colonization of a novel environment are *(i)* the imposition of entirely novel environmental conditions to which the colonizers are not adapted, *(ii)* the effective population size (or N_e) of the colonizing population, *(iii)* the age-specific demographic

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challenges, such as the age-distribution of the colonizers, and *(iv)* hybridization with residents and/or subsequent migrants from the source population. Conversely, when successful colonizers return to their ancestral environment, similar issues will again impinge on their success: *(i)* loss of adaptation to that ancestral environment, *(ii)* N_e of the reverse-colonizers, *(iii)* shifts in their age-specific demography, and *(iv)* adverse effects of hybridization with the endemic ancestral population (Carson & Templeton 1984; Wade & McCauley 1988; Allendorf *et al.* 2013; Santos *et al.* 2013).

The importance of effective population size, N_e

Population size is a major factor affecting the evolutionary dynamics of fitness and its components (Frankham 2005a,b; Allendorf *et al.* 2013). Whether in the wild or in a captive environment, evolving populations are inevitably vulnerable to the following factors: *(i)* inbreeding, *(ii)* loss of genetic diversity, and *(iii)* accumulation of deleterious mutations. Each of these factors can reduce Darwinian fitness, and each is dependent in part on effective population size (N_e). Smaller populations are expected to suffer lower fitness due to inbreeding depression (Charlesworth & Charlesworth 1987), as well as weaker selection against deleterious alleles (Crow & Kimura 1970). The rate of loss of genetic variation is also expected to be higher in smaller populations, leading to the expectation of reduced long-term response to selection (Robertson 1960). The magnitude of inbreeding depends also on the level of environmental stress (Frankham 2005a; Reed *et al.* 2003), which will be exacerbated to the extent to which a population finds itself in an environment to which it is not adapted. Deeper knowledge of the effects of all these factors is fundamental for both evolutionary and conservation biology, and particularly relevant to cases of environmental change and evolutionary rescue. *Chapter Two* will approach this subject in greater detail.

Hybridization and its effects on adaptation

Hybridization most commonly refers to mating between heterospecific individuals. However, the term has also been applied to mating between individuals of different subspecies and populations that, though not from different species, differ to an appreciable extent genetically. For the sake of clarity, we will adopt the definition of Rhymer & Simberloff

(1996): hybridization is *the interbreeding of individuals from genetically distinct populations, regardless of their taxonomic status*.

Successful mating between different species is usually very difficult, but it is not as uncommon in nature as one might think (Mallet 2005). For the last two centuries, biologists tried to study hybridization, although from somewhat different perspectives. Some botanists, like Stebbins (1950), focused on hybridization as a source of genetic diversity. By contrast, zoologists such as Dobzhansky (1951) and Mayr (1942) considered animal hybrids to be rare or exceptional. Thus, they focused on hybridization as a negative selective agent that favored the strengthening of discrimination to maintain species (Stevison 2008), the *reinforcement effect*. Over the last few decades, the study of hybridization has yielded valuable insights, helping researchers to understand the forces limiting hybridization, as well as how gene flow and recombination can act to generate novel haplotypes to facilitate adaptation (Arnold 1997). Hybridization therefore has Janus-faced effects on adaptation. On the positive side, it may supply additional genetic variation, a limiting fuel for the process of adaptation in response to directional selection. On the negative side, when individuals adapted to ancestral conditions cross with colonizers that are adapting to a new environment, their hybrid offspring may have reduced fitness in the new environment (Woodworth *et al.* 2002; Gilligan & Frankham 2003; Frankham 2005a, 2005b, 2008).

The effects of hybridization and effective population size on adaptation have been studied in both animal and plant populations (*e.g.* Klinger *et al.* 1992; Arnold *et al.* 1999; Peters *et al.* 2014), but the effect of hybridization on populations which vary in effective size has not been studied hitherto. Smaller populations are expected to lose more genetic variation at a faster rate. This in turn fosters the expectation that hybridization will have a greater effect on the restoration of genetic diversity in smaller populations. This topic is more thoroughly discussed in *Chapter Three*.

Reverse colonization – trade-offs between novel and ancestral environments

Many of the factors impinging on successful colonization could also play a role on the evolutionary success of former colonizers returning to their ancestral environment. The idea of trade-offs in adaptation to qualitatively different environments is a commonplace in evolutionary reasoning (*e.g.* Leroi *et al.* 1994a,b; Shirley & Sibly 1999; Kawecki & Ebert 2004). Thus, colonizers that are long-adapted to a particular ancestral environment are naturally

supposed to have very low fitness in their new environment. Conversely, once colonizers are well-adapted to their new environment, they may no longer be well-adapted to their ancestral environment. Furthermore, if the colonizers return to depopulated ancestral environments, they will face yet another population size bottleneck, which may reduce both their fitness and their level of genetic variation (see the section above on N_e). On the other hand, hybridization with individuals that remained behind in the ancestral environment may improve the net fitness of those who reverse-colonize the ancestral environment.

The full spectrum of effects on adaptation to qualitatively novel environments has been of major interest within conservation biology, specifically when long-maintained captive populations show deterioration in their ability to thrive in their ancestral wild environments. This is particularly important for the success of *ex-situ* conservation programs that aim to reintroduce into the wild their captive bred individuals (Allendorf *et al.* 2013; Frankham 1995a, 2008, 2009a; Frankham *et al.* 2000, 2002; Woodworth *et al.* 2002; Gilligan & Frankham 2003).

It has been shown that relaxing selection on starvation resistance leads to a clear reversal of the character when N_e is moderately large (*e.g.* Teotónio & Rose 2000; Passananti *et al.* 2004a). The scientific question addressed here was thus whether or not such a clear and immediate response would be exhibited at low values of N_e; in other words, whether reverse evolution is hampered by sustained small population sizes. *Chapter Four* of this thesis presents a reverse colonization experiment where small-sized populations that had undergone strong selection for starvation resistance were returned to their previous, long-standing selection regime, which featured early reproduction, *ad libitum* feeding, and no phase of food supplementation.

The role of age-specific patterns of selection

It is a general evolutionary expectation that adaptation to a novel environment shows strong age-specificity (Mueller *et al.* 2011). The idea stems from Hamilton's theory (1966), in that the forces of natural selection provide age-dependent scaling or weighting factors for the impact of selection on each age-specific component of a population's life history. This weighting is heavy at early ages, then the forces of natural selection decline with adult age until they reach an asymptotic plateau. This in turn leads to the evolution first of aging, and then (at much later adult ages) the evolution of plateaus in life-history characters (Rose *et al.* 2007; Mueller *et al.* 2011). Hamiltonian force-of-selection theory is therefore relevant to the problem

of a population which is transferred to a qualitatively novel environment. The key point is that the age-specific weighting of the force of natural selection leads to the corollary that selection for adaptation to a novel environment will act with full force only at early ages. Even though life-history adaptation to a novel environment proceeds quickly at early ages (*vid.* Matos *et al.* 2000a; Simões *et al.* 2008), there may be age-dependent adaptation with later ages responding less to selection for adaptation to the new environment, at least initially. In *Chapters Five* and *Six* there is a more comprehensive discussion of this topic.

The volte-face of colonization: biological extinctions

There are two main types of threats that cause biological extinction: *deterministic* and *stochastic* (Caughley 1994). *Deterministic* threats include habitat destruction, pollution, overexploitation, species translocation, and global climate change. *Stochastic* threats are random changes in genetic, demographic or environmental factors. Genetic stochasticity (random drift) leads to loss of genetic variation, including beneficial alleles, and an increase in the frequency of harmful alleles. Demographic stochasticity includes variation in sex ratios and age distributions by chance. Environmental stochasticity is simply random environment variation, such as the occurrence of several harsh winters or summers in a row (Allendorf *et al.* 2013).

Extinction is, by definition, a demographic process, since populations are subjected to uncontrollable demographic factors as they become progressively smaller. A major controversy erupted over the role of genetic factors in population viability and extinction risk following Lande's (1988) paper on genetics and demography. The *Lande scenario* is based on the hypothesis that genetic effects are negligible compared to demographic factors, implying that most species would be driven to extinction before genetic factors would have time to impact them. Because small populations are in much greater danger of extinction by purely demographic stochastic effects, they are not very likely to persist long enough to be affected by inbreeding depression (Lande 1988; Pimm *et al.* 1988; Pimm 1991; Young 1991; Wilson 1992; Caro & Laurenson 1994; Caughley 1994; Dobson 1999; Elgar & Clode 2001; Frankham 2003; Sarre & Georges 2009). Other authors have claimed that the genetic variation decrease due to inbreeding in small populations will likely cause a strong reduction in fitness that, in turn, will further reduce a population's size (Frankel & Soulé 1981; Gilpin & Soulé 1986; Dobson *et al.* 1992; Keller 1998; Oostermeijer 2000; Brook *et al.* 2002). This will thereby

reduce the population's ability to persist and drive the population into what is known as the *extinction vortex* (Allendorf *et al.* 2013). In a comprehensive meta-analysis, Spielman *et al.* (2004a) found that the majority of threatened taxa exhibited reduced genetic diversity, leading to a rejection of the *Lande scenario* for most of them. Most threatened taxa were found to be suffering from a reduced ability to evolve, high inbreeding, and impaired fitness, and for these reasons are likely to suffer elevated extinction risk in the future (Reed & Frankham 2003; Frankham 2005b).

All in all, extinction is a demographic process that is likely to be influenced by genetic effects under different circumstances. The main issue is to determine under what conditions genetic concerns are likely to influence a population's persistence and its ability to thrive in the long run (Nunney & Campbell 1993; Allendorf *et al.* 2013).

Experimental Evolution – a powerful tool to study evolutionary constraints

Evolutionary hypotheses are usually tested by studying patterns that reflect past evolution: phylogeny, divergence between groups, variation within populations, genome structure, and genome sequence. Experimental evolution is an alternative research framework that allows us to study evolutionary processes experimentally in real time, making it a very useful tool to study evolutionary constraints (Kawecki *et al.* 2012).

Experimental evolution can be defined as the study of evolutionary changes occurring in experimental populations as a consequence of experimenter-imposed conditions, whether they are environmental, demographic, genetic, social, behavioral, *etc*. In this research approach, sometimes called *laboratory natural selection*, selection can act on any and all traits or nucleic acid sequences relevant to fitness under the environmental regimes. In contrast, artificial selection protocols proceed by breeding individuals explicitly chosen by the experimentalist based on the phenotypic values of defined traits or genotypes; artificial selection is, thus, not usually included in experimental evolution (*cf.* Garland & Rose 2009; Kawecki *et al.* 2012). In some respects, these are disjoint experimental strategies, although they have much in common.

In experimental evolution, populations are studied across multiple generations under defined and reproducible conditions that are most readily achieved in the laboratory (*e.g.* Rose 1984a; Lenski *et al.* 1991; Matos *et al.* 2000a; Chippindale 2006; Simões *et al.* 2007), but sometimes can be approximated in nature (*e.g.* Reznick *et al.* 1990; Ebert *et al.* 2002; Zbinden

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et al. 2008). We can distinguish two chief types of experimental evolution: *(i)* the imposition of new *forward selection* regimes that lead to divergent evolution *(e.g.* Rose 1984a; Rose *et al.* 1992; Chippindale *et al.* 1997; Joshi *et al.* 1996a; Borash *et al.* 2000; Rundle *et al.* 2005; Bennet & Lenski 2007; Hall & Willis 2006; Cooper & Lenski 2010); *versus (ii)* the re-imposition of an ancestral environment on diverged populations, establishing *reverse selection* (Service *et al.* 1988; Teotónio & Rose 2000, 2001; Passananti *et al.* 2004a; Estes & Teotónio 2009). Experimental evolution is a very useful tool for generating contrasting phenotypes by divergent selection. Such divergent phenotypes can then be used to study the biological machinery, whether genetic, developmental or physiological, underlying those contrasts. With either forward or reverse selection, experimental evolution allows biologists to estimate evolutionary rates, trace evolutionary patterns, establish causal relations, and distinguish differentiation due to stochastic effects like genetic drift, from more deterministic mechanisms like natural selection, within the context of better defined historical constraints (Bell 2008; Chippindale 2006; Simões *et al.* 2009; Fragata *et al.* 2014a,b).

Experimental evolution has been used to address diverse questions in many areas of academic evolutionary biology, from speciation to aging (Garland & Rose 2009). It has also been very useful in applications from conservation biology to biotechnology, from medicine to engineering (Kawecki *et al.* 2012). Experimental evolution has been valuable for modern agriculture: the evolution of insect pest resistance to selection imposed by insecticides is one of the best-known examples of an evolutionary response to an agricultural practice (Thrall *et al.* 2011). The serial passage of a pathogen on a particular host often leads to increased specialization and higher virulence on that host thanks to laboratory evolution (Ebert 1998), producing more virulent strains for biological pest control (Kolodny-Hirsch & Van Beek 1997; Dion *et al.* 2011). Experimental evolution has also been used to improve biocatalysts (Kolodny-Hirsch & VanBeek 1997; de Crecy *et al.* 2009). For decades, experimental evolution has been the method of choice for the development of vaccines against viral and bacterial diseases, such as polio, tuberculosis, yellow fever, measles, mumps, and rubella (Ebert 1998; Plotkin & Plotkin 2011), an invaluable contribution to human medicine evolution. More recently, experimental evolution combined with genome sequencing and genetic mapping has been used to identify mutations that confer drug resistance to pathogens, before such mutations appear in nature (*e.g.* Hunt *et al.* 2010). This should foster the rapid diagnosis of resistant infections, and possibly even the development of new drugs (Kawecki *et al.* 2012). From an industrial and

economic perspective, experimental evolution is a useful complement to genetic engineering for the production of biofuels (Arnold 2008; Zuroff *et al.* 2013).

Of greatest relevance for the present research, experimental evolution has been valuable in evolutionary studies on conservation. Among other examples, it has been used in studies of *(i)* the capacity to adapt to environmental changes (Bell & Collins 2008; Bell & Gonzalez 2009; Simões *et al.* 2007; Santos *et al.* 2010), *(ii)* evolution in small/bottlenecked populations (Latter & Mulley 1995; Reed & Bryant 2000; Miller & Hedrick 2001; Reed & Frankham 2003; Reed *et al.* 2003), and *(iii)* the effect of adaptation to captivity with (Margan *et al.* 1998; Woodworth *et al.* 2002; Frankham 2008) and without hybridization (Gilligan & Frankham 2003). Such studies of experimental evolution under conditions of recently imposed captivity may be broadly defined as studies of *Evolutionary Domestication* (see below).

Research on Evolutionary Domestication

A natural experimental paradigm for the study of colonization is the adaptation of experimental populations to laboratory conditions, when these populations are founded with recently caught individuals from the wild. This paradigm is sometimes called *evolutionary domestication* (*e.g.* Simões *et al.* 2009), which has been a major focus of research in the laboratory of Margarida Matos at the University of Lisbon. Nonetheless, the research of Matos *et al.* can be viewed as one particular realization of a fairly broad experimental strategy.

Domestication is historically a very important topic for evolutionary biology. Indeed, domestication can be considered the first evolutionary experiment performed by humans. Early cases of domestication have been dated back more than 10,000 years: dogs and some livestock species were the first animals to be domesticated (Mignon-Grasteau *et al.* 2005; Thalmann *et al.* 2013). Despite some terminological ambiguity, domestication refers (at least in part) to the genetic changes undergone by our commensal species, such as the genetic evolution of dogs from wolves. However, a more useful definition for scientific purposes is that domestication is evolutionary genetic change arising from the transition of a population from nature to deliberate human cultivation (Simões *et al.* 2007). Darwin used the cases of pigeon and dog domestication to argue for the capacity of selection to produce evolutionary change in the very opening chapter of *On The Origin of Species* (1859). Later, he devoted still more attention to it in a book that he wrote specifically on variation under domestication (Darwin 1868).

Domestication typically involves forward directional selection and significant

reductions in effective population size, both of which can reduce Darwinian fitness. Populations under domestication will usually, on the other hand, benefit from relaxation of those selection pressures that arise from predation, interspecific competition, and climatic extremes, particularly if the populations are given artificial housing. However, pastoralists and farmers will impose more stringent selection for other attributes, deliberately or not, particularly tameness (Mignon-Grasteau *et al.* 2005).

Research on domestication in *Drosophila* has become a prominent feature of the scientific literature on the topic: *e.g.* Frankham & Loebel 1992; Latter & Mulley 1995; Hercus & Hoffmann 1999a,b; Matos *et al.* 2000a, 2002, 2004; Sgrò & Partridge 2000; Griffiths *et al.* 2005; Hoffmann *et al.* 2001; Krebs *et al.* 2001; Woodworth *et al.* 2002; Gilligan & Frankham 2003; Reed *et al.* 2003). This is the body of research which constitutes the chief foundations for the present dissertation research.

The Matos laboratory

The Matos lab's characteristic experimental paradigm has featured laboratory populations of *Drosophila subobscura* founded from wild individuals collected from locations within Portugal (*e.g.* Matos *et al.* 2000a, 2002; Simões *et al.* 2008). Once these individuals have been collected from the wild, they and their descendants are maintained with discrete generations of 28 days duration at a steady lab temperature. Care is taken to provide an unchanging food medium and stable population density. Census population sizes are about 600-1,000 individuals during early adulthood (Simões *et al.* 2008).

Figure 1.1 shows the generic type of domestication result generated by the Matos laboratory, in which early fecundity steadily increases over some 100 generations of domestication, relative to a long-established *D. subobscura* stock. Nevertheless, it should be noted that not all functional characters undergo such a clear improvement over the course of domestication. For example, there was no significant change in female starvation resistance among the newly domesticated populations shown in **Figure 1.1,** relative to long-established populations (Simões *et al.* 2009).

Over the last decade, Matos *et al.* have been studying the repeatability of domestication of *Drosophila subobscura* populations over repeated samplings from nearby Portuguese locations (Simões *et al.* 2007, 2008). They have shown that the evolution of newly-sampled populations is repeatable in some respects, with general evolutionary convergence of

phenotypes to those of long-established populations (Simões *et al.* 2008). It has also been found that most of the observed differences in the evolution of replicate populations across experiments were due to genetic sampling effects during the first few generations of lab culture (Santos *et al.* 2012).
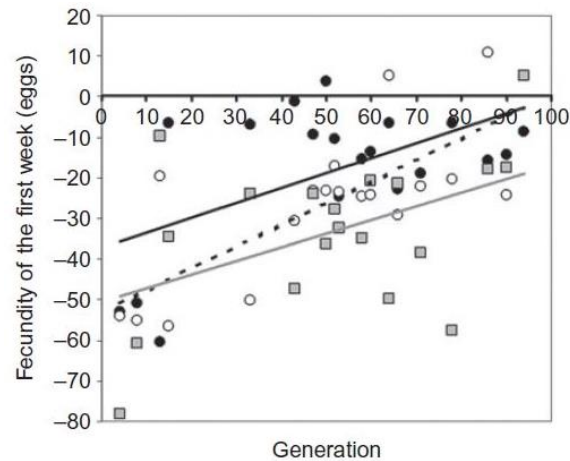
Figure 1.1. Fecundity during the first week of life in populations founded in 1998 (NW) relative to the longer-established (NB) populations. Each data point is the difference between the average absolute values of each population and the same numbered longer-established population. Replicate population 1: black circles, full black line; replicate population 2: open circles, broken line; replicate population 3: gray circles, gray line (Simões *et al.* 2009).

A more recent project addressed the impact of evolutionary history on the dynamics of *D. subobscura* adaptation along the European latitudinal cline (Fragata *et al.* 2014a,b; Matos *et al.* 2015). Fly samples were collected from historically differentiated wild populations from three European locations: Groningen (Netherlands), Montpellier (France), and Adraga (Portugal). Phenotypic analysis showed that initial samples from these populations were clearly differentiated, they subsequently converged for several life-history, physiological, and morphological traits (Fragata *et al.* 2014b). These experimental populations also featured initially strong differentiation in inversion frequencies. But unlike the case of the phenotypes studied, after 40 generations of laboratory evolution, populations remained differentiated at the inversion frequencies level. It seems that, despite the clear evidence for the role of selection in phenotypic convergence, history played an important role in the evolutionary dynamics of inversions (Fragata *et al.* 2014a).

Other work on evolutionary domestication in Drosophila

Sgrò and Partridge (2000) studied domestication in *D. melanogaster* populations, also finding clear changes for some functional characters, but not others. They found increased development time and early fecundity, but some decreases in late fecundity. Using some of the same *D. melanogaster* populations, Hoffmann *et al.* (2001) found that stress resistance was reduced during laboratory adaptation. Griffiths *et al.* (2005) studied laboratory adaptation in *D. birchii* populations derived from crosses of isofemale lines that were in turn derived from wild-caught flies. They found increases in starvation resistance and development time, but reduced recovery from cold shock. In that same study, heat knockdown resistance and wing size did not change with the number of generations of domestication. Comparing the results from these other labs with those from Matos *et al.*, it is apparent that some *Drosophila* characters evolve in a consistent manner across studies of domestication, such as early fecundity. However other characters, such as particular types of stress resistance, exhibit no such consistency across the findings of different labs, or even in the same lab across populations (Simões *et al.* 2008, 2009).

The experimental evolution work of Rose and colleagues

A major problem with early fruit fly research using experimental evolution was insufficient replication (*e.g.* Rose & Charlesworth 1980). With only one or two experimentally evolved populations compared to similar numbers of control populations, these early experiments provided very little statistical power, and thus low confidence in their scientific inferences and very little ability to differentiate between natural selection and genetic drift in selection experiments. Thus, in the early 1980s, a number of *Drosophila* labs began experiments with much greater replication of both deliberately selected and matched control populations (*e.g.* Mueller & Ayala 1981; Luckinbill *et al.* 1984).

Of these labs, the one that eventually produced the greatest number of experimentally evolved *Drosophila* populations was that of Rose (*e.g.* Rose *et al.* 2004), starting with the Rose (1984a) study of the experimental evolution of aging with different age-specific windows for reproduction. At the present time, the Rose lab has a number of *D. melanogaster* stocks that have undergone as much as 38 years of laboratory evolution, where each type of stock usually has five replicate populations that have evolved separately (Rose *et al.* 2004). This research has generally used population sizes large enough to sustain genetic variation genome-wide (N_e

~ 600-1100, Burke *et al.* 2010; Mueller *et al.* 2013). The Rose laboratory has conducted not only forward selection, but also reverse selection in which populations have been returned to their ancestral conditions (*e.g.* Teotónio & Rose 2000). There have been studies of selection with contrasting population sizes in different experiments, both small (*e.g.* Rose 1984b) and moderately large N_e (Rose 1984a). But the Rose lab has not yet studied the impact of varying N_e on the response to selection in a single well-defined experiment.

Some have argued that experimental evolution using long-established laboratory populations are of little use for evolutionary inference (Promislow & Tatar 1998; Harshman & Hoffmann 2000; Linnen *et al.* 2001). This line of criticism is premised at least in part on the notion that laboratory evolution is of value to the extent that its results can be extrapolated to evolution in the wild. Yet it can be argued instead that experimental evolution is about bare possibilities for evolution, not any expected evolutionary process in nature. After all, the laboratory environment is a particular kind of environment, and lab lines are simply natural populations evolving in such environment (Matos *et al.* 2000b; Mueller *et al.* 2005).

Pilot Study of the Impact of N_e on Evolutionary Domestication

The outcome of evolution is a balance between the deterministic effects of natural selection and the stochastic effects of genetic drift. Since genetic drift is more significant in smaller populations, evolutionary dynamics during evolutionary domestication will be affected by population size as it impinges on both genetic drift and the response to selection. Larger populations are thus expected to respond faster when adapting to a novel environment (Hartl & Clark 2007; Woodworth *et al.* 2002), all other things being equal. If multiple populations are maintained under the same selective conditions, genetic drift will cause divergence between them. Finally, effective population size should also affect the likelihood of extinction through both the rate of accumulation of deleterious mutations and demographic stochastic events, such as the loss of most females (Frankham *et al.* 2002, 2005b).

My initial study of laboratory adaptation in the Matos laboratory used populations of contrasting sizes (census ~50 *vs.* ~1200 individuals). The results qualitatively corroborated some of the said theoretical expectations (Santos 2008): smaller N_e populations had both greater between-population heterogeneity of response as well as a slower average rate of improvement in early fecundity (**Figure 1.2**).
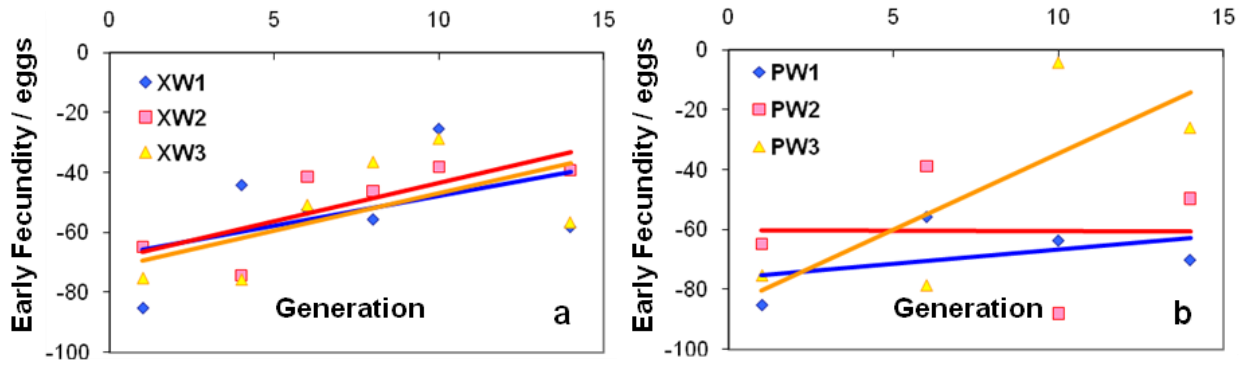
Figure 1.2. Evolutionary trajectories for early fecundity during the initial generations of adaptation to a laboratory environment. **(a)** Large population sizes, **(b)** Small population sizes. All trajectories show the difference between each replicate population and its respective control. From Santos (2008).

Overall Plan of Thesis Research: Combining Matos & Rose Experimental Strategies

This thesis plan results from the combination of Matos and Rose experimental strategies and expertise: a rigorous and highly-replicated design to study adaptation to a novel environment, using *Drosophila melanogaster* stocks with a well-known evolutionary history.

This research was conducted in the Rose laboratory, using six replicate populations made up of outbred *B-type* flies that have been uniformly maintained in vials on a 14-day life cycle for more than 800 generations (*vid.* Rose *et al.* 2004 for more details on their evolutionary history). These populations were maintained at sizes ($N_e \sim 1000$; Mueller *et al.* 2013) large enough to sustain genome-wide genetic variation (Teotónio *et al.* 2009; Burke *et al.* 2010), and have been extensively studied since the 1980's (Rose 1984a,b, Rose *et al.* 1992; Chippindale *et al.* 1997; Teotónio & Rose 2000; Rose *et al.* 2004; Mueller *et al.* 2011; Burke *et al.* 2016; Graves *et al.* 2017). While this system is not in any sense a replication of any evolutionary radiation that exists in the wild, it is suitable for strong-inference tests of general theories of evolutionary genetics (*vid.* Rose *et al.* 2011) from the standpoint of providing six essentially equivalent ancestral sources of *colonists* for the present experiments.

Chapter Two: *Does size really matter? Forward Selection for Starvation Resistance with Varying Effective Population Size*

Despite the fundamental importance of effective population size for evolutionary theory and conservation, its impact on evolution is rarely evaluated under well-defined conditions. *Chapter Two* aims to examine several evolutionary questions concerning a scenario where

populations encounter a novel environment, with and without deliberately reduced effective population size, focusing on the following specific questions. *(i)* How will these populations respond to new demographic conditions? *(ii)* How will life-history evolve in response to such new, harsh conditions? *(iii)* How will the population size differences affect the evolutionary response to selection? *(iv)* Will populations from the same ancestral source population evolve the same way?

Each of the six source populations was used as the ancestor for 10 small-sized populations of about 50 surviving individuals per generation and four large-sized populations maintained at similar moderate N_e levels as their ancestors, for a total of 84 experimental populations. This is the largest laboratory experiment on colonization by Mendelian populations known to us. Of the 14 populations derived from each B_i ancestral population, half were maintained without any strong selection being imposed. The other half were subjected to strong selection for starvation resistance. In this way, the experimental system features both a replicated bottleneck design without drastic selection, and bottlenecking with strong selection, as well as matched large N_e populations. A schematic for this phase of the experimental work is shown in **Figure 1.3**.
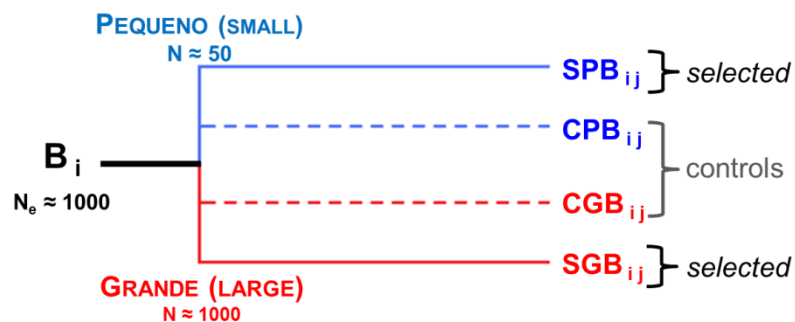
Figure 1.3. Schematic representation of the overall experimental design used to derive the first set of experimental populations. Each B_i ancestor gave origin to 10 small-sized (P; N ≈ 50) and four large-sized populations (G; N ≈ 1000), half of which were selected for starvation resistance (S) while the other half were used as controls (C). Thus, SPB_{2d} refers to the fourth small-sized replicate population derived from ancestral population B_2 that underwent selection for starvation resistance, for example. This phase involved a total of 84 experimental populations.

Chapter Three: Hybridization and Forward Selection with Varying Effective Population Size

Chapter Three examines the effects of *(i)* hybridization on the evolutionary dynamics of populations under forward selection and *(ii)* population size before and after an interpopulation hybridization event.

After 15 generations of selection, both the forward-selected populations and their matched controls were hybridized within each combination of selection, size, and B_i ancestor. This allowed us to compare hybrid vigor in large to small populations, testing for differences in inbreeding depression as a function of N_e. 84 hybrid lines were then created, increasing the total number of experimental populations to 168. Selection was then resumed in order to test for hybrid enhancement of the rate of response to further selection, as well as continuing to test for differences in rate of response as a function of N_e. A schematic for the hybridization component of this phase of the experimental work is shown in **Figure 1.4**.
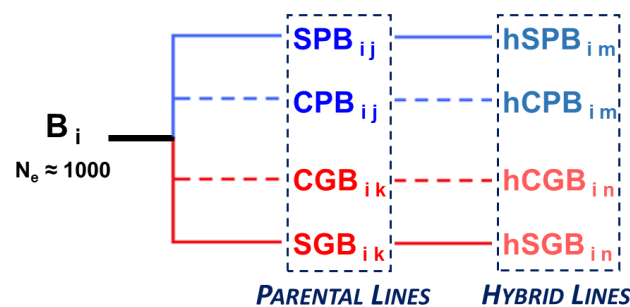
Figure 1.4. Schematic representation of the experimental design used to create the hybrid populations from the first set of 84 lines. For each set of selected and control lines derived from a B_i population, hybridization took place among all replicates, and the resultant hybrids were split into the same number of replicates as their unhybridized ancestors. Starvation resistance selection was then resumed among all the S lines, both hybridized and unhybridized (SPB, SGB, hSPB, and hSGB).

Chapter Four: Reverse Evolution in Small-Sized Populations

The motivation for this experiment was that relaxing selection on starvation resistance leads to a clear reversal of the character when N_e is moderately large (*e.g.* Teotónio & Rose 2000; Passananti *et al.* 2004a). The scientific question was thus whether or not such a clear and immediate response would be exhibited at low values of N_e. In other words, this experiment tested whether reverse evolution was hampered by sustained small population sizes.

After 15 generations of forward selection for starvation resistance, derivatives of the small populations (both selected and control) were created and a reverse-selection experiment was started. Specifically, these populations were returned to 14-day culture in vials, without the dietary changes or selection imposed during the first phase of the experiment (**Figure 1.3**). Nonetheless, these reverse-selected small N_e populations were kept at the same low N_e as was

imposed on them during the first phase of the experimental work, simulating a sustained bottleneck (**Figure 1.5**).
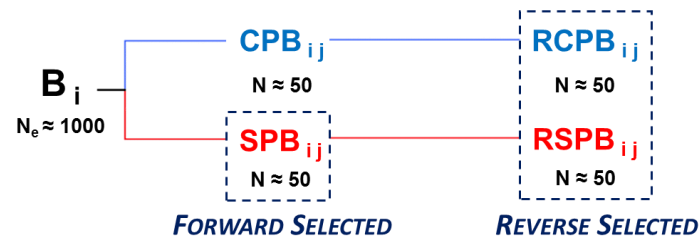
Figure 1.5. Schematic representation of the experimental design used to create the reverse-selected lines. After 15 generations of forward selection, each CPB_{ij} population was used to derive a single $RCPB_{ij}$ population, and each SPB_{ij} population was used to derive one $RSPB_{ij}$ population. These 60 populations were then subjected to 21 generations of reverse selection and sustained small N_e.

Chapter Five: Age-Specific Adaptation to Novel Diets

While some work on quantitative variation in diet has been performed in the Rose laboratory (*e.g.* Chippindale *et al.* 1993, 1997), this thesis presents the first experimental work on the impact of qualitatively different diets on life-history. Such novel diets are likely to arise when populations colonize a new habitat. Of particular interest for the present research was differential impact of dietary change across the ages of adult life-history during new colonization events. *Chapter Five* presents the first experimental test of the evolutionary effect of dietary change on the age-dependent adaptation of *Drosophila melanogaster* populations that have been exposed to a specific type of food for more than 800 generations.

Chapter Six: Aging and Mortality Patterns in Urea-adapted Populations

Adaptation to nitrogenous waste has been an ongoing project of the Mueller laboratory for over 20 years (Joshi *et al.* 1996a,b, 1997, 1998; Borash *et al.* 2000). In the Fall of 1996, Mueller *et al.* created and maintained outbred lines of *Drosophila melanogaster* selected for increased urea tolerance and their life-cycle matched controls (*vid.* Borash *et al.* 2000 for more details). The study of the adult mortality patterns of this unique experimental system enabled us to test several hypotheses that arise from Hamiltonian theory, relevant in a colonization scenario. Specifically, whether *(i)* dietary adaptation is age-specific and *(ii)* long-term adaptation to a new environment (urea) leads to loss of fitness in the ancestral environment. The experimental work on this matter is presented on *Chapter Six*.

Overall creation of a stock system as a better foundation for further experiments

While there are substantive findings from this doctoral research that we believe will be of interest to our colleagues in evolutionary biology, the greater significance of this thesis project may be the creation of this remarkable stock system. As far as we know, this is the largest well-defined and highly-replicated system for studying the impact of different N_e levels across a range of types of laboratory-evolved populations: strongly selected, moderately selected, and reverse-selected populations. The total number of populations that were created for this study was 228, an audacious number for research using outbreeding metazoans. Although not all of these populations are still in culture, frozen samples from all of them are available for sequencing, many of these samples coming from different generations during their experimental evolution. Furthermore, there remain a total of 132 populations still alive, and they constitute an enduring resource for experimental research on the effects of N_e on the response to selection.

Chapter Two.

Does size really matter? Forward Selection for Starvation Resistance with Varying Effective Population Size

ABSTRACT

Effective population size (N_e) is a key factor affecting the evolutionary dynamics of populations. It affects all the major forces that shape evolution: genetic drift, natural selection, and mutation accumulation. Despite the fundamental importance of N_e in evolutionary theory and conservation genetics, it is notoriously difficult to estimate. It is even less often experimentally manipulated in order to study its impact on evolution under well-defined conditions. This chapter presents a highly-replicated and controlled experimental design that aims to examine several evolutionary questions in the context of a colonization scenario, in which populations encounter qualitatively novel selection with and without reduced effective population size. Here we provide strong evidence bearing on the effect of a 20-fold reduction in population size on responses to both weak and strong directional selection. We find significantly impaired responses to selection and increased population differentiation due to drift. We also show that the relative roles of history, chance, and selection are shaped by population size and contingent on the trait under analysis.

RESUMO

O efectivo populacional (N_e) é um factor preponderante na dinâmica evolutiva de uma população, influenciando as principais forças que modelam a evolução: deriva genética, selecção natural e mutação. Apesar da importância fundamental do N_e na teoria evolutiva e na genética da conservação, é um factor extraordinariamente difícil de estimar. Como tal, a sua determinação é muito pouco frequente na literatura. Menos frequentes ainda são a manipulação experimental e o estudo do impacto do N_e em condições experimentais bem definidas. Este capítulo apresenta um delineamento experimental altamente replicado e controlado onde são analisadas várias questões evolutivas no contexto da colonização. Nomeadamente, as populações encontram condições selectivas qualitativamente novas, com e sem redução do efectivo populacional. Aqui apresentamos provas sólidas do efeito duma redução do tamanho populacional (em 20 vezes) na resposta à selecção direccional, forte e fraca. Os dados mostram uma diminuição da resposta selectiva e um aumento da diferenciação populacional devido à deriva genética. Mostramos também que os papéis relativos da história, do acaso e da selecção são moldados pelo tamanho da população e estão dependentes das características em análise.

INTRODUCTION

An evolving population, whether in its natural environment or in a controlled laboratory setting, is a complex system influenced by several evolutionary forces: genetic drift, selection, mutation, and migration. Understanding the effects and interactions of these factors in their full complexity is hard, so experimental evolutionists seek to comprehend their impact, one at a time. Consider the following colonization scenario. After members of a population migrate to a new inhospitable environment they will suffer a reduction in their effective population size as a result of both *(i)* the limited number of migrants, and *(ii)* increased mortality and reproductive failure in the inhospitable environment. Assuming that no other conspecifics are found and no additional migration occurs, the outcome of evolution will be affected by the balance between increased Darwinian fitness (due to the action of natural selection producing adaptation to the new environment) and reductions in fitness that arise from increased expression of homozygous deleterious genetic effects (which arise from initial sampling effects and subsequent genetic drift). This is the evolutionary scenario experimentally addressed in this chapter.

Effective Population Size

One of the fundamental parameters in both conservation and evolutionary biology is *effective population size* (N_e), which integrates the genetic effects of life history variation and total census size on microevolutionary processes. It impinges on both the response to selection and the impact of genetic drift. The concept of N_e was first introduced and developed by Sewall Wright (1931) and Fisher (1930), who defined it as the size (*N*) of an imaginary, theoretically ideal population affected by genetic drift at the same rate per generation as the population being studied. This idealized population size, referred to as a Wright-Fisher population, can take into account factors that increase genetic drift in real populations, such as uneven sex ratio, population size fluctuations, and variance in reproductive success. The comparison with a theoretically ideal population standardizes the rate of genetic drift, not only making N_e comparable across populations with very different life histories, but also reducing to one variable all the factors that contribute to genetic drift (Hare *et al.* 2011). N_e was thus originally developed to adjust for deviations from Wright-Fisher genetic drift (*vid.* Crow 1948), but it has since become a central parameter in evolutionary genetic theory (*e.g.* Ewens 2004; Nagylaki 2011). Effective population size is a major factor affecting the evolutionary dynamics of Darwinian fitness and its components (Reed & Frankham 2003; Frankham 2005b; Reed 2005;

Allendorf *et al.* 2013). We will now address how it affects the major forces that shape evolution.

Inbreeding

The most immediate effect on fitness of a population bottleneck is an increase in the level of inbreeding (*F*), which is defined as the probability of mating between individuals that are more genetically similar than individuals drawn at random from the population (Hedrick & Kalinowski 2000). Since Darwin (1876), if not earlier, inbreeding has long been known to reduce the reproduction and survival of inbred offspring. The decline in fitness due to inbreeding is known as *inbreeding depression* (Charlesworth & Charlesworth 1987; Ralls *et al.* 1988; Lynch 1989; Thornhill 1993; Katju *et al.* 2015; see Charlesworth & Willis 2009 for a review). It has commonly been found in most natural populations that have been tested for it (*e.g.* Jiménez *et al.* 1994; Crnokrak & Roff 1999; Keller & Waller 2002; Reed *et al.* 2003).

The cost of inbreeding can be defined as the magnitude of inbreeding depression. This cost can vary greatly, and is difficult to predict in any given population (Bouzat 2010). This inability to predict accurately this detrimental effect makes it difficult to incorporate inbreeding into models that predict population viability, even when a population's past demographic history and reproductive biology are well known. Nevertheless, substantial inbreeding depression is usually observed among populations that have recently undergone reduced effective population size, especially under stressful conditions, which is of great importance for population management and conservation (Frankham 2010).

Loss of Genetic Diversity and Evolutionary Potential

Loss of genetic diversity is a major concern in both evolution and conservation biology, as genetic diversity is the raw material upon which natural selection acts to produce adaptive evolutionary change. Almost every trait that has been examined shows genetic variation in natural populations (Lewontin 1974). Such genetic variation arises from the combined action of mutation, genetic drift, and natural selection. In the absence of selection, the temporal loss of heterozygosity in a randomly mating diploid population after *t* generations is related to effective population size (N_e), number of generations (*t*), and the inbreeding coefficient (F_t), as follows (see Wright 1969; Falconer & Mackay 1996):

$$\mathbf{H_t \ / \ H_0 = [\ 1 - 1/(\ 2 \ N_e \ ) \ ]^{\ t} = 1 - F_t} \qquad (1.1)$$

Since the middle term in the equation is approximately $e^{-t/2Ne}$, it implies a roughly exponential decline of genetic diversity with time that occurs at greater rates in smaller than larger populations (Frankham 2005b). Thus, among small randomly mating populations in the absence of selection, inbreeding and loss of heterozygosity through drift are unavoidable, the effects being more severe the smaller the population size is. Though the equation applies to neutral variation only, alleles subject to selection may also be lost by drift (Frankham *et al.* 2002; Frankham 2012). This loss of heterozygosity will clearly affect how fast populations respond to selection, and how well they can adapt to environmental changes.

In sum, genetic drift is an important evolutionary process in part because the strength of stochastic genetic processes strongly influences how selection operates. As N_e decreases, genetic drift erodes genetic variation, elevates the probability of fixation of deleterious alleles, and reduces the effectiveness of selection, all of which reduce overall fitness and limit responses to selection (Woodworth *et al.* 2002; Hartl & Clark 2007; Hare *et al.* 2011).

Short and Long-term Responses to Selection

The breeder's equation

$$R = S\,h^2 \tag{1.2}$$

states that the short-term response (R) is proportional to the strength of selection (or *selection differential, S*) and the *narrow-sense heritability (h^2)*, *i.e.* the proportion of additive phenotypic variance of the trait under selection (V_A /V_P) (Falconer & Mackay 1996). According to Robertson (1960), the maximum response to selection (R_{max}) due to pre-existing polymorphisms for additive genes depends on the *effective population size* (N_e), the *standardised selection differential* (i), the *narrow-sense heritability (h^2)*, and the phenotypic standard deviation (σ_P):

$$R_{max} = 2N_e\,i\,h^2\,\sigma_P \tag{1.3}$$

Furthermore, after t generations of selection, quantitative adaptation (R_t) to a given environment is also expected to be proportional to the *selection differential* (S), the initial heritability (h_0^2), and the effective population size (N_e), as follows:

$$R_t = S\,h_0^2\,\sum_{i=1}^{t}[\,1 - 1\,/\,(2N_{e\,i})\,]^{\,t-1} \tag{1.4}$$

Hence, assuming an additive model in the absence of mutation, the temporal loss of genetic diversity leads to the expectation of reduced long-term response to selection. Therefore, for a given value of additive genetic variance (V_A), the long-term response to selection will decrease as N_e declines (Robertson 1960; Hill & Rasbash 1986; Wei *et al.* 1996; Willi *et al.* 2006). Moreover, the selection differential (*S*) itself declines during long-term selection, because the population of selected individuals is highly related and, therefore, smaller than its numbers alone would suggest (Verrier *et al.* 1991). Thus, small populations are expected not only to possess lower levels of genetic variation, but they are also less likely to achieve the maximum response to selection predicted by their genetic variance (Hoffmann *et al.* 2017; but see Wood *et al.* 2016). Finally, the response to selection is less predictable in populations that are small and/or have low genetic variation (Hill 1982; Nomura 1997), as the variance of the response is given by $2FV_A$, where *F* is the inbreeding coefficient and V_A is the initial additive genetic variance. Thus, the response becomes more variable as populations remain small for some time (Willi *et al.* 2006).

These models were developed to predict the response to selection acting on traits that vary primarily due to additive genetic variance, in the absence of mutation, where the rates of loss of molecular and quantitative genetic variation do not differ significantly (Gilligan *et al.* 2005). The fact that characters close to fitness can have high non-additive genetic variance and possible linkage disequilibrium may lead to results that deviate substantially from these theoretical predictions (Frankham *et al.* 2002; Frankham 2005b; Gilligan *et al.* 2005).

Mutation Accumulation

Mutation is the ultimate source of all the genetic variation on which selection may act. Hence it is essential to evolution. Mutations may carry a large cost as many are deleterious and thereby reduce the fitness of the organisms in which they occur (Lynch *et al.* 1999). Mutation is therefore a source of both good and ill for a population (Lande 1995).

The overall effect of mutation on a population is strongly dependent on population size. On the one hand, populations with more individuals have statistically higher probability of acquiring new mutations in each generation and, for the same reason, the absolute number of those mutations that are beneficial is more likely to be higher. On the other hand, larger populations have more effective selection against deleterious mutations, which keeps them at lower frequencies in the balance between the forces of selection and those of mutation (Crow

& Kimura 1970). Consequently, a population with fewer individuals will have lower fitness on average, not only because fewer new beneficial mutations arise, but also because deleterious mutations of sufficiently small effect are roughly selectively neutral and more likely to reach high frequencies through genetic drift. Over long time-spans, this shift in mutation-selection balance can cause numerous deleterious alleles to fix, producing declines in fitness that lead the population to extinction – an outcome referred to as *mutational meltdown* (Lynch & Gabriel 1990; Lande 1994, 1995; Zeyl *et al.* 2001; Whitlock & Bürger 2004; Coron *et al.* 2013).

Effects of Population Size on History, Chance, and Selection signatures

The relative roles of history, chance, and selection in shaping evolution are long-standing topics for debate among evolutionary biologists (Fisher 1930; Wright 1931; Kimura 1968; Gould & Lewontin 1979; Travisano *et al.* 1995; Teotónio & Rose 2000; Joshi *et al.* 2003; Lenormand *et at.* 2009; Losos 2011; Flores-Moya *et al.* 2012; Fragata *et al.* 2014b; Lachapelle *et al.* 2015; Burke *et al.* 2016; Seabra *et al.* 2018). Life-history traits are great characters with which to address this issue, because of their strong association with fitness. This property allows to test for the relative effects of history, chance, and selection in adaptation, relative effects that are strongly dependent on underlying genetic variation (Lande & Arnold 1983; Flatt & Heyland 2011). Also, the widespread pleiotropy and epistasis among these characters (Roff & Emerson 2006) should foster the dependence of selection outcome on genetic background (Whitlock *et al.* 1995; Gavrilets 2010).

In general, the available experimental results show that the relative roles of history, chance and selection are contingent on the trait under analysis. History and chance have a more preponderant role in characters loosely related to fitness (*e.g.* morphological and stress resistance traits). They are more often of less importance for characters more closely related to fitness, like fecundity and mortality (Travisano *et al.* 1995; Teotónio & Rose 2000; Teotónio *et al.* 2002; Joshi *et al.* 2003; but see Fragata *et al.* 2014b). Furthermore, history's signature is more apparent at the genetic level compared to phenotypic level, as historical contingencies can arise due to the effect of mutations on specific alleles that affect molecular evolutionary paths that have equivalent effects on the phenotype (Blount *et al.* 2008; Bedhomme *et al.* 2013; Fragata *et al.* 2014a; Spor *et al.* 2014).

However, the effect of population size on the evolutionary importance of history, chance, and selection has only recently been addressed by Lachapelle and colleagues (2015)

whom studied different-sized experimental populations of *Chlamydomonas reinhardtii* adapting to a high salt environment. They found that adaptation to salt was repeatable (*i.e.* the evolutionary trajectories and outcomes of different lineages in given conditions will be the same) only in populations with $N_e > 50,000$ because of the large contribution of selection. Adaptation was not repeatable in smaller populations ($N_e > 5,000$) because of large constraints from history.

Previous Experiments with Varying N_e

Smaller populations are expected to have lower adaptive potential. Nevertheless, this association is not straightforward in natural populations, as shown by Wood *et al.* (2016) meta-analysis. In part this may be due to low statistical power, focus on traits loosely related to fitness and complexity of natural population meta-structures (Hoffmann *et al.* 2017). Experimental studies may explore different scenarios under controlled conditions and help clarify the actual role of N_e on adaptive potential.

Experiments which have compared selection lines with markedly different N_e consistently yield large contrasts in the phenotypic response to selection (*e.g.* Jones *et al.* 1968; Eisen *et al.* 1973; Madalena & Robertson 1975; Weber 1990; Weber & Diggins 1990). Furthermore, the magnitude of the effect of N_e depends on the level of environmental stress, and thus the intensity of selection. This is particularly relevant in cases of environmental change (*e.g.* global warming, new pests, habitat degradation, and introduced or evolving parasites), because reduced genetic diversity limits the ability of populations to evolve in response to environmental challenges (*e.g.* Lande 1988, Heschel & Paige 1995, Templeton *et al.* 2001, Frankham *et al.* 2002; Reed *et al.* 2003; Reed & Frankham 2003; Spielman *et al.* 2004b; Frankham 2005a). The population's ability to evolve in response to novel environmental conditions is termed *evolvability* (Houle 1992) or adaptive potential.

It is important to take into account how fast populations adapt to adverse or deteriorating environments (*e.g.* Bell & Gonzalez 2009; Carlson *et al.* 2014; Stelkens *et al.* 2014). Because environments undergo stochastic fluctuations, a factor like N_e which reduces fitness will make a population more susceptible to extinction when further perturbations occur (Reed 2005). A population exposed to rapid and sustained environmental change will decline in numbers if it is too maladapted, risking extinction. Before this happens, however, resistant alleles that are already present in the population, or that appear by mutation, may proliferate,

increase the level of adaptation to the novel environment, and restore population growth – this phenomenon is called *evolutionary rescue* (Bell & Gonzalez 2009). Extinction in the face of environmental change is therefore a race between demography and adaptive evolution (Maynard Smith 1989).

Despite the fundamental importance of N_e in evolutionary theory, it is notoriously difficult to assess (Wang 2005). Several techniques have been developed to estimate N_e in natural and artificial populations (*e.g.* Frankham 1995b; Wang 2005; Luikart *et al.* 2010) and in laboratory Mendelian populations (*e.g.* Gilligan *et al.* 2003; Santos *et al.* 2012; Mueller *et al.* 2013), often now using genomic signatures of genetic drift (Teotónio *et al.* 2009, Orozco-terWengel *et al.* 2012). Still less often is N_e experimentally manipulated to study its impact on evolution under well-defined conditions (Frankham *et al.* 1999; Woodworth *et al.* 2002; Reed *et al.* 2003; see review in Frankham *et al.* 2014). There are classic experiments on such characters as *Drosophila* bristle number which illustrate the impact of N_e qualitatively, especially the way low N_e generates genetic drift (*e.g.* Rasmuson 1952). Weber (2004) has supplied a review of the impact of N_e on the response to selection in studies published between 1949 and 1996. Roughly speaking, the response at generation 50 of selection increases as N_e increases, up to a crude plateau for N_e values over 100-300, as shown in **Figure 2.1**.
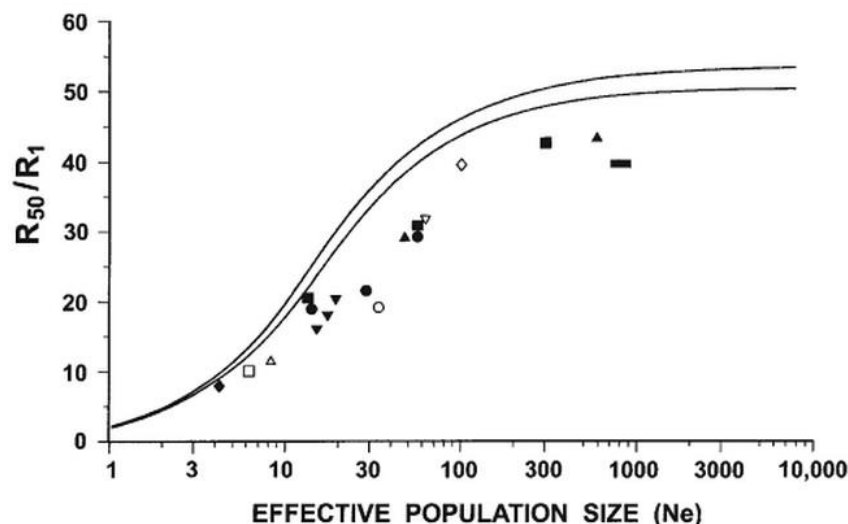
Figure 2.1. A summary of 11 selection experiments showing total response in generation 50, normalized by response in generation one (R_{50}/R_1). The curves show predicted R_{50}/R_1 with and without new mutation at a rate of 0.001 x initial additive genetic variance per generation. © Weber, 2004.

Woodworth *et al.* (2002) studied the joint effect of selection and varying N_e during evolutionary domestication. They founded a total of 23 laboratory populations of *D. melanogaster* at census population sizes ranging from 25 to 500 individuals. After 50 generations in the lab, they compared the performance of flies that had been maintained at different census population sizes, under both benign and crowded conditions. Under benign conditions, individuals from larger N_e populations had better performance compared to individuals from populations with smaller effective size. Under crowded conditions, all laboratory populations had lower performance than recently derived populations.

What do we bring to the table? – The added value of this experiment.

The present study differs from earlier *Drosophila* studies of the effect of effective population size on the response to selection in the following ways: (*i*) the use of 6 ancestral populations with well-characterized histories of maintenance and selection, well-studied functional characteristics, and extensive genome-wide sequencing; (*ii*) the derivation of 84 directly selected and matched control populations; (*iii*) repeated sampling of phenotypic direct and indirect responses to selection over multiple generations.

With this highly-replicated experimental design we aim to respond to several evolutionary questions in the context of colonization, where a population encounters qualitatively novel conditions, suffers demographic perturbations and a reduction in its effective size: *(i)* How will the population respond to these new demographic conditions? *(ii)* How will its life-history evolve in response to such new, harsh conditions? *(iii)* How will the population size differences affect the evolutionary response to the new environment? *(iv)* Will populations from the same source evolve the same way? *(v)* Will the roles of history, chance, and selection shift in populations evolving at different sizes?

MATERIALS AND METHODS

Source of the experimental populations

The experimental populations used in this study were derived from a wild *Drosophila melanogaster* population collected in South Amherst, Massachusetts by Ives in August 1975. The IV population has been maintained ever since at 24-25ºC, large census sizes (N~1,000-2,000), with unlimited food, and 14-day discrete generations. In February 1980, after approximately 130 generations in the laboratory setting, five experimental populations were derived from a single generation of the base population and designated B_{1-5} (where the subscripts indicate the five replicate populations). The B/IV stocks (writing IV as B_0, we have B_{0-5}) have since been assayed maintained in the aforementioned conditions for over 800 generations (*vid.* Rose *et al.* 2004 for more details on their evolutionary history; Kimber & Chippindale 2013, overview their laboratory evolution).

Derivation of the experimental populations

Each of the B replicate populations was used as the ancestor for 10 small-sized populations of about 50 surviving individuals at the time reproduction (5 directly selected + 5 matched controls) and 4 large-sized populations (2 directly selected + 2 matched controls) each maintained with about 1000 surviving individuals at the time of reproduction (see **Table 2.1**), for a total of 84 populations. These census population sizes were sustained throughout the experiment, with increased numbers of adults reared for the replicate populations undergoing selection so as to sustain comparable numbers of surviving females at the time of egg-laying to start the next generation.

Table 2.1. Derivation of the experimental populations. Each B_i population was used to derive 5 selection (S) and 5 control (C) lines (*j* = a, b, c, d, and e), that are kept at small N_e (P for *pequeno*) and 2 selection (S) and 2 control (C) lines *(k* = a, b) at large (G for *grande*) population sizes.

STARVATION RESISTANCE	POPULATION SIZE	
	SMALL (P) ~50 ind.	*LARGE (G)* ~1000 ind.
SELECTED (S)	SPB_{ij}	SGB_{ik}
CONTROLS (C)	CPB_{ij}	CGB_{ik}
Notes	*i = 0,1,2,3,4,5* *j = a, b, c, d, e*	*i = 0,1,2,3,4,5* *k = a, b*

Forward selection procedures for starvation resistance

The eggs from all the populations were collected on regular banana food and given 14 days to develop to adulthood. On day 14, the flies were mixed using CO_2 anesthesia, redistributed in vials (≈50 individuals each), and given 3 days of yeast-supplemented banana food. The control populations were fed with regular banana food, while the populations undergoing selection were given plain agar lacking nutrients during the period of selection. With this protocol, the selected flies had water available from the agar – they were starving but not desiccating. During selection, the census sizes of the cohorts undergoing selection were estimated every 4 hours, until about 80% of each population had starved to death. The surviving flies were then given yeast-supplemented food for 72h. Then the eggs of all populations (selected and controls, both small and large sizes) were synchronously collected to found the new generation.

Figure 2.2 diagrams the starvation-resistance selection protocol. This selection procedure lead to a parallel progressive increase in the age of reproduction for all populations, since the next generation of the controls was initiated at the same time as that of their matched starvation-selected lines. This chapter follows the experimental lines for 25 generations of forward selection to starvation resistance.
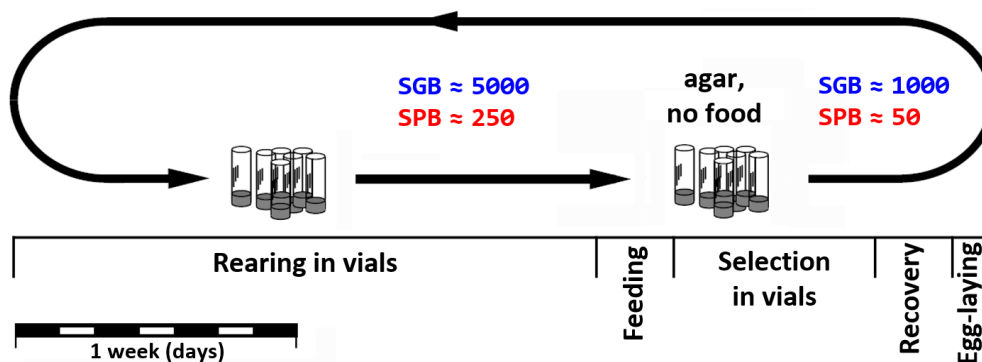
Figure 2.2. Starvation resistant selection protocol in small (**SPB**) and large (**SGB**) populations.

Starvation resistance assay

After one generation with common garden rearing, starvation-assay flies were reared in control conditions. They were given 14 days from egg to develop, after which they were given 72h of yeast-supplemented food. Using CO_2 anesthesia, the flies were paired in couples and flipped into a vial with agar only. Individual mortality was checked every 4 hours, until the last fly died. This trait was assayed at generations 0, 4, 8, 11, 12, 13, 14, 15, 16, 18, 22, and 25 of starvation-resistance selection.

Fecundity assay

After one generation with common garden rearing, fecundity-assay flies were reared for 14 days using the same conditions as those for the starvation resistance assay. Adults were then paired as couples in single vials, with 20 pairs per population. Every 24h for five days, the couple was given fresh charcoal-colored food with yeast paste spread on the surface of the medium. The eggs laid by each female were counted from a photograph of the vial surface (see **Figure 2.3**). This trait was assayed at generations 0, 4, 8, 11, 15, 16, 18, and 22 of starvation-resistance selection.

Figure 2.3. Vial surface photograph showing the eggs resulting from 24h of egg-laying by a single female from the experimental populations.

Statistical data analysis

In all analyses, the normality and homoscedasticity of data were tested by Shapiro-Wilk (1965) and Brown-Forsythe (1974) tests, respectively. After testing by ANOVA and ANCOVA, and when it was appropriate, Tukey HSD (1953) *post-hoc* tests were done. A significance value of 0.05 (α) was used to test all null hypotheses. All analyses were done using *STATISTICA 13* (Dell 2015).

The key statistical tests focused on quantitative measures of how marked N_e disparities affected the direct and indirect responses to selection for increased starvation resistance, *i.e.* estimating the magnitudes of the interactions between these responses and the different N_e values.

The evolutionary trajectories of starvation resistance and fecundity throughout the 25 generations of selection were analyzed according to the following ANCOVA model:

$$Y = \mu + H_i + T_j + S_k + G_l + Rep\{H * T * S\} + H_i * T_j + H_i * S_k + H_i * G_l + T_j * S_k$$
$$+ T_j * G_l + S_k * G_l + H_i * T_j * S_k + T_j * S_k * G_l + H_i * S_k * G_l + H_i * T_j \quad (1.5)$$
$$* G_l + H_i * T_j * S_k * G_l + G_l * Rep\{H * T * S\} + \varepsilon$$

where Y is the trait under analysis (starvation resistance or fecundity); H represents the evolutionary history ($i = 0$–5), random factor; T the population size (j = large or j = small), fixed factor; S the selection regime (k = selected or k = control), fixed factor; G the covariate generation (see which on the assays description), and *Rep* the replicate population (random factor). Generation 0 data were not included in the ANCOVA, since replicates within *History* were still lacking. This is a comprehensive model that includes all variables under study. Analyses within population size or selection regime used adapted versions of the aforementioned model (*e.g.* when comparing small and large populations in control conditions, the factor *Selection* and all its interactions were removed).

When analyzing the selection lines (SGB and SPB), in order to remove the background experimental evolution effect of the changed culture procedures we used data relative to control instead of raw data, *i.e.* we used the difference between the raw value of the character for each individual and the average for the CGB$_i$ controls (or CPB$_i$ depending on the analysis). CGB$_i$ is the average of the two CGB$_{ik}$ lines and CPB$_i$ is the average of the five CPB$_{ij}$ lines. In particular, since we found statistically significant changes in starvation resistance and fecundity without the imposition of selection (**Figures 2.4** and **2.5**, **Table 2.2**), this correction was required for estimating the response to direct starvation selection, stripped of the evolutionary effects of altered nutrition and culture time.

Selection differentials analysis

The selection differential, *S*, was computed for each one of the populations using the breeder's equation: $\boldsymbol{R = S\ h^2}$ (**equation 1.2** of thesis; Falconer & Mackay 1996). R was the cumulative response of female starvation resistance in the first four generations of forward selection (R_4). As a surrogate for h^2 the average heritability estimated for starvation resistance

of B-females by Hutchinson *et al.* (1991) was used. Heritability for starvation resistance has been shown to be high (Service & Rose 1985; Hoffman & Harshman 1999) and it is not expected to change in the first few generations of selection (Jones *et al.* 2003). A two-way mixed-model ANOVA with one fixed factor (*Regime*) and one random factor (*History*) was used to test the differences between the regimes (defined by each combination of size and selection).

Estimation of variance components

This experimental project presents two levels of imbalance in its design: (1) the number of populations within size regime is different (60 populations for small, 24 for large size), and (2) the number of samples per population varies (from 16 to 20 couples). This creates problems when estimating variance components using factorial ANOVA. Joshi, Castillo & Mueller (2003; see Neter *et. al* 1990) presented a solution for a four-way mixed-model ANOVA with a single imbalance at the lowest level. In order to use their approach in our statistical analysis, we split the work into two steps: (1) variance components estimation for *History*, *Size*, and *Selection* (and its interactions), and (2) variance components estimation for *Population* (or *Chance*).

(1) *Variance components estimation for History, Size and Selection:* in this analysis, the average values of each replicate were used, and we had *n* replicate populations in each $H \times T \times S$ combination. Thus, there was only one imbalance due to the number of populations within size regime. The variance components were computed using the expected mean square values of a three-way mixed-model ANOVA with the factors *History* (random, *a=6* levels), *Size* (fixed, *b=2* levels), and Se*lection* (fixed, *c=2* levels), as follows:

History: $\sigma_H^2 = \dfrac{\text{MS H} - \text{MSe}}{\text{b c n}}$ **(1.6)**

Size: "σ_T^2" $= \dfrac{\text{MS T} - \text{MS(H*T)}}{\text{a c n}}$ **(1.7)**

Selection: "σ_S^2" $= \dfrac{\text{MS S} - \text{MS(H*S)}}{\text{a b n}}$ **(1.8)**

*History * Size:* $\sigma_{H*T}^2 = \dfrac{\text{MS(H*T)} - \text{MSe}}{\text{c n}}$ **(1.9)**

*History * Selection:* $\sigma_{H*S}^2 = \dfrac{\text{MS(H*S)} - \text{MSe}}{\text{b n}}$ **(1.10)**

*Size * Selection:* "σ_{T*S}^2" $= \dfrac{\text{MS(T*S)} - \text{MS(H*T*S)}}{\text{a n}}$ **(1.11)**

*History * Size * Selection:* $\sigma_{H*T*S}^2 = \dfrac{\text{MS(H*T*S)} - \text{MSe}}{\text{n}}$ **(1.12)**

Error: $\sigma_e^2 = \text{MS e}$ **(1.13)**

Because the number of replicates per cell (interaction H*T*S) varied, *n* was substituted by *n'*, given by:

$$n' = \frac{1}{(a\,b\,c\,) - 1} \left[\sum_{i=1}^{abc} n_i - \frac{\sum_{i=1}^{abc} n_i^2}{\sum_{i=1}^{abc} n_i} \right] \tag{1.14}$$

(2) *Variance components estimation for Chance:* in this analysis, the absolute value for each individual was used. We had *d* replicate populations in each $H \times T \times S$ combination and *n* individual values in each *{Pop}*$H \times T \times S$ combination. Thus, there were two levels of imbalance: due to the number of populations within size regime and due to the number of samples per population. The variance components for chance were computed using the expected mean square values of a four-way mixed-model ANOVA with the factors *History* (random, *a=6* levels), *Size* (fixed, *b=2* levels), Se*lection* (fixed, *c=2* levels), and *Population* (nested within *H*T*S*, *d* levels), as follows:

$$\text{Population (chance): } \sigma^2_{Pop\{H*T*S\}} = \frac{\text{MS Pop}\{H*T*S\} - \text{MSe}}{n} \tag{1.15}$$

Because the number of individuals per population varied, *n* was substituted by *n'*, given by:

$$n' = \frac{1}{(abcd) - 1} \left[\sum_{i=1}^{abcd} n_i - \frac{\sum_{i=1}^{abcd} n_i^2}{\sum_{i=1}^{abcd} n_i} \right] \tag{1.16}$$

The variance components of large and small populations were also analyzed separately using adapted versions of the previous models.

Population differentiation analysis

Population differentiation was assessed by comparing the variances for starvation resistance and early fecundity between the four regimes (CGB, CPB, SGB, and SPB) after 25 and 22 generations of forward selection, respectively. For each regime the variance was computed using the mean square of error in a one-way ANOVA (*History* has a random factor) of the populations' average value. The variance was then standardized by dividing the average value of the character. This standardization was chosen because the relationship between variance (σ^2) and mean (μ) in the characters under study was more linear than the relationship between variance (σ^2) and square of mean (μ^2). The significance of the difference between each pair of variances was then compared, using the ratio of the greater variance over the smaller (*Fs,* Sokal & Rohlf 1995).

RESULTS

General linear model assumptions

As a consequence of the Central Limit Theorem, moderate violations of normality have little material effect on the robustness of analysis of variance, as long as homoscedasticity is guaranteed (Sokal & Rohlf 1995). Small deviations from normality were accepted, and homoscedasticity was verified by the Brown-Forsythe test, which has great robustness and statistical power even when significant deviations from normal distributions occur (Olejnik & Algina 1987). Our distribution tests showed that all populations were homoscedastic and generally normal (data not shown).

Adaptation to the culture regime

To analyze the effect of the handling procedure on the characters of interest, regardless of any imposed selection, both small and large control populations were assessed. During the 25 experimental generations, a general increase in starvation resistance and decrease of early fecundity was seen. Starvation resistance of large *N* controls (CGB) significantly increased from 28.7 to 35.9 hours, in males, and from 49.9 to 62.5 hours, in females. The small *N* controls (CPB) saw their starvation resistance significantly increased from 28.7 hours to 40.2 hours, in males, and from 49.9 to 74.3 hours, in females. In terms of early fecundity, CGB populations suffered a statistically significant decline, from 244 to 176 eggs. A similar effect was shown by the small controls, CPB, which started with 244 eggs and ended with 144 eggs. The pattern of life-history trait change over the length of the experiment is shown on **Figures 2.4** and **2.5** for CGB and CPB, respectively. **Table 2.2** summarizes the ANCOVA results.

Table 2.2. Summary of the mixed-effects ANCOVAs used to analyze the evolutionary trajectories of small and large controls. Data shows the F statistic and respective p-value for the facto*r Generation* of each size regime and life-history trait analyzed. All changes through time were significant.

Regime	Male starvation resistance		Female starvation resistance		Early fecundity	
	F statistic	*p-value*	*F statistic*	*p-value*	*F statistic*	*p-value*
CGB	89.7852	0.000221	59.0850	0.000594	8.2936	0.034581
CPB	24.7176	0.004136	14.2969	0.012834	33.407	0.002179
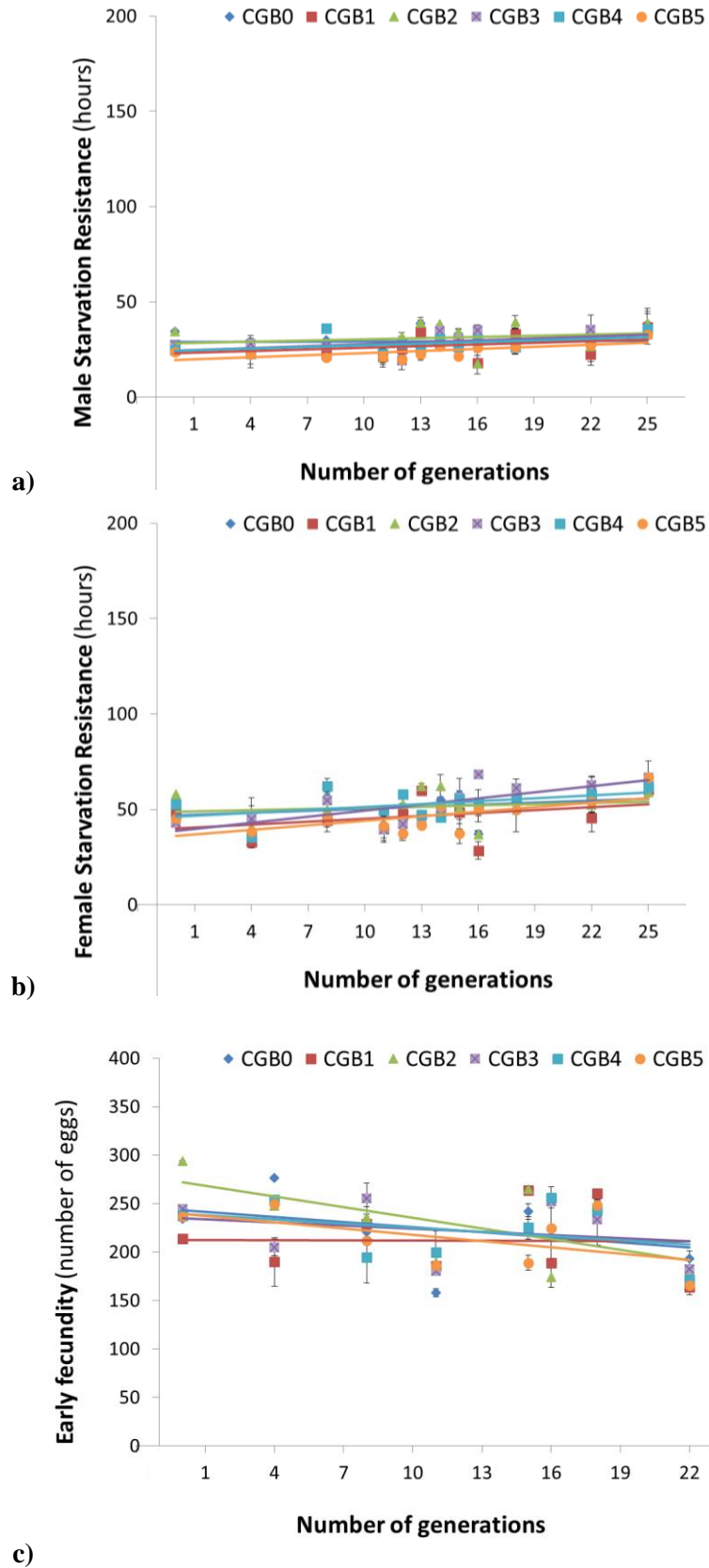
Figure 2.4. Evolutionary trajectories for **a)** male starvation resistance, **b)** female starvation resistance, and **c)** early fecundity of large control populations (CGB). Average population values for each B_i ancestor are shown. Error bars denote standard error of mean (computed as differences between replicate populations).
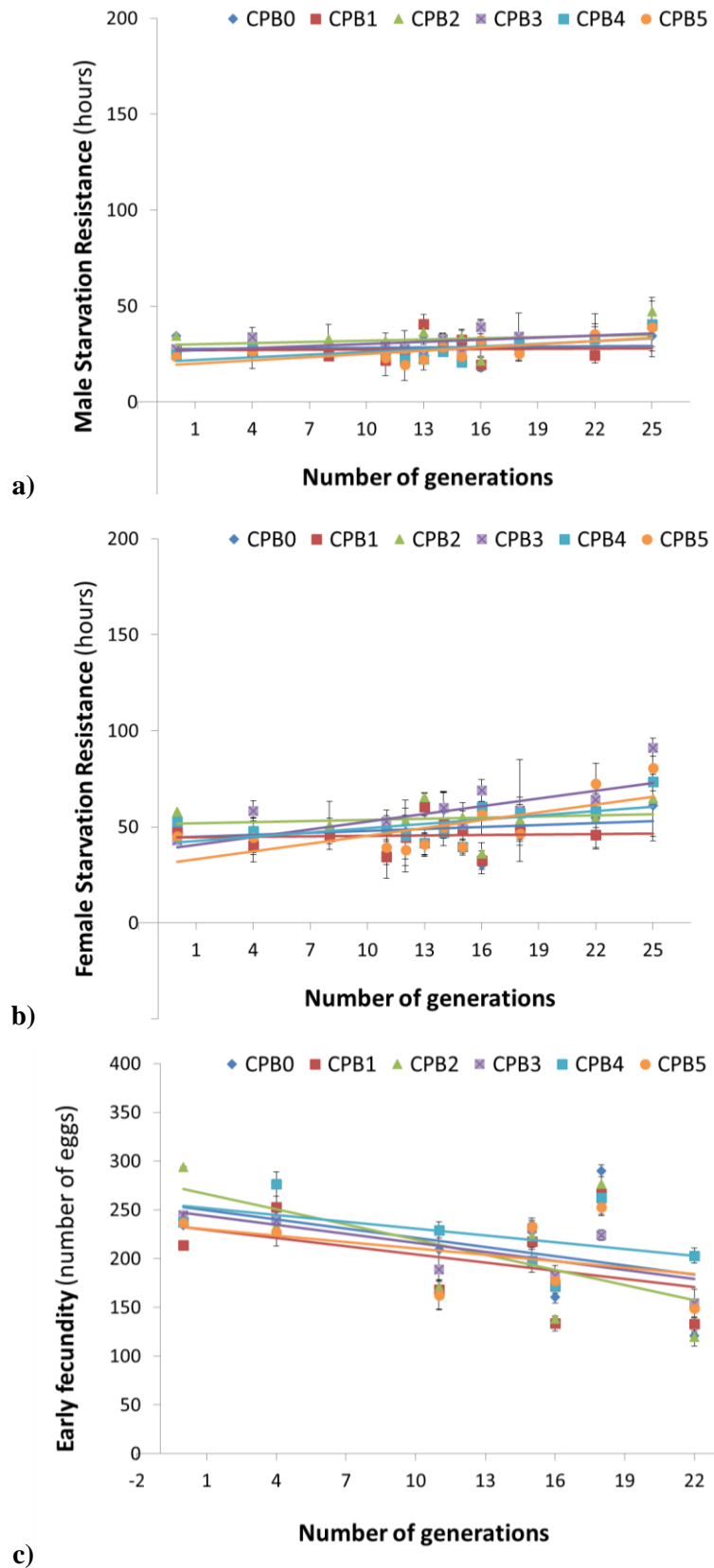
Figure 2.5. Evolutionary trajectories for **a)** male starvation resistance, **b)** female starvation resistance, and **c)** early fecundity of small control populations (CPB). Average population values for each B_i ancestor are shown. Error bars denote standard error of mean (computed as differences between replicate populations).

Effect of population size reduction in controlled laboratory conditions

As seen in the previous section, throughout this experiment, both large and small control populations significantly increased their starvation resistance and decreased their early fecundity (**Figures 2.4** and **2.5**, **Table 2.2**). The evolutionary rates between the two control regimes were then compared. In terms of starvation resistance, there were no significant differences between regimes. However, the early fecundity decline was significantly more abrupt in the small populations (CPB) than in their larger counterparts (CGB). **Table 2.3** summarizes the ANCOVA used to compare the evolutionary trajectories.

Table 2.3. Summary of the mixed-effects ANCOVAs used to compare the evolutionary trajectories of small and large controls. Data shows the F statistic and respective p-value for the interaction *Size*Generation* of each life-history trait analyzed. Significant changes are highlighted in bold.

Male starvation resistance		Female starvation resistance		Early fecundity	
F statistic	*p-value*	*F statistic*	*p-value*	*F statistic*	*p-value*
0.0028	0.959778	0.6146	0.468503	**15.9569**	**0.010354**

Effect of population size under strong forward selection

To analyze the effect of sustained small population size on the rate of response to strong selection we compared the evolutionary trajectories of SGB and SPB regimes using the control-corrected data (that is, the difference from their respective CGB_i control, see *Material and Methods*). This correction was required for focal statistical analysis of the effects of direct starvation selection, since we found statistically significant changes in starvation resistance and fecundity among the control lines (**Figures 2.4** and **2.5**, **Table 2.2**). Additionally, we repeated all the analyses using CPB_i as controls for SPB_{ij} and obtained similar results (data not shown).

All selected populations, both in large (SGB) and small size (SPB), substantially increased their starvation resistance and declined their early fecundity over the course of the experiment (**Figures 2.6** and **2.7**). After 25 generations of forward selection, the SGB populations had their male starvation resistance (relative to CGB) increased by 128.5 hours, being a four-fold higher than the controls. SGB females also had their starvation significantly increased to 152.9 hours, three times higher than CGB. By the end of the selection experiment, SPB male starvation resistance averaged 44.4 hours (two times larger than CGB) and SPB females increased 66.7 hours (also twice the controls). Both starvation-selected stocks suffered

a fecundity decrease through the experiment, although only SPB showed a significant decline (54 eggs less than CGB by generation 22, or 70% of the latter output). **Table 2.4** summarizes the ANCOVA results.

Table 2.4. Summary of the mixed-effects ANCOVAs used to analyze the evolutionary trajectories of small and large selected lines. Data shows the F statistic and respective p-value for the facto*r Generation* of each size regime and life-history trait analyzed. Significant changes are highlighted in bold.

Regime	Male starvation resistance		Female starvation resistance		Early fecundity	
	F statistic	p-value	F statistic	p-value	F statistic	p-value
SGB	**279.4444**	**0.000013**	**845.4445**	**0.000001**	0.514836	0.505121
SPB	**36.72685**	**0.001765**	**73.02790**	**0.000361**	**9.554997**	**0.027037**

After analyzing the evolutionary trajectories of each regime, we tested for differences in the response of starvation resistance and fecundity between the SGB and SPB stocks. As expected, in both males and females, the rate of response of the large lines was significantly higher than the one observed for small N_e populations. In terms of early fecundity, no significant difference between SGB and SPB lines were found (see **Table 2.5**).

Table 2.5. Summary of the mixed-effects ANCOVAs used to compare the evolutionary trajectories of small and large selected stocks. Data shows the F statistic and respective p-value for the interaction *Size*Generation* of each life-history trait analyzed. Significant changes are highlighted in bold.

Male starvation resistance		Female starvation resistance		Early fecundity	
F statistic	p-value	F statistic	p-value	F statistic	p-value
62.6444	**0.000516**	**95.5595**	**0.000188**	0.50520	0.508980

a)

b)

c)
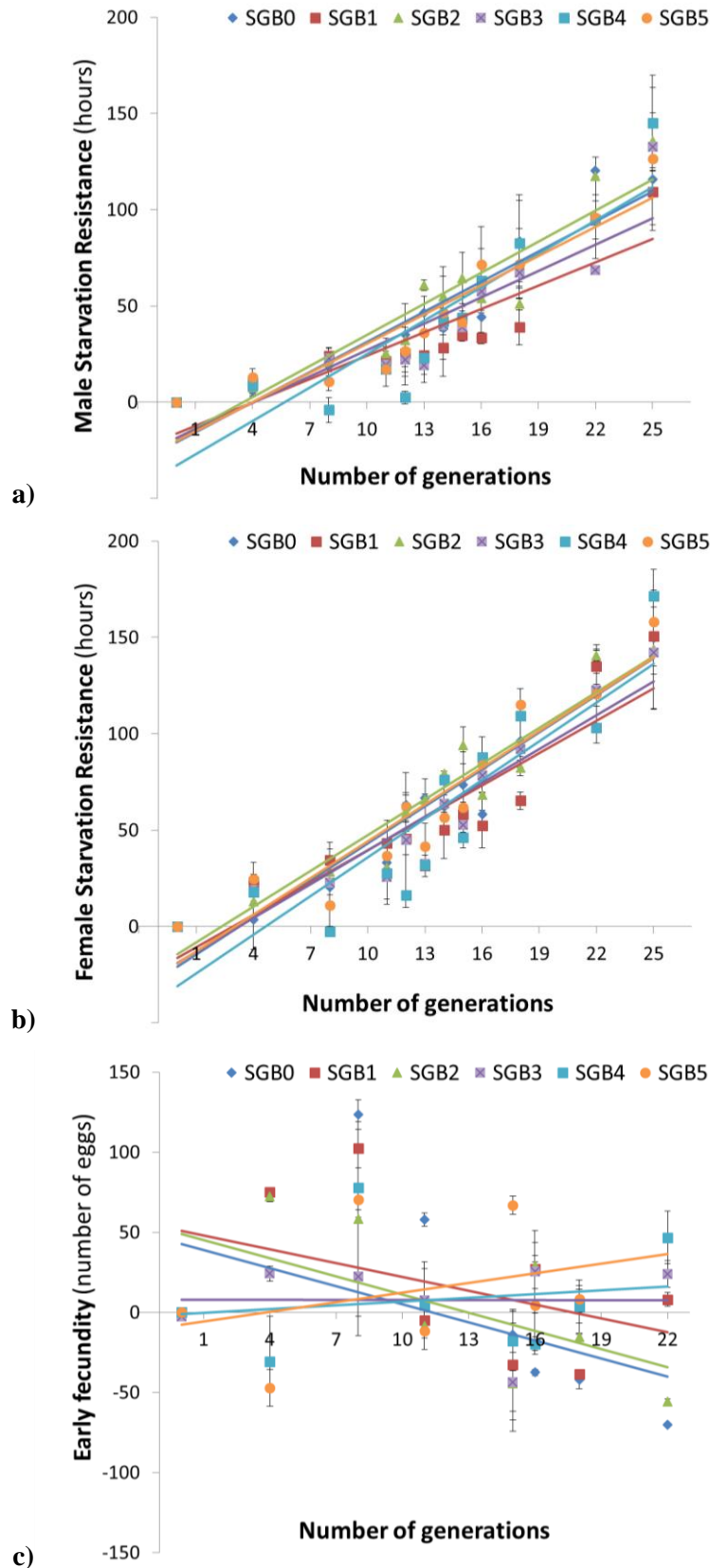
Figure 2.6. Evolutionary trajectories for **a)** male starvation resistance, **b)** female starvation resistance, and **c)** early fecundity of large selected populations (SGB). Average population values (CGB$_i$-corrected) per B$_i$ ancestor are shown. Error bars denote standard error of mean (computed as differences between replicate populations).
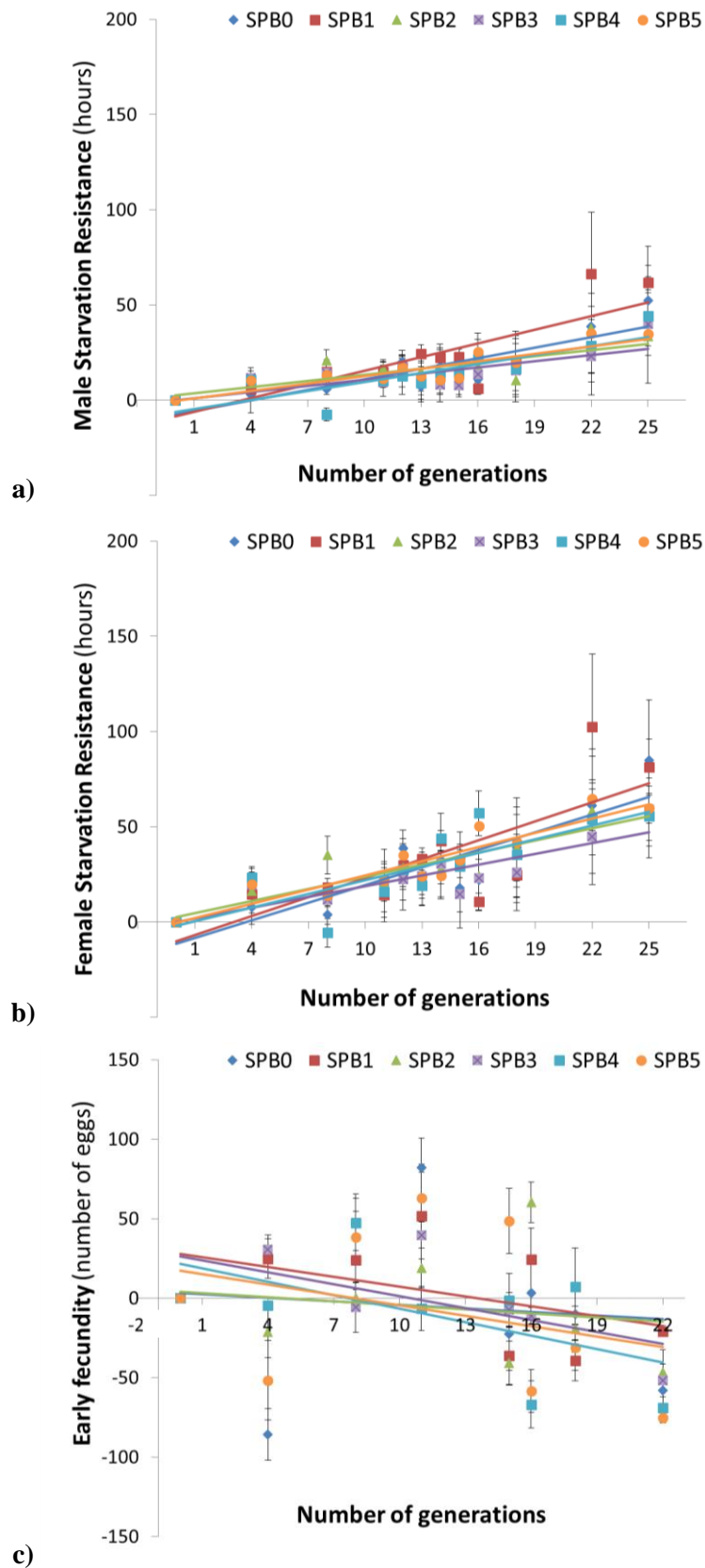
Figure 2.7. Evolutionary trajectories for **a)** male starvation resistance, **b)** female starvation resistance, and **c)** early fecundity of small selected populations (SPB). Average population values (CGB_i-corrected) per B_i ancestor are shown. Error bars denote standard error of mean (computed as differences between replicate populations).

Effect of strong directional selection during a sustained bottleneck

In order to evaluate the effect of sustaining a strong selection protocol when the populations undergo a long-term bottleneck, we compared the small-selected stocks (SPB, **Figure 2.7**) with their small-controlled counterparts (CPB, **Figure 2.5**). Because of the evolutionary response to the culture regime, these two small stocks were compared using the relative data to the average CGB_i. As expected, both male and female starvation resistances were significantly higher in the selected lines. In terms of fecundity, no significant difference was found between CPB and SPB evolutionary trajectories (see **Table 2.6**).

Table 2.6. Summary of the mixed-effects ANCOVAs used to compare the evolutionary trajectories of SPB and CPB. Data shows the F statistic and respective p-value for the interaction *Selection*Generation* of each life-history trait analyzed. Significant changes are highlighted in bold.

Male starvation resistance		Female starvation resistance		Early fecundity	
F statistic	*p-value*	*F statistic*	*p-value*	*F statistic*	*p-value*
34.53016	**0.002023**	**49.56771**	**0.000890**	0.00722	0.935585

Effect of Size on Selection Differentials

Selection differentials were computed based on the cumulative response of female starvation resistance in the first four generations of forward selection (**Table 2.7**). First, selected lines were compared to controls within population size regime, and significant differences were found in both large (p<0.002) and small sizes (p<0.002). This implies that the selection protocol is causing a difference between the base populations and the selected individuals. Second, the effect of size was assessed, by comparing large selected populations (SGB) with the small selected populations (SPB). In this case, no significant differences were found (p>0.9), *i.e.* the differences in selective response between SGB and SPB were due to the effect of size and not to different selection pressures from the start of the experiment.

Table 2.7. Selection differentials computed for female starvation resistance. Data shows the mean selection differential for each regime (*size*selection* interaction) and the respective standard error.

Regime	Size	Selection	Mean value	Standard error
CGB	Large	Control	-6.574	6.742
CPB	Small	Control	-1.973	7.578
SGB	Large	Selected	4.367	8.279
SPB	Small	Selected	4.448	7.698

Relative Effect of History, Chance, Size, and Selection during Forward Selection

The evaluation of the relative effects of *History*, *Chance*, *Size* and *Selection* was done using a variance components analysis through time. This analysis provided estimates of the relative amount of variance that could be attributed to each one of these factors. The initial differences between the experimental populations were attributed exclusively due to the effect of *History* only (differences in B_i ancestor), which accounts for 100% explained variance for *History* in generation zero.

Male and female starvation resistance showed a very similar pattern of variance component change – see **Figure 2.8 a)** and **b)**. The effect of *History* suffered a very sharp decrease during the first 5 generations, showed some variation during the following 12, and by generation 18 (and from there on) was null. The effect of *Chance* increased for 5-6 generations, reaching 40% of explained variance; it then dropped slower until generation 15, and stayed steadily low for the rest of the experiment (about 5% of explained variance). The effect of *Size* was only apparent by generation 12, when it explained 20% of the total variance. That value remained steady for the rest of the experiment. Lastly, *Selection* exhibited a rapid increase in its relative effect up to generation 12, when it explained about 80% of the variance encountered. It stayed around that value for the remainder of the experiment, albeit with some variation.

As expected, the temporal change of variance components for early fecundity was very different from starvation resistance – see **Figure 2.8 c)**. First, *Selection* had a lower impact, explaining at most only 20% of the total variance. There was a slow increase until generation 18, with a sudden drop at generation 15 and a smaller decrease at generation 22. The effect of *Size* was only apparent from generation 6 on, reaching 40% towards the end of the experiment. It increased with time and showed two drops to zero on generations 15 and 18. *History* dropped abruptly and stayed close to zero from the first few generations on (with slight increases on generations 16 and 22). Finally, *Chance* seemed to be the most important factor for early fecundity variance. It showed somewhat erratic fluctuation. It was around 90% of explained variance from generation 4 to 15. It then started decreasing, showing a fast drop and then recovered at generation 16.

Male Starvation Resistance

a)

Female Starvation Resistance

b)

Early Fecundity

c)
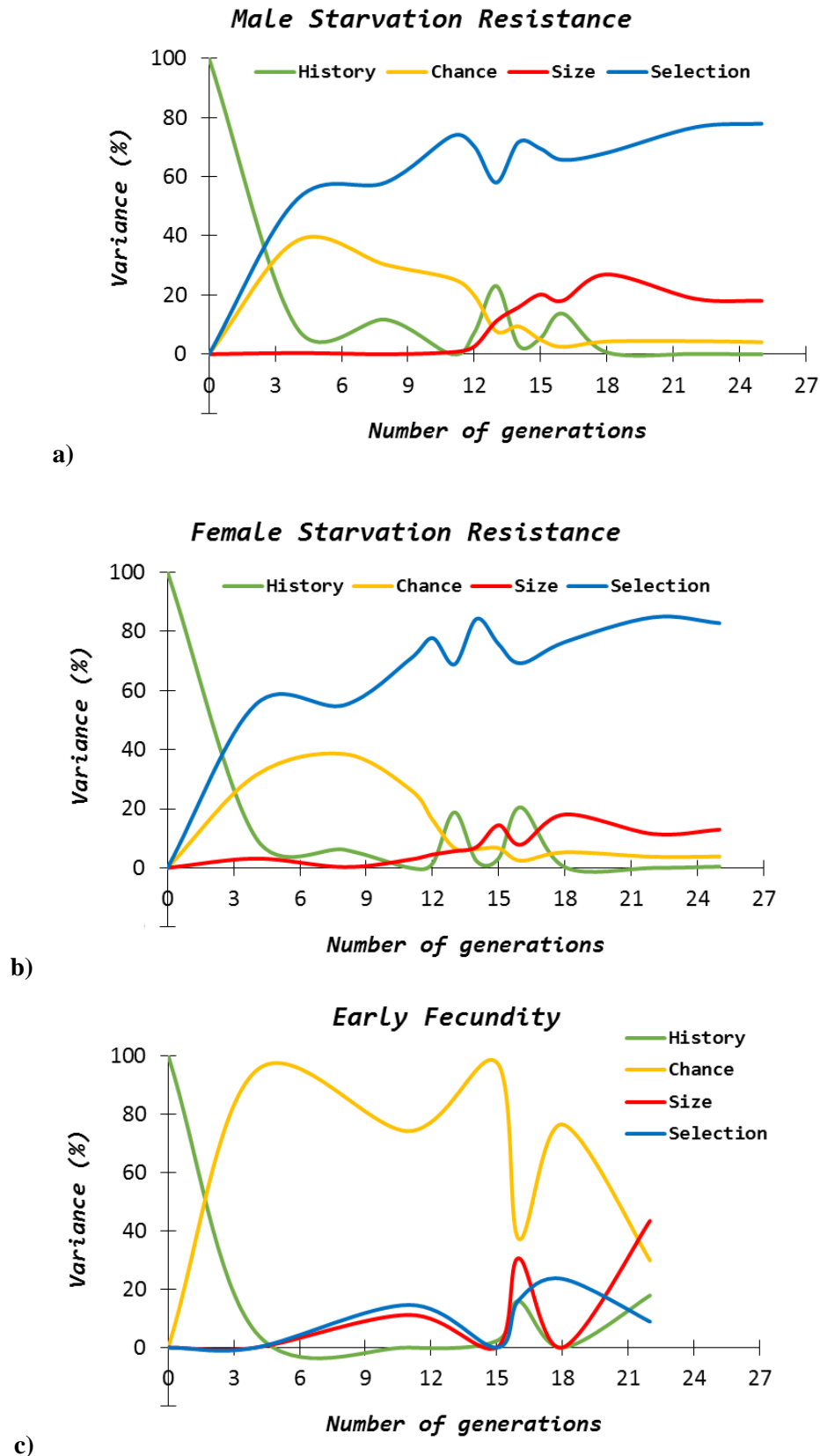
Figure 2.8. Evolution of variance components for **a)** male starvation resistance, **b)** female starvation resistance, and **c)** early fecundity, expressed as percent of explained variance.

Effect of Size on History, Chance, and Selection signatures

The evolution of variance components of large and small populations was analyzed separately and compared. Once again, the initial differences between the populations were exclusively due to the differences in B_i ancestor, accounting for 100% of explained variance for *History* in generation zero.

The patterns for male and female starvation resistance were again very similar (**Figure 2.9** and **2.10**, respectively). The effect of *History* dropped slower, and it was always higher, in the small populations. Similarly, the % of variance explained by *Chance* in small populations was generally higher, and the differences were aggravated with time. The effect of *Selection* showed a sharper increase in the large populations and it was always higher when compared to its effect on the small populations.

The temporal change of variance components for early fecundity was clearly different from that for starvation resistance; it is depicted in **Figure 2.11**. The effect of *History* dropped sharply and it was generally lower in small populations. Except for a sudden drop in generation 16, the effect of *Chance* was always more pronounced in small than in large populations. Finally, the effect of *Selection* was barely noticed in large populations, explaining from 20 to 40% of the character variance in small populations.

Figure 2.9. Variance components in large and small populations for male starvation resistance: **a)** history, **b)** chance, and **c)** selection, expressed as percent of explained variance.

Importance of History in Female Starvation Resistance
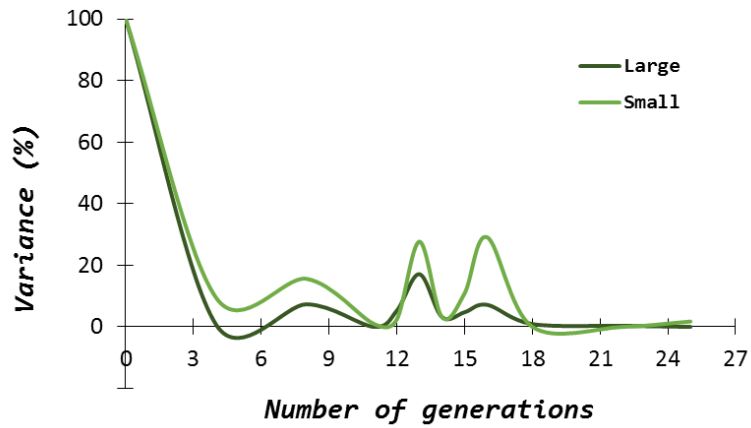
a)
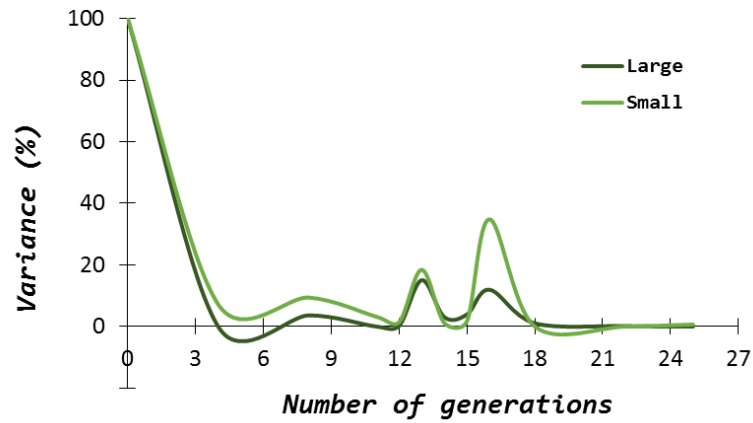
Importance of Chance in Female Starvation Resistance

b)
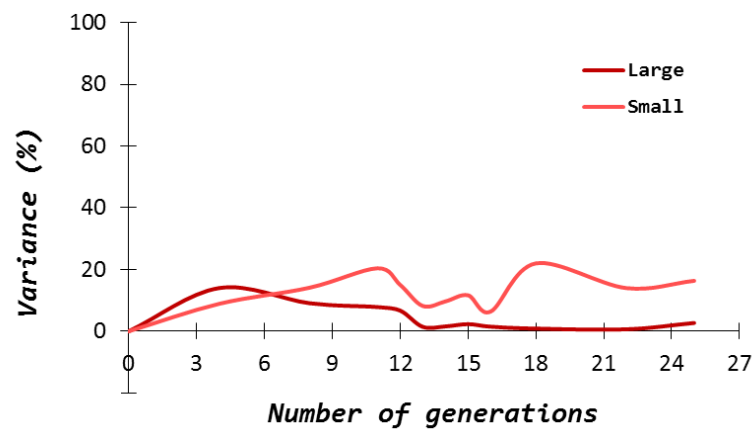
Importance of Selection in Female Starvation Resistance

c)
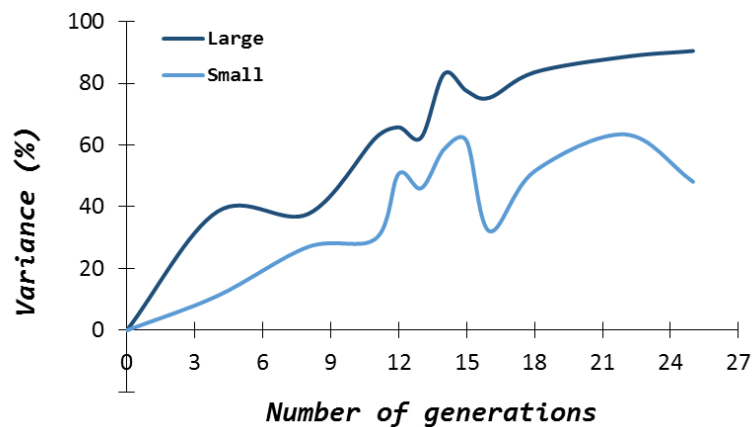
Figure 2.10. Variance components in large and small populations for female starvation resistance: **a)** history, **b)** chance, and **c)** selection, expressed as percent of explained variance.
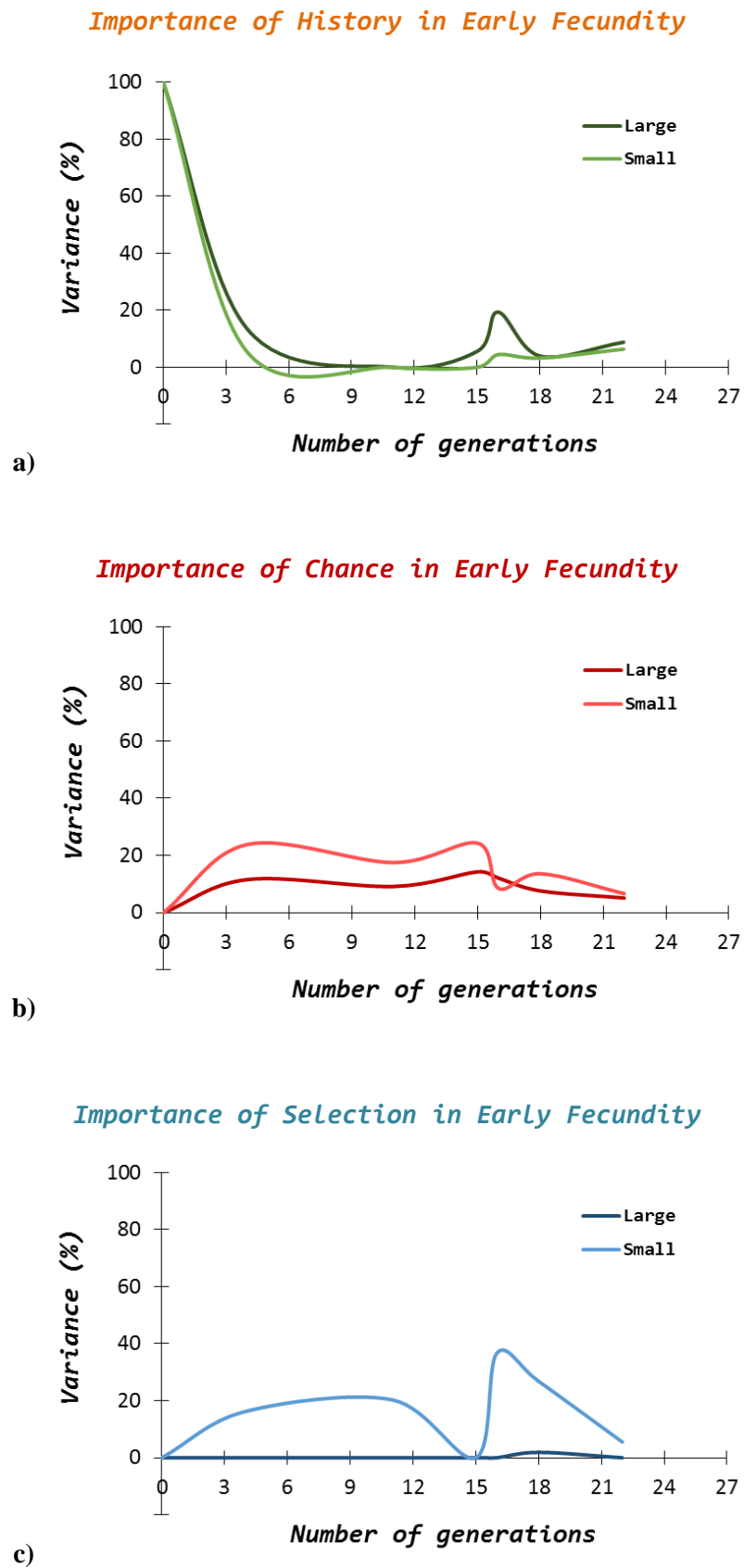
Figure 2.11. Variance components in large and small populations for early fecundity: **a)** history, **b)** chance, and **c)** selection, expressed as percent of explained variance.

Selection, Drift, and Population Differentiation

At the end of the forward selection experiment, the population differentiation was assessed by computing and comparing the nested-population (or *"chance"*) variance in each of the four regimes (CGB, CPB, SGB, and SPB) in terms of starvation resistance at generation 25 of selection and fecundity at generation 22. In the absence of selection, the small populations showed a significantly higher variance in starvation resistance, but not in male starvation resistance or fecundity. Under selection, there was no variance difference between SPB and SGB. Selection seemed to cause an increase of population differentiation among the large selected populations only (SGB), with a significantly higher heterogeneity in female starvation resistance relative to large unselected populations. **Table 2.8** shows the *F-ratios* (Sokal & Rohlf 1995) and their respective statistical significance.

Table 2.8. Effect of size and selection on population differentiation. Each comparison shows the variance of each regime standardized (divided by the mean). Significance of *F-ratios*: n.s. $p > 0.05$; * $0.01 < p < 0.05$; ** $p < 0.01$.

Comparison	*CPB / CGB*	*SPB / SGB*	*SGB / CGB*	*SPB / CPB*
Effect	Size in control conditions	Size under forward selection	Selection in large size	Selection in small size
Male starvation resistance	5.95 / 0.95 *	4.26 / 3.20 n.s.	3.20 / 0.95 n.s.	4.26 / 5.95 n.s.
Female starvation resistance	5.45 / 0.23 **	3.72 / 3.21 n.s.	3.21 / 0.23 **	3.72 / 5.45 n.s.
Early Fecundity	2.45 / 1.28 n.s.	2.07 / 1.02 n.s.	1.02 / 1.28 n.s.	2.07 / 2.45 n.s.

DISCUSSION

Adaptation to the culture regime

An important feature of any experimental evolution design is the effect of the handling procedure on the characters of interest, irrespective of any deliberately imposed selection. There are significant differences between the general handling procedures imposed on the B_i ancestors and those imposed on the control and starvation-selected populations in this experiment. Specifically, the generation length and the pattern of nutrition were both changed for all populations. Generation length was increased at first to 19 days, but progressively extended to 31 days over the course of the 25 generations of selection, due to the increasing average starvation resistance of the selected lines. The nutritional change consisted of yeast supplementation on the surface of the vials in which adults were kept, as described in the *Materials and Methods* section. The secular increase in starvation resistance and decrease in fecundity among the large-sized controls (the CGB populations, **Figure 2.4**) were strong evidence of this phenomenon. While the changes in starvation resistance were statistically significant (**Table 2.2**), they were of relatively small magnitude, particularly compared to the changes in starvation resistance shown by the selected lines (**Figures 2.6** and **2.7**). These patterns of increased starvation resistance and antiparallel decline in early fecundity with postponed reproduction were previously observed (*e.g.* Service & Rose 1985; Hutchinson *et al.* 1991; Rose *et al.* 1992).

Effect of population size reduction in controlled laboratory conditions

The comparison of replicated experimental stocks kept simultaneously in conditions that only differed with respect to their population size regimes, provides a powerful test for the evolutionary effect of sustained small N_e. Evidence for the impact of reduced N_e on function was supplied by the significant difference between small (CPB) and large stocks (CGB) in early fecundity. However, there were no detectable effects on male or female starvation resistance (**Figures 2.4** and **2.5**, **Table 2.3**). Because fecundity is a character closer to fitness than starvation resistance, we have a greater expectation that weaker selection and more rapid genetic drift allowed recessive deleterious alleles to rise to high frequencies for fecundity in these populations.

Effect of population size under strong forward selection

With strong selection for starvation resistance, the response to the imposed stress is expected to be proportional to the strength of selection differential, the initial heritability, and the effective population size (Frankham & Kingsolver 2004). The evolutionary trajectories of both small (SPB) and large-sized populations (SGB) showed a substantial increase in starvation resistance with time, but significantly lower magnitude responses to selection in the small-selected stocks (**Figures 2.6** and **2.7, Tables 2.4** and **2.5**). These findings matched conventional expectations, since lower N_e is expected to reduce overall fitness and limit the response to selection, because the smaller lines are subjected not only to more loss of heterozygosity in turn due to more rapid genetic drift, but also to less effective selection (Woodworth *et al.* 2002; Hartl & Clark 2007; Hare *et al.* 2011; Hoffmann *et al.* 2017, but see Wood *et al.* 2016).

Furthermore, the selection lines were significantly different from their controls with respect to the selection differentials for female starvation resistance (**Table 2.7**). This means that the selection protocol is causing differences between the base populations and the selected individuals, as expected. In addition, the lack of initial differences between SGB and SPB selection differentials, implies that the phenotypic differences between small and large stocks were due to the effect of population size and not to different selection pressures.

In previous studies, selection for starvation resistance has shown to increase mean longevity and reduce early fecundity, indicating a positive genetic correlation between these stress-resistance traits and longevity, and a negative genetic correlation between such survival-related characters and early fecundity (Rose *et al.* 1992; Leroi *et al.* 1994a,b; Chippindale *et al.* 1996). Our findings corroborate these results, as both small and large-sized selection stocks saw their fecundity decline through time (**Figures 2.6c** and **2.7c, Table 2.4**). We would expect the small stocks to suffer a less pronounced decline in fecundity due to the said trade-off, but a sharper one due to inbreeding depression. Indeed, SPB fecundity decreased abruptly with time, while SGB did not (**Table 2.5**). SPB decline could be solely explained by the detrimental effects of inbreeding in small stocks, but it was not: when SPB fecundity was corrected using CPB controls (to account for the effects of inbreeding) there was still a significant decline with time. The lack of difference between SPB and SGB was probably due to low statistical power in large stocks, given the high variance observed. The relative lack of replication in the large populations can contribute for these results. But an open question remains: considering previous studies in the Rose lab why is it that the trade-off is not visible in the large populations?

Effect of strong directional selection during a sustained bottleneck

When populations are kept at small N_e for extensive periods of time, their fitness is expected to decline due to the detrimental effects of inbreeding (Charlesworth & Willis 2009). As our results showed, those populations that underwent a sustained bottleneck saw their evolutionary response to starvation impaired, as well as their fecundity (**Figures 2.5** and **2.7**, Tables **2.2** and **2.4**).

We also wanted to determine how sustained bottlenecks interact with directional selection. The *a priori* expectation was that all small-sized populations would suffer a decline in fecundity due to inbreeding, but that this decline would be exacerbated by starvation resistance selection and its trade-off with early fecundity (*e.g.* Chippindale *et al.* 1996). Our results showed that selected lines had a significantly higher starvation resistance than the controls, which means that, although impaired, the effect of selection was strong enough to counteract the inbreeding caused by small N_e. In terms of fecundity, selected females from small populations laid on average 23 eggs less than their matched unselected counterparts, but this difference was not statistically significant (**Table 2.6**). It seems that selection for starvation resistance, by itself, was not strong enough to cause a significant decline in fecundity.

Size and its Effect on History, Chance, and Selection signatures

At the beginning of this experiment, variation among all populations was due to the secular drift of the source stock, as the B_i ancestors were kept separate for over 800 generations. For male and female starvation resistance, the strongly selected traits in SPB and SGB, the signature of *History* was quickly erased by *Selection* (**Figures 2.8a,b**), as shown in other studies (Travisano *et al.* 1995; Joshi *et al.* 2003; Fragata *et al.* 2014b). The signature of *Selection* was always relatively weaker and the one of *Chance* always stronger in small populations, compared to their effects on the large populations (**Figures 2.9** and **2.10**), as expected. In fact, in large populations *Chance* was important only for the first few generations, probably due to sampling effects (Santos *et al.* 2012). For early fecundity (**Figure 2.11**), an indirectly selected trait in regimes selected for starvation resistance, and in large-sized populations, the importance of *History* dropped fast and abruptly a few generations into the experiment, while *Selection* had little impact throughout the generations. Conversely, *Chance* seemed to be the most important factor for fecundity variance. These results corroborate theoretical expectations, since *Selection* is not acting directly on this trait across regimes.

Furthermore, in small populations, the effect of *History* was generally lower and the signature of both *Chance* and *Selection* was always more pronounced. Overall, our results show that the relative roles of history, chance and selection are contingent on the trait under analysis, in accordance with other studies (Travisano *et al.* 1995; Joshi *et al.* 2003; Fragata *et al.* 2014b). Additionally, *History* and *Chance* play more preponderant roles in smaller populations but are quickly overrun by selection, particularly in larger populations.

Selection, Drift, and Population Differentiation

In the absence of mutation and directional selection, genetic variation is randomly lost by drift from individual populations, but between-population differentiation increases, as a result of genetic drift (*e.g.* Hänfling & Brandl 1998). Our results showed that, in the absence of strong directional selection, as occurs for starvation resistance in the control populations, the between-population variance was significantly higher in small populations than large, most likely due to differences in genetic drift. Under selection, the variance between small replicates seemed to be reduced, which supports the thesis that strong directional selection can reduce between-population variance. In the large outbred lines, selection seemed to cause an increase of population differentiation on the starvation resistance of females that showed to have a significantly higher heterogeneity when compared to their matched controls (**Table 2.8**). This later result, though not the former, is consistent with the findings of Cohan & Hoffmann (1989), where selection led their *Drosophila* populations to diversify. The causes of such contrasting patterns among populations of different sizes are elusive. Future genomic analysis in these populations may help clarify this issue.

Final remarks

In the absence of strong directional selection, one impact of a 20-fold reduction in population size was a significant decline in fecundity, showing clearly the loss of function caused by small N_e. On the other hand, the response to strong directional selection for starvation resistance was significantly reduced in magnitude in the smaller population-size lines. These results are consistent with the overall fitness decline and reduced response to selection expected with lower N_e, as smaller-sized populations are subjected not only to more severe loss of heterozygosity and inbreeding due to stronger drift, but also to less effective selection.

The relative roles of history, chance, and selection in shaping evolution are a long-standing topic for discussion among evolutionary biologists and, in general, the present results show that they are contingent on the trait under analysis. Our experimental results also show that history and chance are more important with smaller than with larger N_e.

Chapter Three.

Hybridization and Forward Selection with Varying Population Size

ABSTRACT

The complex interplay of gene flow, mutation, drift, and selection in natural populations hinders the prediction of which evolutionary force will be predominant at any given time or place. It has been clearly shown that inbreeding resulting from genetic drift (especially in small populations) can strongly reduce a population's fitness and increase extinction risk. Several natural and experimental studies have demonstrated that small, inbred populations can have their fitness increased and inbreeding depression alleviated by introducing genetic variation, in a process called genetic rescue. Here we present a comprehensive study of the effect of the genetic rescue using inter-population hybridization in different evolutionary scenarios: *i)* under a sustained bottleneck, *ii)* under strong directional selection, and *iii)* under strong directional selection and small population size. Our major findings suggest that (1) hybridization can have very strong effects on a population's subsequent evolution, especially under a sustained bottleneck; (2) the effects of heterosis are pervasive and generally diluted with succeeding generations; (3) some characters are more susceptible to hybridization than others (fecundity and starvation resistance, respectively). We conclude that the complexity of life-history genetic architecture and the multitude of factors that can interfere with the outcome of hybridization, make it very unpredictable. As such, the classical theoretical expectations may not be obtained, at least over our range of time and effective population sizes. Thus, the evolutionary consequences of hybridization are of such magnitude and unpredictability that its use on conservation management should be done with caution, especially in small, endangered populations.

RESUMO

A complexa interacção do fluxo de genes, mutação, deriva e selecção em populações naturais dificulta a previsão de qual a força evolutiva predominante num determinado momento ou cenário. Já foi claramente demonstrado que a consanguinidade resultante da acção da deriva genética (especialmente em populações pequenas) pode reduzir fortemente a performance adaptativa duma população e aumentar o seu risco de extinção. Vários estudos em populações naturais e experimentais demonstraram que populações pequenas e consanguíneas podem aumentar sua performance adaptativa e diminuir os efeitos da depressão consanguínea por introdução de variabilidade genética, num processo denominado resgate genético. Neste manuscrito apresentamos um estudo abrangente do efeito do resgate genético usando hibridação interpopulacional em diferentes cenários evolutivos: *i)* sob constante efeito de gargalo, *ii)* sob forte selecção direccional e *iii)* sob forte selecção direccional e tamanho populacional pequeno. Os nossos resultados sugerem que (1) a hibridação pode ter efeitos muito fortes sobre a subsequente evolução da população, especialmente sob um constante efeito de gargalo; (2) os efeitos da heterose são generalizados e geralmente diluídos com o passar das gerações; (3) alguns caracteres são mais susceptíveis à hibridação do que outros (fecundidade e resistência à inanição, respectivamente). Concluímos que a complexidade da arquitectura genética de características da história da vida e a multiplicidade de factores que podem interferir com a hibridação, tornam os seus efeitos muito imprevisíveis. Assim, as expectativas teóricas clássicas podem não ser atingidas, pelo menos com o nosso intervalo de gerações e de efectivos populacionais. Deste modo, as consequências evolutivas da hibridação são de tal magnitude e imprevisibilidade que o seu uso como estratégia de gestão populacional em conservação deve ser feito com cautela, especialmente em populações pequenas, em risco de extinção.

INTRODUCTION

Hybridization provides an exceptionally tough set of problems for evolutionary and conservation biologists (Ellstrand *et al.* 2010; Fitzpatrick *et al.* 2015). The issues are complex and very controversial, beginning with the seemingly simple task of defining hybridization (Harrison 1993). It has been used to refer to the interbreeding between species, but this taxonomically restrictive use of hybridization can be problematic, especially since it is sometimes difficult to agree on what a species is (Allendorf *et al.* 2013; Harrison & Larson 2014; Fitzpatrick *et al.* 2015). Here we use a more general definition, formulated by Rhymer & Simberloff (1996): *hybridization is the interbreeding of individuals from genetically distinct populations, regardless of their taxonomic status.*

In the mid-nineteenth century, Darwin developed the modern view of species by arguing that mating between them can be difficult (Darwin 1859). Detection of hybridization is troublesome, although it is becoming much easier through the application of molecular techniques. Since Darwin, and with the help of improved molecular data, researchers have been able to uncover many examples of hybridization across multiple taxa. Although interpreting the evolutionary significance of hybridization and determining the role of hybrid populations can be challenging, over the last 50 years the study of hybridization has yielded valuable insights, providing a better understanding of the forces that shape adaptation and evolution (*e.g.* Arnold 1997; Pekkala *et al.* 2012, 2014; Allendorf *et al.* 2013).

Primordial thoughts on the role of hybridization in systematics and evolution go back to Linnaeus and Darwin (Arnold 1997). On the one hand, botanists have generally accepted hybridization as a pervasive and important aspect of evolution (*e.g.* Grant 1963, Stebbins 1950, 1959) because many plant taxa have hybrid origins; hybridization can produce both new species and novel adaptations (Mallet 2007). On the other hand, evolutionary biologists working with animals have been more interested in how the evolution of reproductive isolation leads to speciation (Mayr 1942; Dobzhansky 1951). Hybrid offspring are often relatively unfit, among animals, fostering the development of reproductive isolation, and eventually speciation.

Many studies in both conservation and evolutionary biology have studied the role of hybridization in the evolution of genetically differentiated populations (*e.g.* Mayr 1970; Rieseberg 1995; Rhymer & Simberloff 1996; Margan *et al.* 1998; Allendorf *et al.* 2001, Barton 2001; Mallet 2005; Frankham 2008; Frankham *et al.* 2011; Pekkala *et al.* 2012). The introduction of genetic material from other populations has often been proposed as an aid to

conservation and resource management, because of its presumed benefits for the viability of *inbred* populations, leading to *genetic rescue* or *genetic restoration* (cf. Tallmon *et al.* 2004; Hedrick 2005; see also Edmands 2007; Frankham *et al.* 2011; Hedrick *et al.* 2011; Frankham 2015). There is now good experimental evidence for the genetic benefits of interpopulation hybridization (reviewed in Tallmon *et al.* 2004), as interbreeding by individuals from genetically differentiated populations often leads to *heterosis* – the increased fitness of hybrid offspring when compared to non-hybridized parents.

Heterosis and inbreeding depression reversion

Inbreeding can be defined as the probability of two genes at any given locus being identical by descent and *inbreeding depression* as a reduction of life-history traits in crosses between relatives (Falconer & Mackay 1996; Lynch & Walsh 1998). The amount of *inbreeding depression* will be determined by the state of dispersion of gene frequencies in a population. In the absence of selection, the coefficient of inbreeding can be a measure of this state of dispersion. Since inbreeding tends to reduce fitness, natural selection is likely to oppose the inbreeding process by favoring the least homozygous individuals, making inbreeding depression dependent on the rate of inbreeding (Falconer & Mackay 1996). The effect of inbreeding on a given character depends on the proportion of directional dominance: the higher the dominance genetic variance, and the lower the additive genetic variance, the greater the effect of inbreeding (Falconer & Mackay 1996; Roff 1997; Lynch & Walsh 1998). Traits closely related to fitness often show large dominance variance at evolutionary equilibrium, making them more susceptible to inbreeding depression. It is expected that the closer a character is to fitness, the greater the impact of inbreeding depression when closely related individuals mate (Roff 1997, 1998; DeRose & Roff 1999; Wright *et al.* 2008; Charlesworth & Willis 2009; Pekkala *et al.* 2014).

The temporal loss of heterozygosity in closed, randomly mating populations is contingent on the population's effective size, its inbreeding level, and the number of generations involved (Wright 1969; Falconer & Mackay 1996; see the *Introduction* section of *Chapter Two* for more details). In the absence of selection, the decrease in heterozygosity will be more severe in populations of smaller size, where genetic drift has a stronger effect. The stochasticity of genetic drift through time also causes randomness in the direction of gene frequency change, thus different small populations will lose (or fix) different alleles. This will

produce both (1) specific maladaptive changes in individual populations and (2) differentiation of populations. Both will be more severe in smaller populations, compared to larger ones. Sufficiently strong directional selection can counteract the stochastic effects of drift. Except for *loci* that feature some type of balancing selection, selection is expected to reduce heterozygosity because of the fixation of alleles that confer a fitness advantage. In the absence of genetic drift, selection drives gene frequencies toward stable equilibria at values which would be the same for all populations under the same conditions (Falconer & Mackay 1996).

Heterosis in crosses between populations, just like inbreeding depression, depends on dominance: *loci* without dominance cause neither inbreeding depression nor heterosis. Also, the amount of heterosis following a cross between two populations depends on the difference of gene frequency between the populations. Heterosis at the level of a cross will be greatest when, at *loci* which show overdominance, one allele is fixed in one parent population and the other allele in the other parent population. When considering the joint effects of all *loci* at which the two parent populations differ (and assuming no epistasis), the heterosis of the cross will be the sum of net dominance effects across all *loci*. When there is no overdominance, if some *loci* are dominant in one direction and some in the other, their effects may largely cancel out, leading to an absence of cross-level heterosis. Hence, the occurrence of heterosis upon crossing is not only dependent on dominance, but also on its direction (Falconer & Mackay 1996). Finally, because heterozygosity peaks in the first hybrid generation and is diluted thereafter, cross-heterosis should also peak in the first generation and decrease when hybrid offspring mate randomly with each other in subsequent generations (Lynch 1991; Lynch & Walsh 1998).

Hybridization restores heterozygosity and heterosis is thought to reverse inbreeding depression through three different, though not exclusive, mechanisms. First, the *dominance model* proposes that the *hybrid vigor* is due to the effects of recessive deleterious alleles being largely masked in the heterozygote. Second, the *overdominance model*, suggests that synergistic allelic interactions at overdominant *loci* lead to superior performance in the progeny, since crossing increases levels of heterozygosity. Finally, the *epistasis model* presumes that the establishment of new favorable interactions between *loci* and disruption of negative interactions that may have been fixed by genetic drift are responsible for the hybrid superiority (Lewontin 1964; Lynch 1991; Lynch & Walsh 1998; Whitlock *et al.* 2000; Erickson & Fenster 2006; Lippman & Zamir 2007; Charlesworth & Willis 2009; Edmands *et al.* 2009).

The risk of outbreeding depression

Hybridization between differentiated populations can, however, result in a decrease in fitness of the hybrid offspring below that of the non-hybridized parents due to several kinds of genetic incompatibilities – so-called *outbreeding depression* (*e.g.* Parker 1992; Gharrett *et al.* 1999; Fenster & Galloway 2000; Edmands & Deimler 2004; Galloway & Etterson 2005; Burton *et al.* 2006). Three main mechanisms have been proposed to explain outbreeding depression: (1) chromosomal incompatibilities, (2) adaptive differentiation among populations, and (3) genetic drift. We address each of these in succession. First, it has long been known that fixed chromosomal differences (such as polyploidy, translocations, centric fusions, inversions...) between two interbreeding populations may cause karyotypic imbalance and incompatibility, reducing the hybrid's capacity to mature into a healthy, fit adult (*e.g.* Dobzhansky 1951; Wilson *et al.* 1974; Barton & Hewitt 1981). Second, when isolated populations evolve in different local environments, selection might lead to the evolution of different *multi-locus* genotypes that work well together – *coadapted gene complexes* (Templeton 1986; Lynch 1991; Fenster *et al.* 1997; Lynch & Walsh 1998). Hybridization can break up these complexes, causing a decrease in the hybrid population's fitness. Furthermore, bringing together alleles that are neutral or beneficial individually but have deleterious effects when combined can also result in outbreeding depression (Phillips & Johnson 1998; Orr & Turelli 2001; Edmands 2007; Presgraves, 2010). Third, genetic drift has been proposed as a mechanism leading to reproductive isolation because drift's random effects may lead to high levels of population differentiation. Through time, two closed populations can suffer so much genetic change that they become incompatible, and hybrid progeny have very low fitness (Ralls *et al.* 2013).

Whether hybridization has a positive or a negative impact on population viability will depend on the relative magnitudes of heterosis and outbreeding depression which, in turn, are influenced by the level of genetic divergence between the populations (Lynch 1991; Falconer & Mackay 1996; Lynch & Walsh 1998; Whitlock *et al.* 2000; Pekkala *et al.* 2012). In the absence of selection, heterosis should increase linearly with the divergence of the populations, whether the underlying genetics involve overdominance or recessive deleterious genetic effects. This is particularly relevant in evolution under small effective population size. By contrast, outbreeding depression due to epistasis is expected to develop slowly in the first stages of population divergence, but then accelerate as populations become increasingly diverged (Orr, 1995; Orr & Turelli, 2001).

Hybridization can, thus, be a double-edged sword for adaptation. On the positive side, it may supply additional functional genetic variation, the limiting fuel for the process of adaptation. Theoretical and experimental studies suggest that some threatened species may be able to adapt to environmental change on a sufficiently fast time-scale to prevent their extinction, so-called "evolutionary rescue" (Carlson *et al.* 2014; Stelkens *et al.* 2014) The probability of rescue will depend on the severity of the stress, the effective population size, and the level of genetic variance present in the evolving population (Lande & Shannon 1996; Barrett & Schluter 2008; Bell & Gonzalez 2009; Gonzalez *et al.* 2013). On the negative side, adaptation to a given environment may reduce fitness in other environments. Hybrids between an ancestral and a newly-adapted population may reduce fitness in the ancestral environment or the new environment, perhaps even both environments. For instance, when individuals that are adapting to novel conditions cross with others that are adapted to the ancestral environment, their hybrid offspring may have reduced fitness in the novel environment (Woodworth *et al.* 2002; Gilligan & Frankham 2003; Frankham, 2005a, 2005b, 2008). In sum, several factors can change the outcome of a hybridization event, making it difficult to predict the evolutionary consequences of interpopulation crosses in a given evolutionary scenario. Nonetheless, disentangling the influence of various factors on the long-term effects of hybridization is very important, not only for the advancement of evolutionary theory, but also for creating informed policies of endangered populations' management.

Hybridization, effective size, and adaptation

The effects of hybridization and effective population size on adaptation have been studied in both animal and plant populations (*e.g.* Klinger *et al.* 1992; Arnold *et al.* 1999; Peters *et al.* 2014), but the effect of hybridization on experimental populations that are evolving at different effective population sizes has rarely been studied (see Margan *et al.* 1998; Pekkala *et al.* 2014). Despite the need for this kind of knowledge, long-term studies on the effects of hybridization are still scarce (but see Edmands *et al.* 2005; Erickson & Fenster 2006; Bijlsma *et al.* 2010; Hwang *et al.* 2011).

According to classical population genetic theory, the capacity of a population to respond to selection depends on its level of genetic variation for the traits undergoing selection (Falconer & Mackay 1996). Genetic variation within randomly mating populations is generally increased by mutation and gene flow but decreased by drift and directional selection in the

absence of heterozygote advantage (Willi *et al.* 2006). Neutral models usually characterize genetic variation in terms of mean heterozygosity or allelic diversity, being the latter considered a better measure of the adaptive potential under environmental change (Allendorf 1986). When only drift and mutation occur, heterozygosity increases monotonically with population size, because (1) the magnitude of genetic drift is inversely proportional to N_e and drift results in a decrease of heterozygosity at a rate of $1/[2N_e]$ per generation (Kimura 1955, Wright 1931), and (2) fewer mutations appear in small populations per generation. Consequently, larger populations are always expected to have higher heterozygosity in neutral evolutionary genetic theory. Likewise, under these assumptions of neutrality and additive gene effects, the additive genetic variance (V_A) of a quantitative trait increases linearly with N_e (Lynch & Hill 1986) and the amount of new genetic variance introduced by mutation. Another effect of N_e in its relationship with drift is that populations of small size will tend to differentiate through time as a consequence of random fixation of different alleles (Falconer & Mackay 1996). In fact, the effect of $1/[2N_e]$ per generation (mentioned above) also applies to the evolutionary dynamics of population differentiation (F_{ST}, see Wright 1951; Hartl & Clark 2007). Consequently, through migration or hybridization, populations may recover the lost heterozygosity by drift. The outcome of neutral evolution becomes less predictable at low effective population sizes and, therefore, small populations should exhibit widely varying amounts of genetic variation. This suggests that their direct and correlated responses to selection will be more variable (Willi *et al.* 2006).

For polygenic traits under selection, the genetic variance is supposed to depend on the type and intensity of selection, the rate and effect of mutations, and the number of *loci* involved (Houle 1989). As in neutral models, the additive genetic variance is predicted to be higher at larger N_e with simple directional selection, because of the combined effects of more mutations and weaker drift. Particularly, under strong directional selection there is no theoretical limit to this contribution (Keightley & Hill 1987), because mutations with positive effect on the phenotype are always favored (Willi *et al.* 2006). In any case, under strong directional selection (and assuming additive gene effects only) genetic variation is expected to decline through time, because selection increases the likelihood that the allele with the highest fitness will be fixed (Willi *et al.* 2006). With the assumption of this type of directional selection, selection should be more efficient in large than in small populations, because the probability of elimination of a deleterious mutation (whether recessive or not) shows a positive sigmoidal relationship with the product of N_e and the selection coefficient (Robertson 1960). Thus, according to classical

population genetics theory, as population size declines, stronger selection is required to prevent the loss of rare beneficial mutations due to drift, and to ensure elimination of deleterious mutations (Willi *et al.* 2006). Once again, interpopulation hybridization may allow the increase of genetic variants, which will eventually promote a higher evolutionary response (Tallmon *et al.* 2004; Hedrick 2005; Edmands 2007; Hedrick *et al.* 2011).

This chapter's experiments constitute an attempt to link the classic evolutionary studies that address the effects of hybridization and effective population size separately. Furthermore, we will address a neglected issue in the literature, the impact of hybridization as a function of evolutionary scenario. To be specific, we performed a massive hybridization that involved the 84 populations created for the forward selection experiment (see *Chapter Two* for more details). After 15 generations of forward selection for starvation resistance, each combination of selection, size, and B_i ancestor were crossed, creating 84 new hybrid populations. Selection was then resumed for ten more generations. Our highly replicated experimental design allowed us to study the effects of hybridization after many generations under different selection and effective population size conditions. We were also able to analyze how much these hybrids differed from their parental populations right after hybridization, as well as the effect of hybridization on the population's evolutionary dynamics. Most importantly, we were able to determine how much the aforementioned effects depended on population size *versus* both past and present selective regime.

MATERIALS AND METHODS

Hybridization and derivation of the experimental populations

The experimental populations used in this study were derived from the 84 populations described in *Chapter Two*, after 15 generations of forward selection for starvation resistance. The creation of these new 84 populations involved crosses within each B_i group formed by a combination of size and selection. For instance, SPB0a, SPB0b, SPB0c, SPB0d, and SPB0e were all crossed together to derive $hSPB_{0\alpha}$, $hSPB_{0\beta}$, $hSPB_{0\gamma}$, $hSPB_{0\delta}$, and $hSPB_{0\varepsilon}$. Similarly, SGB_{0a} and SGB_{0b} were crossed to derive $hSGB_{0\alpha}$ and $hSGB_{0\beta}$. A schematic for this phase of the experimental work is shown in **Figure 1.4** of *Chapter One*, which is here reproduced for the sake of convenience.
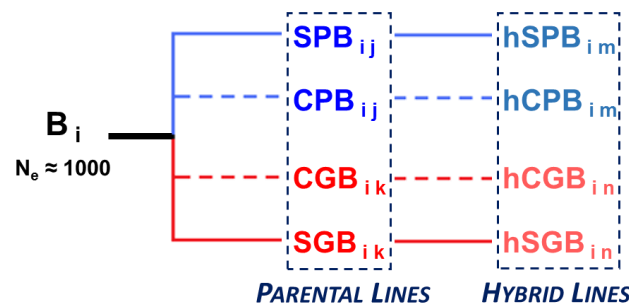
Figure 1.4. Schematic representation of the experimental design used to create the hybrid populations from the first set of 84 lines. For each set of selected and control lines derived from a B_i population, hybridization took place among all replicates, and the resultant hybrids were split into the same number of replicates as their unhybridized ancestors. Starvation resistance selection was then resumed and imposed to all the *S* lines (SPB, SGB, hSPB, and hSGB).

For each of the small hybrid populations, 5 male and 5 female virgins from each parental line were placed into <u>one vial</u>; *e.g.* 5m + 5f from each of SPB0a, SPB0b, SPB0c, SPB0d, and SPB0e resulting in a total of 50 flies (see **Figure 3.1a**). This was repeated <u>five times</u> to create the five hybrid populations $hSPB_{0\alpha}$, $hSPB_{0\beta}$, $hSPB_{0\gamma}$, $hSPB_{0\delta}$, and $hSPB_{0\varepsilon}$. Large hybridized populations were created using 10 virgin males and 10 virgin females that were collected into <u>25 vials</u> for each population *e.g.* (10m + 10f) from SGB0a and SGB0b to total 1000 flies. This was repeated <u>two times</u> to generate the hybrid populations $hSGB_{0\alpha}$ and $hSGB_{0\beta}$ (see **Figure 3.1b**). During the hybridization generation, selection was relaxed to forestall differential representation of lines that had responded more to selection.

Continued forward selection procedures for starvation resistance

The *hSPB$_{im}$* and *hSGB$_{in}$* hybrid populations, as well as their unhybridized *SPB$_{ij}$* and *SGB$_{ik}$* ancestors, underwent continued selection for ten more generations, with the four sets of lines handled in parallel. The selection protocol is described in the Materials and Methods section of *Chapter Two* of this thesis.

Starvation resistance and fecundity assays

Both of these traits were assayed according to the protocol described on *Chapter Two*. Starvation resistance and fecundity were assayed at generations 1, 3, and 7 after the hybridization event (corresponding to generations 16, 18 and 22 from the start of the experiment). This means that the first hybrid assay was done after one generation of selection. An additional starvation resistance assay was done at generation 10 (generation 25 from the beginning of the study).

Statistical data analysis

In all analyses, the normality and homoscedasticity of data were tested by Shapiro-Wilk (1965) and Brown-Forsythe (1974) tests, respectively. After testing by ANOVA and ANCOVA, and when it was appropriate, Tukey HSD (1953) *post-hoc* tests were done. A significance value of 0.05 (α) was used to test all null hypotheses. All analyses were done using *STATISTICA 13* (Dell 2015).

The key statistical tests focused on quantitative measures of how marked N_e disparities affect the direct and indirect responses to forward selection for increased starvation resistance, after the hybridization event. The magnitudes of the interactions between the evolutionary responses, the hybridization state, and different N_e values were estimated.

The evolutionary trajectories of starvation resistance and fecundity throughout the 10 generations of selection following hybridization were analyzed according to the following ANCOVA model:

$$
\begin{aligned}
Y = \mu &+ H_i + T_j + S_k + C_l + G_m + H_i * T_j + H_i * S_k + H_i * C_l + H_i * G_m + T_j * S_k + T_j * C_l \\
&+ T_j * G_m + S_k * C_l + S_k * G_m + C_l * G_m + H_i * T_j * S_k + H_i * S_k * C_l + H_i \\
&* C_l * G_m + H_i * T_j * C_l + H_i * T_j * G_m + H_i * S_k * G_m + T_j * S_k * C_l + T_j * C_l \\
&* G_m + T_j * S_k * G_m + S_k * C_l * G_m + H_i * T_j * S_k * C_l + H_i * S_k * C_l * G_m \\
&+ H_i * T_j * C_l * G_m + H_i * T_j * S_k * G_m + T_j * S_k * C_j * G_m \\
&+ Rep\{H * T * S * C\} + G_m * Rep\{H * T * S * C\} + \varepsilon
\end{aligned}
\tag{3.1}
$$

where *Y* is the trait under analysis (starvation resistance or fecundity); *H* represents the evolutionary history ($i = 0$-5), random factor; *T* the population size (j = large or j = small), fixed factor; *S* the selection regime (k = selected or k = control), fixed factor; C the cross type (l = parental or l = hybrid), fixed factor, *G* the covariate generation (see which on the assays description), and *Rep* the replicate population (random factor). This is a comprehensive model that includes all variables under study. Analyses within population size or selection regime used adapted versions of the aforementioned model (*e.g.* when comparing small and large populations in control conditions, the factor *Selection* and all its interactions were removed). A significance value (α) of 0.05 was used to test all null hypotheses.

Because we found statistically significant changes in starvation resistance and fecundity of the control lines CGB and CPB (**Figure 3.2** and **Table 3.2**), when analyzing the selection lines (SGB, SPB, hSGB, and hSPB), we used data relative to control instead of raw data; *i.e.* the difference between the raw value of the character for each individual and the average for the CGB_i controls. This correction was required for estimating the response to direct starvation selection, stripped of the evolutionary effects of altered nutrition and culture time (see *Chapter Two* for a more detailed explanation). Additionally, we repeated all the analyses using CPB_i as controls for SPB_{ij} and obtained similar results (data not shown).
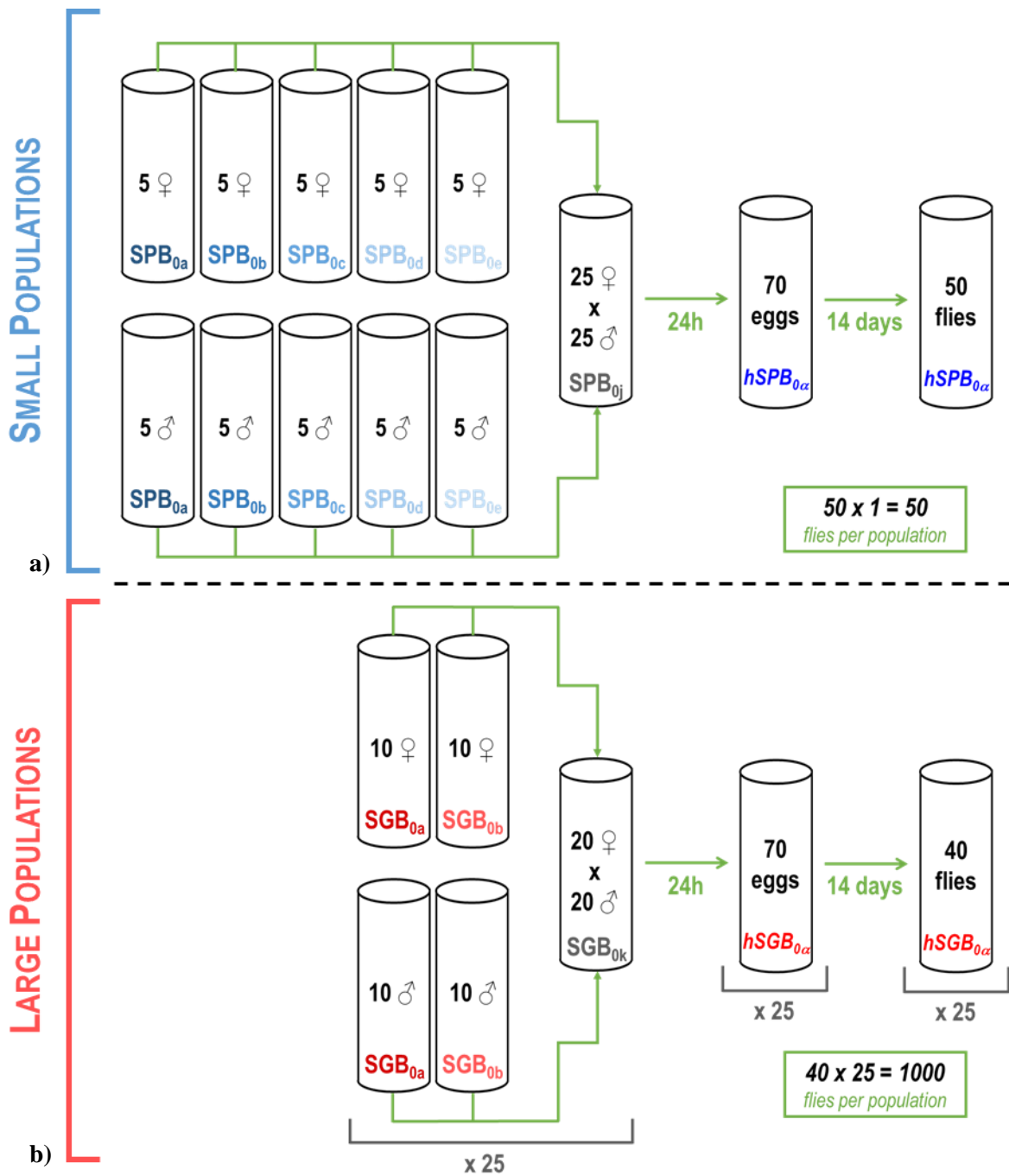
Figure 3.1. Hybridization and derivation of the new experimental populations of **a)** small and **b)** large size, using the B_0 derivatives as an example. This protocol was followed 5 times for the small lines and twice for the large lines, within each B_i ancestor, over all six B-type evolutionary radiations.

RESULTS

General linear model assumptions

Small deviations from normality were accepted, and homoscedasticity was verified by the Brown-Forsythe test. Our distribution tests showed that all populations were homoscedastic and generally normal (data not shown).

Detection of inbreeding depression

The decline in fitness due to inbreeding was tested by comparing the performance of hybrid populations with their respective parental lines, two generations after hybridization. In terms of starvation, both male and female hybrids were generally more resistant than the respective parental lines (except for *hSGB* females), but no significant differences were found within any of the *size*selection* regimes (p>0.05). The early fecundity of hybrids lines was lower than that of the respective parental stocks, but only significantly so for the small controls (CPB-hCPB comparison, p<0.002). **Table 3.1** shows the comparison between the two types (parental and hybrid) of each *size*selection* regime regarding starvation resistance and fecundity.

Table 3.1. Comparison of parental and hybrid lines for inbreeding depression detection. Data are given as the mean value ± standard error of mean (as differences between replicates) of each *size*selection*type* regime, for each life-history trait analyzed. Starvation resistance values are in hours and early fecundity in number of eggs laid per female. Significant differences between each *size*selection* parental-hybrid pair are highlighted in bold.

Size*selection comparison	Regime	Male starvation resistance *(hours)*	Female starvation resistance *(hours)*	Early fecundity *(eggs per female)*
Large controls	CGB	24.3 ± 2.18	45.6 ± 4.07	220 ± 10.4
	hCGB	28.8 ± 3.55	49.2 ± 5.66	211 ± 12.3
Small controls	CPB	26.8 ± 1.54	47.5 ± 3.01	**163 ± 4.6**
	hCPB	26.8 ± 1.01	47.9 ± 2.42	**116 ± 3.1**
Large selected	SGB	78.4 ± 5.75	117.1 ± 7.79	224 ± 10.4
	hSGB	79.1 ± 5.63	113.5 ± 7.06	198 ± 12.9
Small selected	SPB	39.5 ± 2.43	74.4 ± 5.13	212 ± 7.4
	hSPB	44.9 ± 1.93	84.5 ± 3.61	175 ± 5.7

Evolution after hybridization I: Controlled laboratory conditions

After the hybridization event, parental and hybrid control lines were maintained under control conditions and followed for ten more generations. **Figures 3.2** and **3.3** depict the evolutionary trajectories for starvation resistance and early fecundity of parental and hybrid controls, respectively.

The parental control lines (CGB and CPB) showed a significant increase in their average starvation resistance. CGB starvation resistance increased from 24.3 to 35.9 hours ($p<0.008$) in males, and from 45.6 to 62.5 hours ($p<0.01$) in females. CPB saw its starvation resistance increased from 26.8 to 40.2 hours ($p<0.02$) in males, and from 47.5 to 74.3 hours ($p<0.0004$) in females. Although both stocks showed a reduction in early fecundity, only CGB's decline was significant (from 220 to 176 eggs, $p<0.007$). Even though CPB went from an average of 163 to 144 eggs, the trend was not linear ($p>0.1$). See **Figure 3.2** for their evolutionary trajectories and **Table 3.2** for the ANCOVA results.
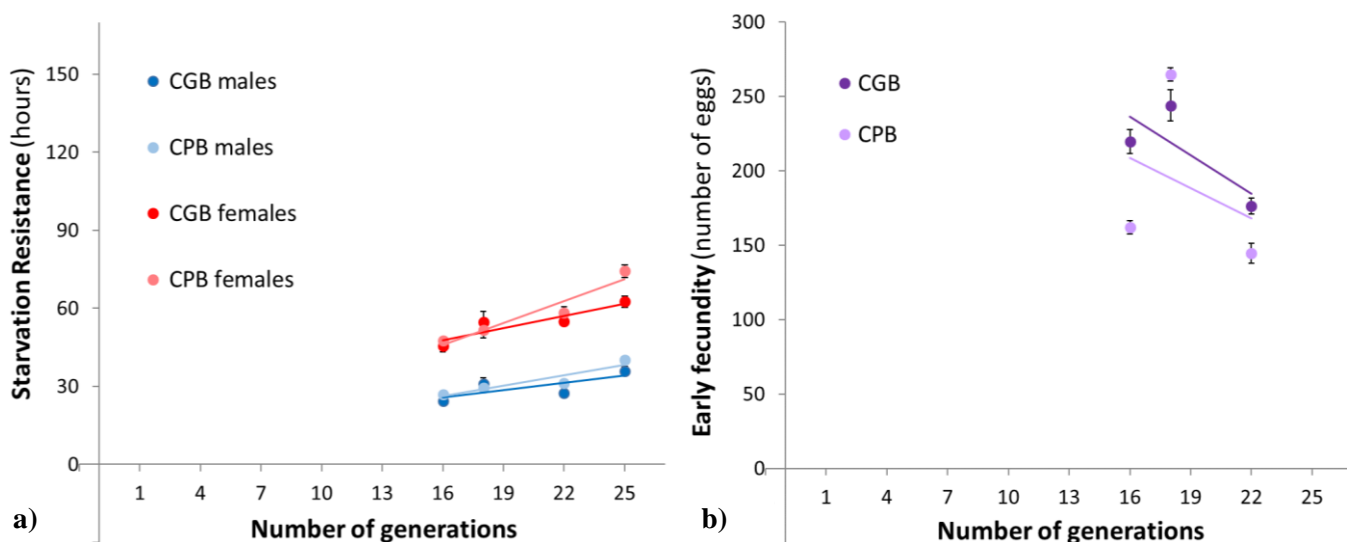
Figure 3.2. Evolutionary trajectories for **a)** male and female starvation resistance, and **b)** early fecundity of the parental control populations. Average values for small (CPB) and large (CGB) regimes are shown. Error bars denote standard error of mean (as differences between replicates).

Similar to the results for the parental lines, the hybrid controls showed a general increase of starvation resistance, but these results were not always statistically significant. hCGB went from 28.8 to 36.3 hours ($p>0.2$) in males, and from 49.2 to 65.3 hours ($p<0.02$) in females. The small hybrid lines (hCPB) showed non-significant increasing trends in both male

(from 26.8 to 36.5 hours, p>0.2) and female (from 47.0 to 67.4 hours, p>0.1) starvation resistances. In terms of fecundity, the large hybrids (hCGB) showed a non-significant decline with time, from 211 to 189 eggs (p>0.6) and hCPB a significant increase, from 116 to 154 eggs (p<0.009). See **Figure 3.3** for the evolutionary trajectories and **Table 3.2** for the summary of ANCOVA results.
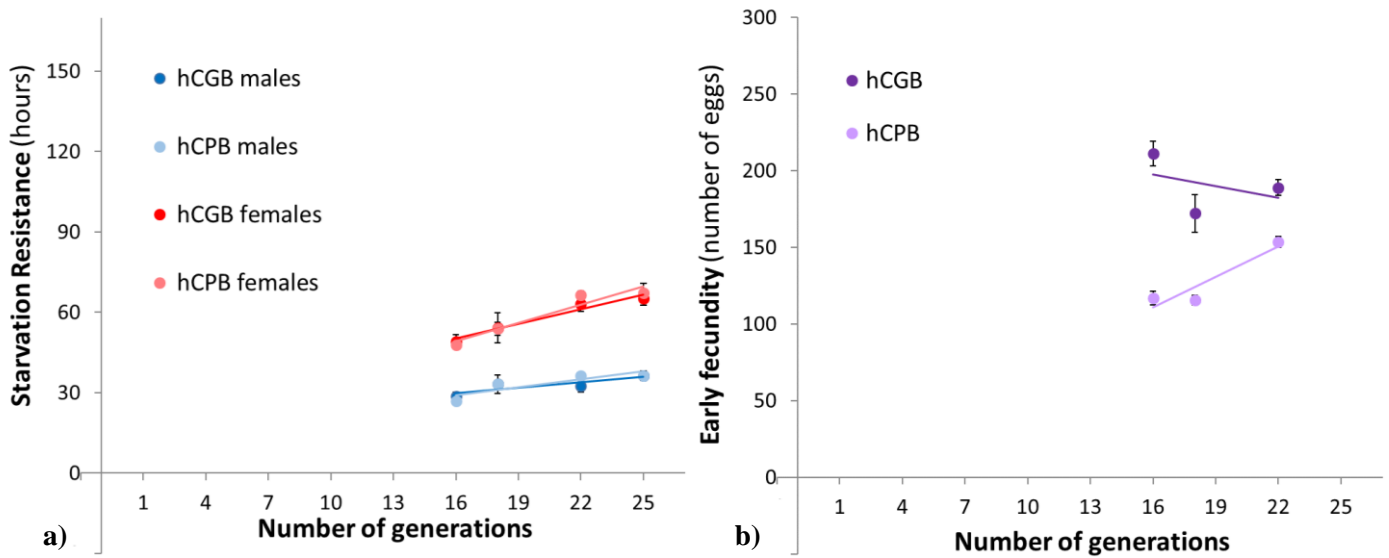
Figure 3.3. Evolutionary trajectories for **a)** male and female starvation resistance, and **b)** early fecundity of the hybrid control populations. Average values for small (hCPB) and large (hCGB) regimes are shown. Error bars denote standard error of mean (as differences between replicates).

Table 3.2. Summary of the mixed-effects ANCOVAs used to analyze the evolutionary trajectories of parental and hybrid controls. Data shows the F statistic and respective p-value for the facto*r Generation* of each *size*type* regime and life-history trait analyzed. Significant changes through time are highlighted in bold.

Size * Type regime	Male starvation resistance		Female starvation resistance		Early fecundity	
	F statistic	*p-value*	*F statistic*	*p-value*	*F statistic*	*p-value*
CGB	**18.40258**	**0.007784**	**16.66240**	**0.009521**	**19.62947**	**0.006821**
CPB	**11.25586**	**0.019910**	**68.80258**	**0.000349**	2.83051	0.152870
hCGB	2.063342	0.210369	**12.85771**	**0.015778**	0.20595	0.668673
hCPB	1.51147	0.272897	3.243847	0.131096	**17.45012**	**0.008666**

The effect of hybridization in control stocks was then tested, comparing the evolutionary trajectories of parentals and hybrids within each size regime: large, CGB *vs.* hCGB and small, CPB *vs.* hCPB. In terms of starvation resistance, for both large and small controls, no significant differences were found in males or females' performances. Also, fecundity trajectories within the large lines did not differ significantly (p>0.1). However, the evolution of fecundity in the small lines was significantly different: CPB decreased and hCPB increased with time. See **Figure 3.4** for the comparison per trait and **Table 3.3** for the summary of ANCOVA results.

Table 3.3. Summary of the mixed-effects ANCOVAs used to analyze the effect of hybridization in control conditions. Data shows the F statistic and respective p-value for the interaction *Type*Generation* of each size regime and life-history trait analyzed. Significant changes are highlighted in bold.

Comparison	*Male starvation resistance*		*Female starvation resistance*		*Early fecundity*	
	F statistic	*p-value*	*F statistic*	*p-value*	*F statistic*	*p-value*
CGB – hCGB (large controls)	0.795229	0.413364	0.92540	0.380239	3.34851	0.126354
CPB – hCPB (small controls)	1.25331	0.313249	2.02562	0.212965	**11.14260**	**0.020479**

Finally, the effect of hybridization under contrasting population size was analyzed, comparing hybrid and parental lines evolving in different size regimes (**Figure 3.4**). For all the traits under study, no interaction between hybridization and population size was detected (**Table 3.4**), *i.e.* under control conditions, hybridization seems to have the same influence on the evolutionary rate of small and large populations.

Table 3.4. Summary of the mixed-effects ANCOVAs used to analyze the effect of hybridization in control conditions, under different population sizes. Data shows the F statistic and respective p-value for the interaction *Size*Type*Generation* of each life-history trait analyzed.

Male starvation resistance		*Female starvation resistance*		*Early fecundity*	
F statistic	*p-value*	*F statistic*	*p-value*	*F statistic*	*p-value*
0.37139	0.568788	3.77947	0.109091	0.5850	0.478828
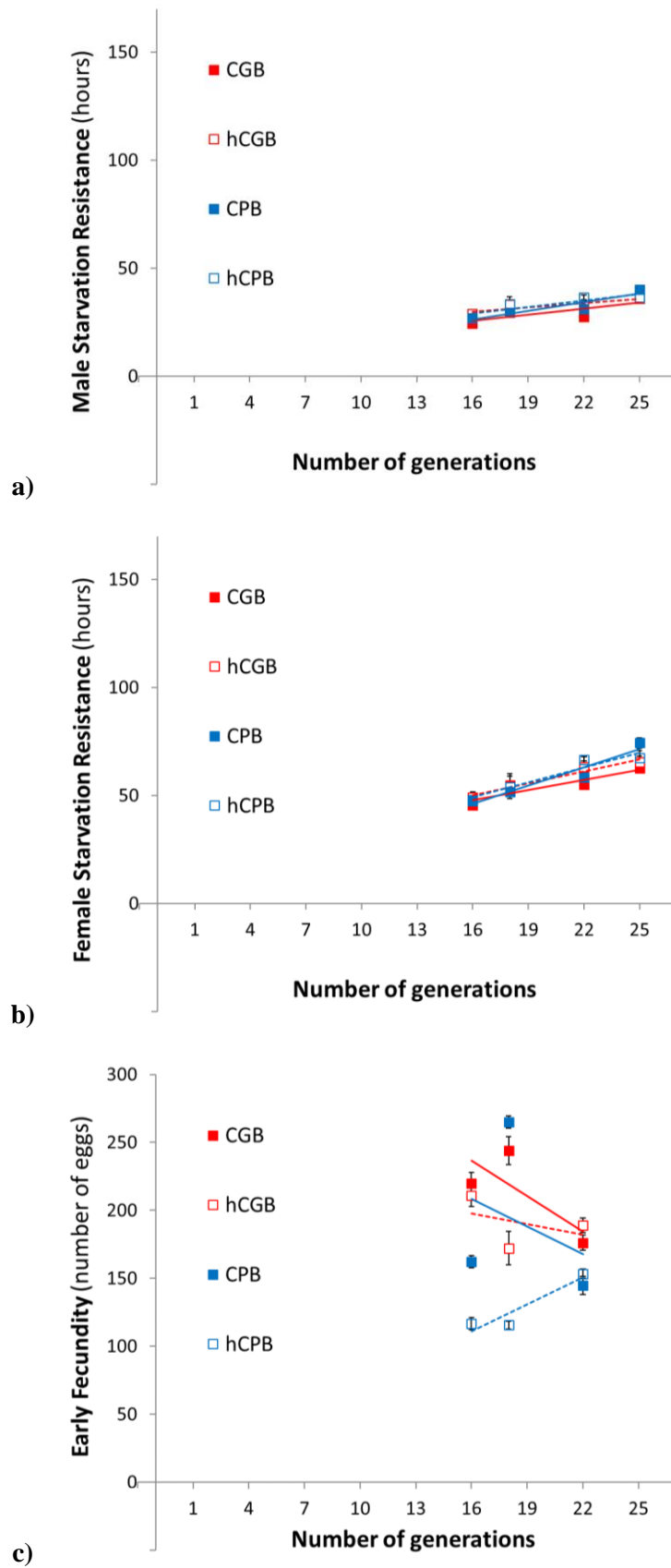
Figure 3.4. Evolutionary trajectories for **a)** male starvation resistance, **b)** female starvation resistance, and **c)** early fecundity of the hybrid (dashed) and parental (solid) controls, under both size regimes. Average values for the four size*type regimes are shown. Error bars denote standard error of mean (as differences between replicates).

Evolution after hybridization II: strong directional selection

After the hybridization event, selection for starvation resistance in the previously selected lines was resumed, yielding ten more generations of evolutionary trajectories for the four *Size*Type* selected regimes. Since we found statistically significant changes in starvation resistance and fecundity among the control lines, and similar to what was done in *Chapter Two*, we used the control-corrected data (that is, the difference from their respective CGB_i control, see *Material and Methods*) to build and analyze the evolutionary trajectories of parental and hybrid selected lines (see **Figures 3.5** and **3.6**). Therefore, if a population's trait average value is lower than the controls, we will obtain a negative value for that character, whether it is starvation or fecundity.

Both large-population-size (SGB) and small-population-size (SPB) parentals showed a significant increase in their average starvation resistance with continued selection. SGB starvation resistance increased from 53.9 to 128.5 hours (p<0.00001) in males, and from 71.5 to 166.7 hours (p<0.0001) in females. Their small counterparts, SPB, saw their starvation resistance increased from 15.5 to 44.4 hours (p<0.006) in males, and from 29.4 to 129.2 hours (p<0.0004) in females. Even though both small and large parental stocks showed decreases in early fecundity, none were statistically significant. SGB went from 5 to -3 eggs on average (p>0.7) and SPB from -8 to -53 eggs (p>0.05). See **Figure 3.5** for the evolutionary trajectories and **Table 3.5** for the ANCOVA results.

The hybrid selected lines also had a significant increase on their starvation resistance. The large hybrids (hSGB) went from 54.7 to 122.8 hours (p<0.00006) in males, and from 67.9 to 150.7 hours (p<0.00009) in females. The small hybrid (hSPB) males started with 20.4 and ended with 42.3 hours (p<0.03) and the females started with 38.9 and ended with 61.3 hours, p<0.03). In terms of fecundity, both large and small hybrids showed a significant increase with time: hSGB went from -16 to 30 eggs (p<0.03) and hSPB from -47 to -18 eggs (p<0.02). See **Figure 3.6** for the evolutionary trajectories and **Table 3.5** for the ANCOVA results.
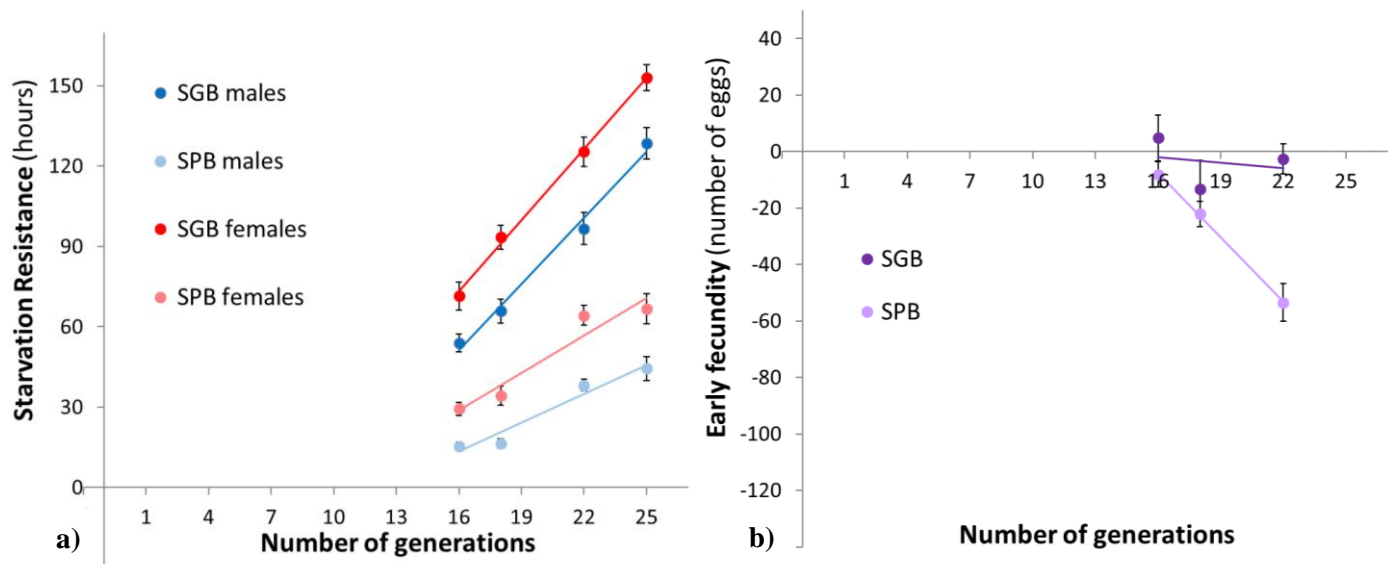
Figure 3.5. Evolutionary trajectories for **a)** male and female starvation resistance, and **b)** early fecundity of the parental selected populations. Average CGB$_i$-corrected values for small (SPB) and large (SGB) regimes are shown. Error bars denote standard error of mean (as differences between replicates).
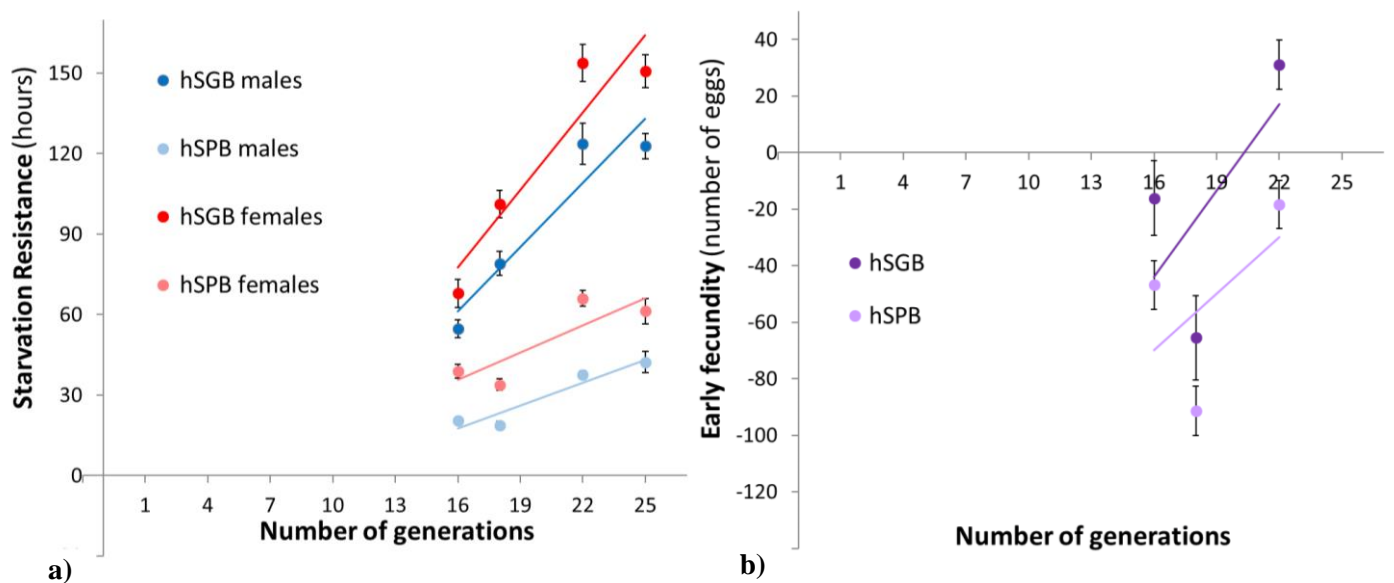
Figure 3.6. Evolutionary trajectories for **a)** male and female starvation resistance, and **b)** early fecundity of the hybrid selected populations. Average CGB$_i$-corrected values for small (hSPB) and large (hSGB) regimes are shown. Error bars denote standard error of mean (as differences between replicates).

Table 3.5. Summary of the mixed-effects ANCOVAs used to analyze the evolutionary trajectories of parental and hybrid selected lines. Data shows the F statistic and respective p-value for the facto*r Generation* of each *size*type* regime and life-history trait analyzed (CGB$_i$-corrected data). Significant changes through time are highlighted in bold.

Size * Type regime	Male starvation resistance		Female starvation resistance		Early fecundity	
	F statistic	p-value	F statistic	p-value	F statistic	p-value
SGB	**113.1945**	**0.000007**	**94.30370**	**0.000012**	0.077236	0.791046
SPB	**21.82289**	**0.005467**	**15.09473**	**0.011571**	6.337088	0.051690
hSGB	**164.9600**	**0.000051**	**134.1379**	**0.000084**	**10.33925**	**0.023577**
hSPB	**10.79742**	**0.021811**	**11.04759**	**0.020921**	**11.87343**	**0.018281**

We then compared the evolutionary trajectories of parentals and hybrids within each size regime: SGB *vs*. hSGB (large) and SPB *vs*. hSPB (small), in order to test for the effect of hybridization in starvation selected stocks. Similar to what was found in the control regimes, no significant differences were found in males or females' starvation resistance, for both large and small selected lines. The evolutionary trajectories for early fecundity of hybrids were significantly different from the parental lines, in both large ($p<0.002$) and small ($p<0.009$) regimes: the parental lines experienced a decrease in fecundity and the hybrids saw their fecundity increased. See **Figure 3.7** for the comparison per trait and **Table 3.6** for the summary of ANCOVA results.

Table 3.6. Summary of the mixed-effects ANCOVAs used to analyze the effect of hybridization under selection for starvation resistance. Data shows the F statistic and respective p-value for the interaction *Type*Generation* of each size regime and life-history trait analyzed. Significant changes are highlighted in bold.

Comparison	Male starvation resistance		Female starvation resistance		Early fecundity	
	F statistic	p-value	F statistic	p-value	F statistic	p-value
SGB – hSGB (large controls)	3.9710	0.082442	0.0024	0.962733	**25.92458**	**0.001156**
SPB – hSPB (small controls)	0.65032	0.456618	1.21653	0.320266	**17.03969**	**0.008822**

Finally, the effect of hybridization under contrasting population sizes was analyzed, comparing hybrid and parental lines evolving in different size regimes (**Figure 3.7**). For all the traits under study, no interaction between hybridization and population size was detected (**Table 3.7**), *i.e.* under selection for starvation resistance, hybridization seems to have the same influence on the evolutionary rate of small and large populations.

Table 3.7. Summary of the mixed-effects ANCOVAs used to analyze the effect of hybridization under selection for starvation resistance and different population sizes. Data shows the F statistic and respective p-value for the interaction *Size*Type*Generation* of each life-history trait analyzed.

Male starvation resistance		Female starvation resistance		Early fecundity	
F statistic	p-value	F statistic	p-value	F statistic	p-value
0.71889	0.431487	0.69880	0.437210	0.23125	0.648100
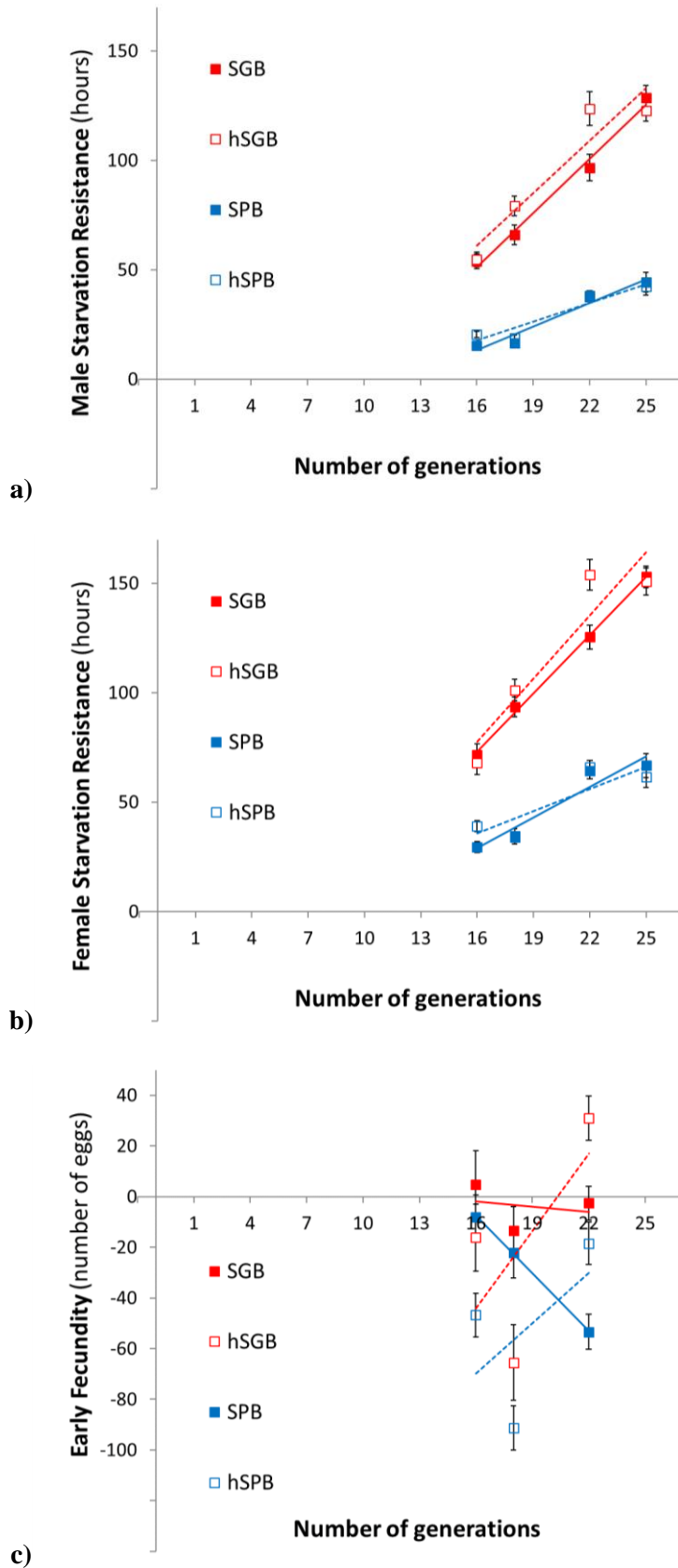
Figure 3.7. Evolutionary trajectories for **a)** male starvation resistance, **b)** female starvation resistance, and **c)** early fecundity of the hybrid (dashed) and parental (solid) selected lines, under both size regimes. Average CGB_i-corrected values for the four size*type regimes are shown. Error bars denote standard error of mean (as differences between replicates).

DISCUSSION

The complex interplay of gene flow, mutation, drift, and selection in natural populations makes it difficult to predict which evolutionary force will be most important at any given time or place (Grant & Grant 2002). It has been unequivocally shown that inbreeding resulting from genetic drift, especially in small populations, can strongly reduce a population's fitness and increase extinction risk (*e.g.* Frankham 2005a,b; Hedrick & Kalinowski 2000; Allendorf *et al.* 2013; Pekkala *et al.* 2014). Several natural and experimental studies have demonstrated that small, inbred, endangered populations can have their fitness increased and inbreeding depression alleviated by introducing genetic variation through immigrants, in a process called *genetic rescue* (Richards 2000; Ingvarsson 2001; Tallmon *et al.* 2004; Frankham 2015; Whiteley *et al.* 2015). Furthermore, Margan *et al.* (1998) showed that several small pooled populations were similar or superior to a single large population of equivalent size in terms of inbreeding, fitness, and genetic diversity, as genetic theory would predict (Kimura & Crow 1963; Robertson 1964; Maruyama 1970; Varvio *et al.* 1986; Chesser 1991; Nei & Takahata 1993). Here we present a comprehensive study of the effect of the genetic rescue using inter-population hybridization in different evolutionary scenarios: *i)* under a sustained bottleneck (CPB and SPB), *ii)* under strong directional selection (SGB and SPB), and *iii)* under strong directional selection and small population size (SPB).

Inbreeding depression and heterosis

Inbreeding, the mating of individuals that are related by ancestry, and *inbreeding depression*, the decline of the phenotypic value of fitness-related traits due to crosses between relatives, are expected to be stronger in smaller populations (Falconer & Mackay 1996). After the first 15 generations of this experiment, a general increase in homozygosity by descent and population differentiation would be expected, with a much stronger expression in the lines under a sustained bottleneck (CPB and SPB). The mass hybridization event was then expected to restore some of the lost heterozygosity due to drift and selection, causing a general increase of characters' mean value (*heterosis*).

Regardless of size or selection regime, the experimental lines generally improved their resistance to starvation stress following hybridization (**Table 3.1**). Although the increased starvation trend was consistent across stocks, hybrid females of the large selected lines (*hSGB*) showed a slight decrease in stress resistance. This result can be explained by means of

outbreeding depression: the combined effects of drift and selection could have led to the evolution of different genotypes combinations that work well together (the *coadapted gene complexes*). The hybridization event could have broken up these gene complexes, resulting in lower fitness of the resulting hybrids (Templeton 1986; Lynch 1991; Fenster *et al.* 1997; Lynch & Walsh 1998). Interestingly, none of these differences proved to be statistically significant. This may be owing to (1) opposed directions in dominance of some *loci* which effects may have cancelled out (Falconer & Mackay 1996) and/or (2) the peaking of heterosis in the first generation (Lynch 1991; Lynch & Walsh 1998) and the traits being assayed two generations after hybridization, due to experimental limitations.

All hybrid populations showed a decline in early fecundity when compared to their parentals, irrespective of size or selection regime (**Table 3.1**). If all effects were due only to inbreeding depression, fecundity should increase following heterozygotic restoration by hybridization (heterosis). Notwithstanding, declining fecundity as a result of increasing starvation resistance has been previously observed (*e.g.* Hutchinson *et al.* 1991; Rose *et al.* 1992; *Chapter Two* of thesis), providing evidence for antagonist pleiotropy between survival and reproduction. Because heterosis depends on directional dominance (Falconer & Mackay 1996; Roff 1997; Lynch & Walsh 1998) and fecundity is in a trade-off with starvation resistance, a decline in hybrid fecundity is to be expected if the genes that are responsible for the trade-off have dominance effects *in the same direction*: the heterozygous value for both starvation resistance and fecundity is closer to the same homozygous genotype (**Figure 3.8**). Only in hybrids of small controls (*hCPB*) was the decline in early fecundity statistically significant. Hybridization may have broken up advantageous parental gene combinations and exposed harmful epistatic interactions involving recessive alleles, which were differentially fixed by the individual CPB lines. This, when combined with the decline in early fecundity due to the fecundity-starvation trade-off, could have further reduced early fecundity in *hCPB* lines.
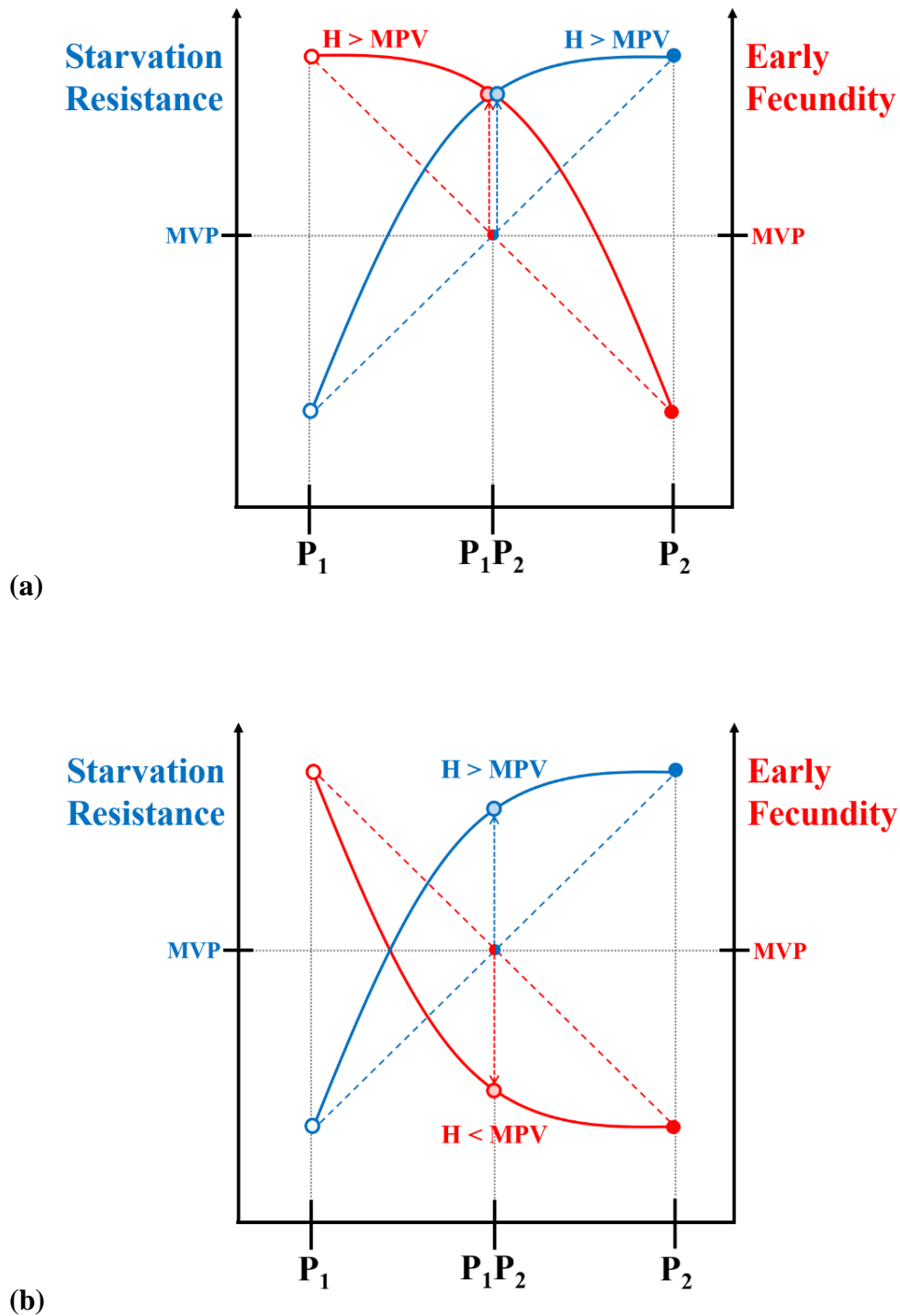
Figure 3.8. Direction of dominance effects of two characters in a trade-off: **(a)** <u>Different direction</u>: the heterozygote is closer to P_2 in starvation and to P_1 in fecundity. **(b)** <u>Same direction</u>: the heterozygote is closer to the same homozygote (P_2) in both starvation and fecundity. ◉ ◉ P_1 phenotypic values. ◉ ◉ Hybrid (P_1P_2) phenotypic values. ● ● P_2 phenotypic values. ● Mid-parent values (MPV).

Evolutionary consequences of hybridization

For many decades, there has been little doubt that the existence of genetic variation is advantageous to the evolutionary survival of a population: it confers the ability to evolve rapidly and so to meet the needs of a changing environment, both through the course of time and in the colonization of new locations (Frankham 1996; Reed & Frankham 2003; Willi *et al.* 2006; Johansson *et al.* 2007; Hoffmann & Sgrò 2011). Interpopulation crossing as a way to introduce genetic variation has proved to improve the viability of endangered populations (see Tallmon *et al.* 2004; Edmands 2007; Frankham *et al.* 2011; Hedrick *et al.* 2011). In the previous section, we analyzed the effects of hybridization on life-history immediately after an interpopulation cross; now we will refer to the longer-term consequences of the hybridization event. A population's ability to respond to selection depends on its level of genetic variation, for the traits undergoing selection. This variation can be characterized in terms of *heterozygosity*, the frequency of heterozygotes at any given time, or *allelic diversity*, the number of different allelic types segregating in the population (Falconer & Mackay 1996). According to neutral evolutionary genetic theory, when only drift and mutation occur, heterozygosity increases monotonically with population size (Kimura 1955, Wright 1931). Larger populations are expected to have higher heterozygosity and, thus, higher response to selection than smaller populations (Falconer & Mackay 1996). For polygenic traits under strong selection, the genetic variation is also expected to be higher at larger N_e because of the combined effects of more mutations and weaker drift. Hybridization may allow the recovery of genetic variants previously lost, leading to an increase of evolutionary response (Tallmon *et al.* 2004; Hedrick 2005; Edmands 2007; Hedrick *et al.* 2011).

The impact of hybridization without directional selection

In the absence of strong directional selection and as far as starvation resistance concerns, our results corroborated the predicted increase of resistance to stress, associated with postponed reproduction, as previously found (*e.g.* Hutchinson *et al.* 1991; Rose *et al.* 1992; *Chapter Two* of this thesis). Nevertheless, neither small nor large lines showed significant differences due to hybridization (**Figure 3.4a,b** and **Tables 3.3** and **3.4**). It seems that hybridization caused little to no effect on the experimental lines under control conditions, which may be due to a lack of differential loss of genetic variance in the different populations. This is not surprising in large populations due to the inverse relationship between

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heterozygosity loss, as well as population differentiation, and N_e (Wright 1951; Hartl & Clark 2007). But it is somewhat more surprising in small lines, although there were no differences in the evolutionary responses of starvation resistance between small and large controls prior to hybridization (see *Chapter Two*).

In terms of early fecundity, the large populations showed an expected decline over time due to the trade-off with postponed reproduction and, once again, there were no significant differences from the uncrossed controls. The small hybrid lines, however, showed an immediate drop in early fecundity (compared to their parentals) followed by a sharp temporal increase, slightly exceeding that of the matched parental stock, but always below the large population's fecundity (**Figure 3.4c** and **Table 3.3**). One possible explanation is that the hybridization event had a very strong effect, causing an immediate and substantial decline in fecundity. The interpopulation cross may have broken up advantageous parental gene combinations and exposed harmful epistatic interactions involving recessive alleles (outbreeding depression; see Edmands 2007; Frankham *et al.* 2011; Ralls *et al.* 2013), which were differentially fixed by the individual CPB lines. As time went by, the negative effects of hybridization may have been alleviated, because the negative epistatic relationships created during the intercross were broken up and the deleterious alleles were eliminated. This could account for the hybrid's recovery to non-hybridized values. Nevertheless, outbreeding depression is not a classical expectation when populations of small sizes under similar selection regimes are considered (Ralls *et al.* 2013).

The impact of hybridization under strong directional selection

Under strong directional selection for starvation resistance, both large and small experimental populations showed, as expected, a significant temporal increase of the trait's mean value. However, there was no significant effect of hybridization (**Figure 3.7a,b** and **Tables 3.5** and **3.6**). Due to strong selection, the loss of genetic variation for starvation resistance could have been consistent across lines, making the populations very similar and, consequently, indifferent to the effect of hybridization, even in the smaller lines. Nonetheless, during forward selection, small populations showed an increased differentiation through time, higher than large populations (see *Chapter Two*), which contradicts the hypothesis stated above. A possible explanation is that small populations lose *environmental homeostasis* due to inbreeding, increasing both within and between population environmental variance (Fox &

Reed 2010). For instance, Reed *et al.* (2003) found strong lineage effects across environments in inbred *D. melanogaster* populations. In our system, such effect might have led to higher interpopulation variance in small *vs.* large populations, without necessarily increase additive variance after hybridization. Noteworthy, most of the alleles that are likely to be lost in small populations are those rare alleles that are neutral or slightly deleterious (Kimura 1983). Such alleles are considered by many to play a little role in adaptation to novel or changing environments. For quantitative traits, several empirical studies have shown that some morphology, behavior, and fitness traits' heritabilities can remain high, or even increase, despite reduction of the population to as small as a single pair (*e.g.* Bryant *et al.* 1986; Lopez-Fanjul & Villaverde 1989; Bryant & Meffert 1993; Wade *et al.* 1996; Cheverud *et al.* 1999). Hence, the effects of population size and fluctuations in population size on genetic variation are not completely clear (Reed *et al.* 2003). Moreover, the evolutionary dynamics of outbred sexual populations in a polygenic scenario may contribute to the lack of effective fixation of certain alleles (Chevin & Hospital 2008; Burke *et al.* 2010; Phillips *et al.* 2016; Graves *et al.* 2017; Seabra *et al.* 2018), which makes the small population size less important for the evolutionary outcome than what is predicted by conventional population genetic theories.

In terms of fecundity, both small and large lines suffered an initial post-hybridization decline, followed by a significant increase (**Figure 3.7c** and **Table 3.5**), marking a substantial impact of hybridization on this character (**Table 3.6**). As mentioned before, the initial decline in hybrid fecundity can be explained by the survival-reproduction trade-off, if the genes that are involved have dominance effects in the same direction (**Figure 3.8**). Over the course of subsequent generations, several factors could have accounted for the recovery of the character: (1) the effect of heterosis is diluted, (2) some negative epistatic interactions created by hybridization are broken up through recombination, (3) some of the restored heterozygosity allow the establishment of new, favorable gene interactions. These are *a posteriori* hypotheses that might by worthy to further explore. Once again, these experimental results do not follow the classical expectations of the effects of hybridization in finite, randomly mating populations.

Whether a stronger bottleneck and/or a longer-term experiment would change the impact of hybridization that we report here remains unknown. Also, a population expansion after hybridization could foster reshuffling between variants across populations (*e.g.* fast breaking of epistatic combinations) leading to increased additive variance and further selective response (Reed *et al.* 2003; Allendorf *et al.* 2013). Founder-flush experiments have indeed showed that bottlenecks followed by population expansion may increase additive genetic

variance (*e.g.* Bryant & Meffert 1993). But a pertinent experiment that is lacking, to the best of our knowledge, would be to impose a prolonged bottleneck followed by extensive expansion and gene flow.

Final remarks

Interpopulation hybridization has often been suggested as a way to improve the viability of *inbred* populations and decrease extinction risk (Tallmon *et al.* 2004; Hedrick 2005; Edmands 2007; Frankham *et al.* 2011; Hedrick *et al.* 2011). Because of the complex genetic architecture of fitness-related traits and the multitude of factors that can interfere with the outcome of a hybridization event, it is very difficult to predict the evolutionary consequences of interpopulation cross in any given scenario. With our highly-replicated experimental design, we tried to shed light on the evolution of populations under different combinations of directional selection and population size regimes. Our major findings suggest that conventional theoretical expectations of the effects of hybridization are not generally obtained, at least over the range of time and effective population sizes used by us. We further note that no comparably scaled experiments have been done by other laboratories under such carefully controlled conditions. One interpretation of this is that conventional theory (*e.g.* Falconer & Mackay 1996; Hartl & Clark 2007) concerning the effects of hybridization and population size may have survived up to this point chiefly because of a lack of experiments of sufficiently powerful design to test its predictions.

Chapter Four.

Reverse Evolution in Small-Sized Populations

ABSTRACT

Extensive research on experimental evolution of Mendelian populations showed that contrasting selection regimes rapidly cause phenotypic divergence and that populations under the same selection regime quickly converge on common phenotypes. A population's evolutionary history is very important for its subsequent evolution and fate, namely, in a scenario of reverse colonization, *i.e.* migration back to the ancestor environment. Prior research in the Rose laboratory has shown clear reversal of fitness-traits when N_e is moderately large; here we focused on whether reverse evolution is impaired (or not) by a sustained bottleneck. Our major findings show that populations kept in a small N_e can reproduce the possible patterns of reversion to the ancestor, contingent to character and/or population: complete reversion, partial convergence, and steep convergence with superior outcome than the ancestor. We further found the history signature to be strong with respect to the *tempo* of reverse evolution, smoothed but not erased by reverse selection, over the time period studied.

RESUMO

Diversos estudos de evolução experimental em populações mendelianas mostraram que a aplicação de diferentes regimes de selecção provoca rápida divergência fenotípica e que populações sob o mesmo regime selectivo convergem rapidamente num fenótipo semelhante. A história evolutiva de uma população é muito importante para sua subsequente evolução, nomeadamente, num cenário de colonização reversa (*i.e.,* migração para o ambiente ancestral). Estudos anteriores no laboratório de Michael R. Rose mostraram uma clara reversão de características da história da vida quando N_e é moderadamente grande; neste projecto estudamos se a evolução reversa é (ou não) comprometida por um efeito de gargalo continuado. Os nossos resultados sugerem que as populações mantidas em N_e reduzido reproduzem os possíveis padrões de reversão ao estado ancestral, dependendo da característica e/ou da população: reversão completa, convergência parcial e convergência abrupta ultrapassando o ancestral. Adicionalmente, verificou-se que a assinatura da história é muito marcada em termos da taxa evolutiva, podendo ser atenuada, mas não apagada por selecção reversa durante o período de tempo estudado.

INTRODUCTION

In research over the last three decades, it has been found that laboratory selection can quickly and reproducibly shape population phenotypes (see Garland & Rose 2009). Experimental evolution in Mendelian populations that have not been inbred shows the following. (1) Phenotypic divergence occurs rapidly when these populations are subjected to new types of selection (*e.g.* Luckinbill *et al.* 1984; Rose *et al.* 1992; Chippindale *et al.* 1997; Zhou *et al.* 2007; Turner *et al.* 2011; Turner & Miller 2012; Burke *et al.* 2016). (2) Independent replicate populations under the same regime quickly converge on common phenotypes (Teotónio & Rose 2000; Simões *et al.* 2008; Fox *et al.* 2011; Fragata *et al.* 2014b; Burke *et al.* 2016; Simões *et al.* 2017).

Mendelian populations that have not been inbred maintain considerable amounts of standing genetic variation, reshuffled every generation by recombination. It has been found that, under uniform conditions, previous selection histories will be erased quickly, if N_e is kept moderate to high (Burke *et al.* 2016). Populations of *Drosophila* with different genetic backgrounds and varied census sizes sometimes converge when given common selection regimes, but not always (Cohan & Hoffman 1989; Griffiths *et al.* 2005; Simões *et al.* 2008; Santos *et al.* 2010; Fragata *et al.* 2014a,b; Simões *et al.* 2017). The evolutionary rates at which convergence occurs can also be trait-specific, as the relative contributions of history and selection may vary across traits. Fitness-related traits, as defined by a particular selection regime, are expected to converge faster and more consistently than characters less associated with fitness, where history can have a more preponderant role (Travisano *et al.* 1995; Teotónio & Rose 2000; Teotónio *et al.* 2002; Joshi *et al.* 2003; but see Fragata *et al.* 2014b).

One particular case of convergence is reverse evolution. For the sake of clarity, we use the Bull & Charnov (1985) definition of reverse evolution as *the reacquisition by derived populations of the same character states, including fitness, as those of ancestor populations.* The (ir)reversibility of evolution has long received the attention of evolutionary biologists (Darwin 1859; Dollo 1893; Gregory 1936; Muller 1939; Simpson 1953; Lewontin 1966; Gould 1970; Maynard-Smith 1970; Wright 1977; Wagner 1982; Bull & Charnov 1985; Service *et al.* 1988; Marshall *et al.* 1994; Gayon 1998; Teotónio & Rose 2000, 2001; Teotónio *et al.* 2002, 2009; Whiting *et al.* 2003; Passananti *et al.* 2004a,b; Bridgham *et al.* 2009; Desai 2009; Duncan *et al.* 2011; Klimov & OConnor 2013). There has been considerable controversy about the reversibility of evolution over shorter time spans, (Gayon 1998). But it was long thought that long-term evolution was not likely to be reversible at all levels of biological organization,

because retracing several evolutionary events over long periods of time was considered improbable (Teotónio & Rose 2001). Irreversible evolution is now viewed as an *extreme type of evolutionary restriction*, one that renders a population incapable of reacquiring an ancestral state (Bull & Charnov 1985). Thus, a major question of interest is the degree to which evolutionary history constrains reverse evolution (Maynard Smith 1970; Bull & Charnov 1985; Loeschcke 1987; Gould 1989; Williams 1992; Travisano *et al.* 1995; Losos *et al.* 1998; Teotónio & Rose 2000, 2001; Bell 2008).

Reverse evolution was, for a long time, approached through comparative biology, embryology, and paleontology (*e.g.* Simpson 1953; Lande 1978; Bull & Charnov 1985; Wake 1991; Sanderson & Hufford 1996). Although it is a widely used and very useful method to understand the phylogenetic history of the compared taxa, it cannot unveil the specific genetic mechanisms that drive reverse evolution (Teotónio & Rose 2001). Real-time experimental evolution, notwithstanding its limits with respect to observable evolutionary time, is the tool that we use to address these issues here.

Experimental studies of reverse evolution are fairly scarce. Most of them have used somewhat inbred lines that were artificially selected for a morphological trait. In those cases, reverse selection was usually able to revert character states to levels close to the ancestral one (reviews in Wright 1977; Falconer & Mackay 1996). Over the last three decades, several reverse evolution experiments have been attempted with both sexual and asexual populations (*e.g.* Lenski 1988a,b; Service *et al.* 1988; Graves *et al.* 1992; Bull *et al.* 1997; Lenski 1998; Rainey & Travisano 1998; Burch & Chao 1999; Crill *et al.* 2000; Moore *et al.* 2000; Teotónio & Rose 2000; see Teotónio & Rose 2001 for a review; also Passananti *et al.* 2004a,b).

Reverse evolution can be achieved with experimental evolution by imposing an ancestral laboratory environment on populations that have been subjected to different culture regimes since their last common ancestors. Because this requires the use of the ancestral population as a control, a sample of the ancestral (kept in a state of suspended animation) or an equivalent population (direct descendants of the ancestral kept in ancestral conditions) must be available. Examples of the use of this experimental design using *Drosophila melanogaster* are the studies of Service *et al.* 1988, Graves *et al.* 1992, and Teotónio & Rose 2000. Service and colleagues took a 5-fold replicated stock of *Drosophila melanogaster* that had been selected for increased late-life reproduction and re-imposed the same environment as that of their common ancestral population, which featured early reproduction (Rose 1984a; Service *et al.* 1988). The late-reproduction populations were differentiated for several characters, including

increased late fecundity, longevity, and stress resistance, as well as depressed early fecundity. During the first 20 generations, starvation resistance and early fecundity showed a rapid, although incomplete, reverse evolution. Conversely, ethanol tolerance and desiccation resistance showed no significant response to reverse selection over those same 20 generations (Service *et al.* 1988). The experiment was extended by Graves and collaborators for more 100 generations; convergence to the ancestral values became apparent in all traits in that study. The rapid reversion observed for starvation resistance and early fecundity indicates that these characters were under the influence of pleiotropic alleles generating a large negative genetic correlation between the two traits. Because of this correlation, both traits rapidly moved toward their ancestral values during reverse evolution, as selection focused on early fecundity (Graves *et al.* 1992).

Teotónio & Rose (2000) performed a similar 50-generation reverse experiment on a much larger scale, assessing a greater number of fitness-related traits in several populations of diverse evolutionary histories. The patterns of reverse evolution varied among evolutionary histories and characters: except for early fecundity, populations with different histories had statistically heterogeneous responses to reverse selection. Four kinds of evolutionary trajectories were observed. (1) Reversion to ancestral character values with full convergence after ~20 generations of reverse selection. (2) Linear response without convergence within the 50 generations of the experiment. (3) Initial rapid reversion, followed by a stalling of evolution, without full convergence to ancestral character values. And, finally, (4) no significant change throughout the 50 generations of evolution.

These studies showed that reverse evolution is neither inevitable nor impossible, but contingent on the number of generations exposed to the novel environment, the degree of differentiation from the ancestral population, and the genetic variation of the character as well as its relation to fitness. This range of evolutionary responses of differentiated populations that share common origin to the ancestral environment as well as basic underlying genetic mechanisms are summarized in **Figure 4.1**.
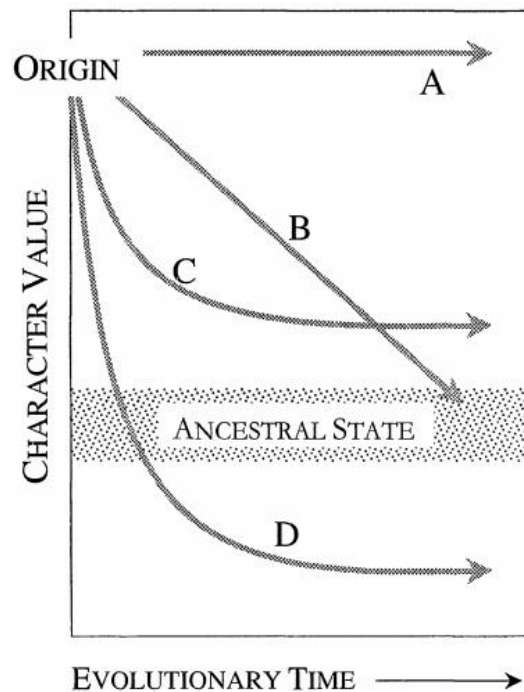
Figure 4.1. Possible evolutionary responses of differentiated populations of common origin to the ancestral environment. **A:** The character does not change because it is effectively neutral or there is no available genetic variability. **B:** When the character is directly related to fitness, or is fitness itself, there is response with concomitant convergence to the ancestral character state by pleiotropy. **C:** Two-phased response: (1) rapid initial response through pleiotropy with fitness is accomplished but convergence is only partial. (2) no change through time because the character is effectively neutral or all standing genetic variability was exhausted during the first phase. **D:** Response that surpasses the ancestral character state – the genetics of the derived population may allow the attainment of a previously inaccessible character level. © Teotónio & Rose (2001)

Previously in this thesis, two major constraints that can shape colonization and the colonizing populations' subsequent evolution were addressed: effective population size (*Chapter Two*) and hybridization and its effects on adaptation to a novel and challenging environment (*Chapter Three*). This section of the thesis will focus on factors influencing the evolutionary success of reverse colonization: *(i)* loss of adaptation to that ancestral environment, *(ii)* N_e of the reverse-colonizers, *(iii)* shifts in their age-specific demography, and *(iv)* adverse effects of hybridization with the endemic ancestral population.

Factors that might affect colonization and reverse-colonization have been of major interest within conservation biology, especially whether long-maintained captive populations show deterioration in their ability to thrive in their ancestral wild environments. This is particularly important for *ex-situ* conservation programs that aim to reintroduce into the wild their captive bred individuals (Allendorf *et al.* 2013; Frankham 1995b, 2008, 2009b; Frankham *et al.* 2000, 2002; Woodworth *et al.* 2002; Gilligan & Frankham 2003).

Prior research from the Rose Laboratory has shown that relaxing selection on starvation resistance leads to a clear evolutionary reversion of the character when N_e is moderately large (*e.g.* Teotónio & Rose 2000; Passananti *et al.* 2004b). The scientific question addressed in this chapter is whether such clear response would be exhibited at low values of N_e, specifically whether reverse evolution is impaired by sustained small population sizes. This is tested using a reverse colonization experiment where small-sized populations that had undergone strong selection for starvation resistance were returned to their previous, long-standing selection regime, which featured early reproduction, *ad libitum* feeding, and no phase of food supplementation.

MATERIALS AND METHODS

Derivation of the reverse-selected experimental populations

The experimental populations used in this study were derived from the 84 populations described in *Chapter Two*. After 15 generations of forward selection for starvation resistance, derivatives of the small populations (both selected and control) were created and a reverse-selection experiment was started. Each of the 30 CPB_{ij} lines was used to derive a single $RCPB_{ij}$, and each of the 30 SPB_{ij} populations was used to derive one $RSPB_{ij}$. The creation of the reverse-selected small stocks increased the experiment's replication size to 228. **Figure 1.5** of *Chapter One*, which is here reproduced, shows a diagram of the derivation process.
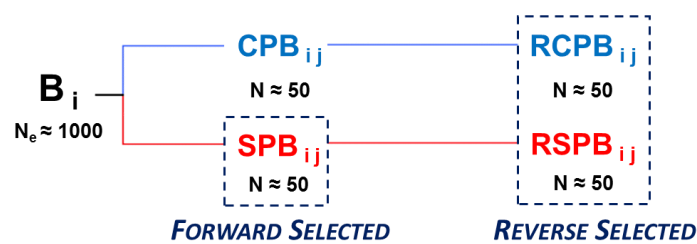
Figure 1.5. Schematic representation of the experimental design used to create the reverse-selected lines. After 15 generations of forward selection, each CPB_{ij} population was used to derive a single $RCPB_{ij}$ population, and each SPB_{ij} population was used to derive one $RSPB_{ij}$ population. These 60 populations were then subjected to 21 generations of reverse selection and sustained small N_e.

Reverse selection procedures for starvation resistance

These reverse-selected populations were returned to a 14-day culture in vials, without the dietary changes or selection imposed during the first phase of the experiment (*Chapter Two* of this thesis). These stocks were kept at the same low N_e as was imposed on them during the first phase of the experimental work, simulating a sustained bottleneck in effective population size (**Figure 1.5** of *Chapter One*, which is here reproduced).

Starvation resistance and fecundity assays

Both life history traits were assayed using the protocols described in *Chapter Two*. Starvation resistance and fecundity were assayed at generations 0, 5, 12, and 21 after the derivation of the R-selected lines.

Statistical data analysis

In all analyses, the normality and homoscedasticity of data were tested by Shapiro-Wilk (1965) and Brown-Forsythe (1974) tests, respectively. After testing by ANOVA and ANCOVA, and when it was appropriate, Tukey HSD (1953) *post-hoc* tests were done. A significance value

of 0.05 (α) was used to test all null hypotheses. All analyses were done using *STATISTICA 13* (Dell 2015).

The key statistical tests focused on quantitative measures of how reverse selection affects starvation resistance and fecundity, after the 15 generations of forward selection for increased starvation resistance. The magnitudes of the interactions between the evolutionary responses and the selection regime were estimated.

The evolutionary trajectories of starvation resistance and fecundity throughout the 21 generations of reverse selection were analyzed according to the following ANCOVA model:

$$Y = \mu + H_i + S_j + G_k + H_i * S_j + H_i * G_k + H_i * S_j * G_k + Rep\{H * T\} + G_k \\ * Rep\{H * T\} + \varepsilon \tag{4.1}$$

where Y is the trait under analysis (starvation resistance or fecundity); H represents the evolutionary history ($i = 0\text{-}5$), random factor; S the prior selection regime ($j = $ selected or $j = $ control), fixed factor; G the covariate generation (see which on the assays description), and *Rep* the replicate population (random factor). This is a comprehensive model that includes all variables under study. Analyses within prior selection regime or within a single generation used adapted versions of the aforementioned model (*e.g.* when analyzing the trajectories of selected or control lines, the factor *Selection* and all its interactions were removed). The slopes of the evolutionary trajectories of each population were compared using the respective slopes as raw data (Y in the model).

Ancestral population state proxy

Because a sample of the ancestral *Drosophila melanogaster* population could not be kept in a state of suspended animation, this experiment required the use of its direct descendants which were kept in ancestral conditions, *i.e.* the B flies. Due to experimental constraints, the B populations were not assayed in synchrony with generation 0 of the experimental stocks; for the initial differentiation comparisons data from the earliest time point assayed (generation 5) was used instead.

Differences to control as raw data

Since statistically significant changes in starvation and fecundity were found among the control lines (**Table 4.3** and **Figure 4.3**), to remove confounding effects due to environmental stochasticity, we used the control-corrected data (difference from the respective B_i control) to perform all the statistical analyses.

RESULTS

General linear model assumptions

Small deviations from normality were accepted, and homoscedasticity was verified by the Brown-Forsythe test. Our distribution tests showed that all populations were homoscedastic and generally normal (data not shown).

Initial differentiation from the ancestral population

The initial differentiation of the reverse-selected populations from the ancestral stock was tested. The RCPB flies showed significantly higher starvation resistance in both males ($p<0.02$) and females ($p<0.02$) and lower early fecundity, although not significantly ($p>0.2$). The RSPB lines showed significantly higher starvation resistance in both males ($p<0.01$) and females ($p<0.001$), and significantly lower early fecundity ($p<0.05$). The mean values and standard errors for each character and regime are detailed in **Table 4.1**.

Table 4.1. Comparison of the ancestral B stock with the reverse-selected lines (RCPB and RSPB) for initial differentiation testing. Data are given as the initial (generation 5) mean value ± standard error of mean (as differences between replicates) of each regime, for each life-history trait analyzed. Starvation resistance values are in hours and early fecundity in number of eggs laid per female. Significant differences to the ancestral population (B) are highlighted in bold.

Regime	Male starvation resistance (hours)	Female starvation resistance (hours)	Early fecundity (eggs per female)
B	27.2 ± 1.94	43.2 ± 2.01	210.0 ± 15.04
RCPB	**41.1 ± 2.55**	**67.2 ± 3.72**	170.6 ± 6.56
RSPB	**59.4 ± 2.80**	**102.6 ± 3.36**	**172.6 ± 3.77**

Differentiation from the ancestor after 21 generations of reverse selection

After 21 generations under ancestral conditions, the differentiation of the reverse-selected populations from the ancestral stock was tested. The RCPB flies showed higher starvation resistance than the B counterparts, significantly higher in females ($p<0.02$) but not in males ($p>0.05$); on average, RCPB early fecundity was higher than B but the difference was not statistically significant ($p>0.4$). The RSPB lines showed not only significantly higher starvation resistance in both males ($p<0.01$) and females ($p<0.001$), but also in early fecundity ($p<0.03$). The mean values and standard errors for each character and regime are detailed in **Table 4.2.**

Table 4.2. Comparison of the ancestral B stock with the reverse-selected lines (RCPB and RSPB) after 21 generations of reverse selection. Data are given as the final (generation 21) mean value ± standard error of mean (as differences between replicates) of each regime, for each life-history trait analyzed. Starvation resistance values are in hours and early fecundity in number of eggs laid per female. Significant differences to the ancestral population (B) are highlighted in bold.

Regime	Male starvation resistance (hours)	Female starvation resistance (hours)	Early fecundity (eggs per female)
B	22.1 ± 1.81	41.7 ± 2.33	171.3 ± 9.21
RCPB	29.9 ± 1.38	**58.2 ± 2.18**	184.8 ± 7.33
RSPB	**36.2 ± 1.54**	**75.5 ± 2.70**	**204.8 ± 5.43**

The average initial (G5) and end-of-experiment (G21) values for the life-history traits assayed as well as the relevant statistical tests of differentiation to the ancestral stock are summarized in **Figure 4.2**.

Evolutionary change of the ancestral population

During this experiment, the B flies saw their starvation resistance significantly decreased in males (from 27.2 to 22.1 hours, $p < 0.03$) but not in females (from 43.2 to 41.7 hours, $p > 0.7$). At the start of the experiment, the early fecundity of each control female averaged 210 eggs, and at the end of the experiment it averaged 171 eggs ($p < 0.03$). **Table 4.3** summarizes the ANCOVA results from the B evolutionary trajectories' analyses. The pattern of change of starvation resistance and fecundity over the experiment is shown on **Figure 4.3**.

Table 4.3. Summary of the mixed-effects ANCOVAs used to analyze the evolutionary trajectories of B controls. Data shows the F statistic and respective p-value for the factor *Generation* of each life-history trait analyzed. Significant changes through time are highlighted in bold.

Male starvation resistance		Female starvation resistance		Early fecundity	
F statistic	p-value	F statistic	p-value	F statistic	p-value
9.7996	**0.025945**	0.1039	0.760224	**9.7925**	**0.025848**
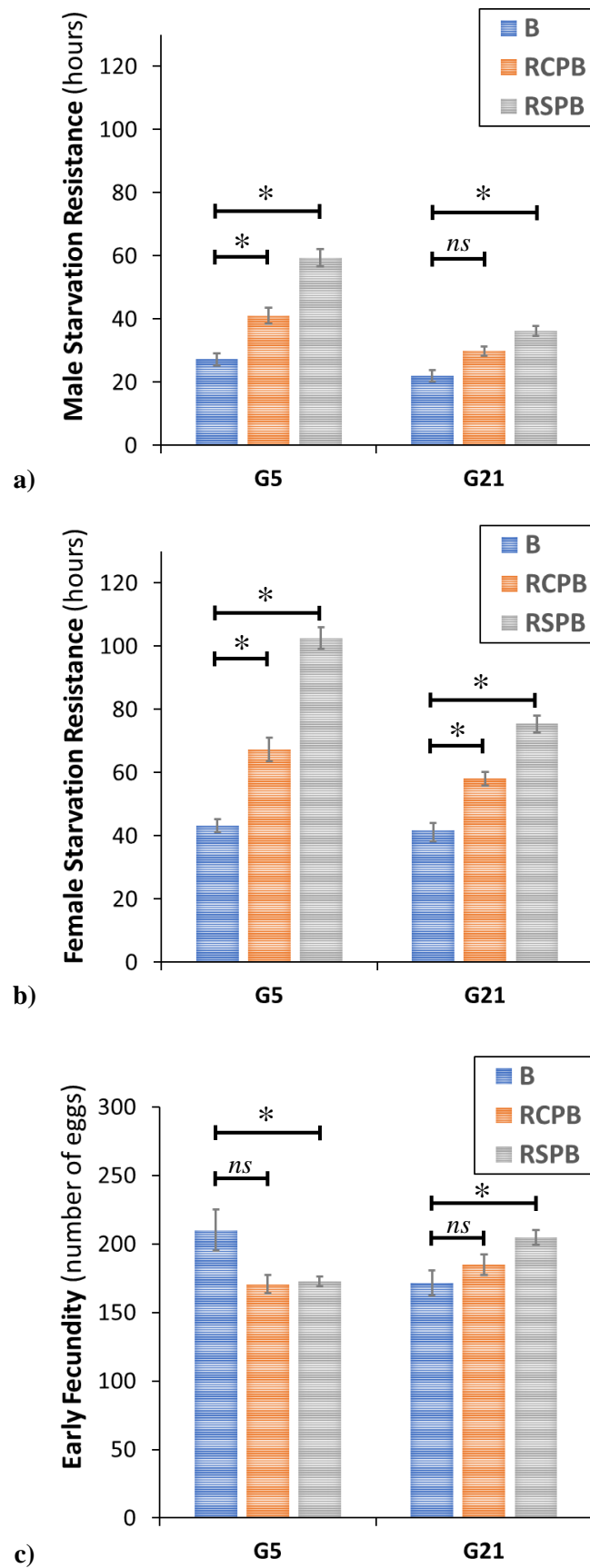
Figure 4.2. Initial (G5) and final (G21) state of the experimental populations for **a)** male, **b)** female starvation resistance, and **c)** early fecundity. Average stock values are shown and error bars denote standard error of mean. p≤0.05 is marked with * and p>0.05 with *ns*.
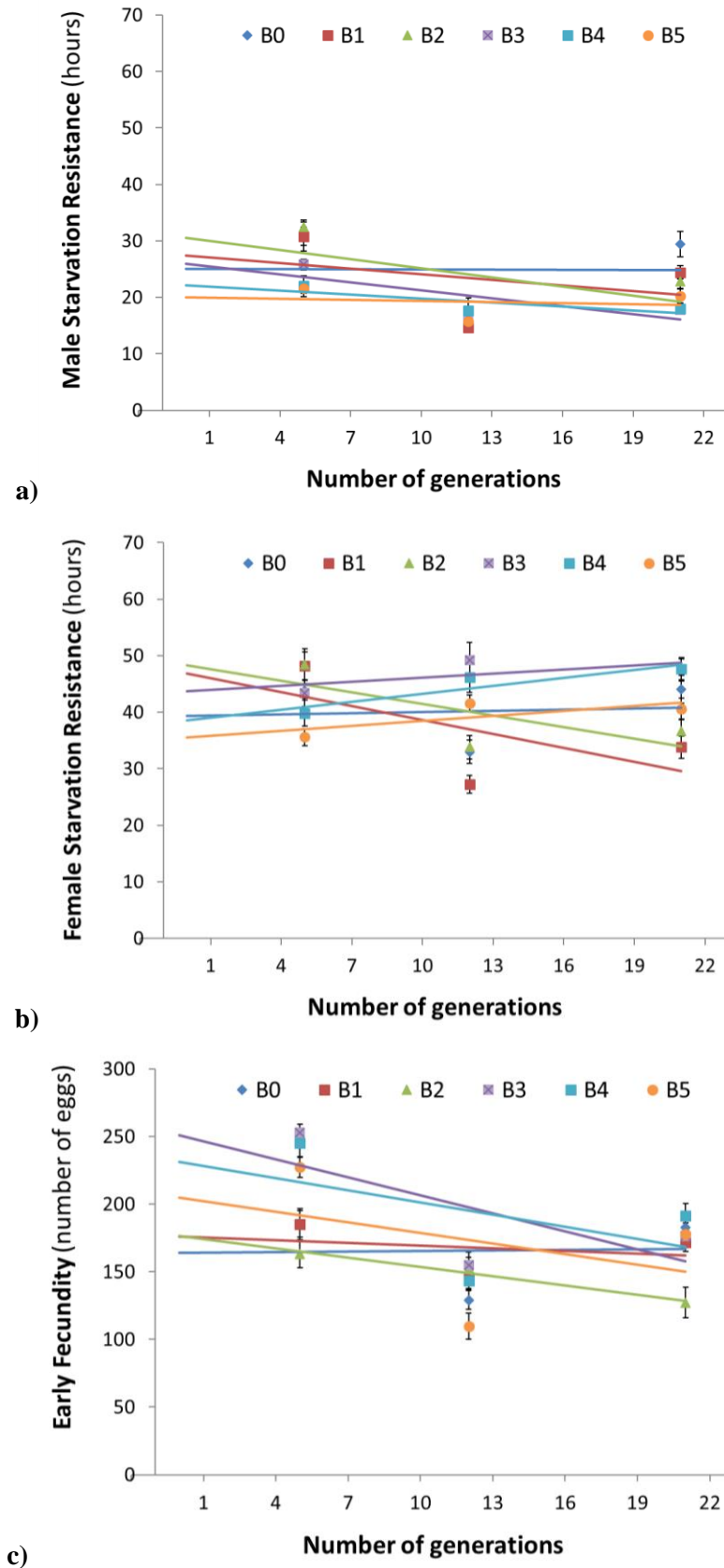
Figure 4.3. Evolutionary trajectories of the ancestral populations (B) for **a)** male, **b)** female starvation resistance, and **c)** early fecundity. Average population values are shown. Error bars denote standard error of mean.

Evolution under reverse selection

The evolutionary trajectories of the experimental lines were analyzed over 21 generations of reverse selection. Because the general expectation is the reversion to B state, using relative-to-control data to analyze evolutionary trajectories, the predicted trend is toward zero. *E.g.*, if a population's trait average value is lower than the controls, a negative value for the trait is obtained, and a positive slope is expected (see **Figures 4.4** and **4.5**).

Through the length of the experiment, RCPB starvation resistance decreased in males and females though not significantly (**Table 4.4** and **Figure 4.4**). The formerly forward-selected counterparts, RSPB, saw their male and female starvation resistances significantly decreased (**Table 4.4** and **Figure 4.5**). In terms of early fecundity, both RCPB and RSPB stocks showed a significant increase in their average values (**Table 4.4**). Interestingly, RSPB started with lower fecundity than the control and by generation 21 presented higher values (**Figure 4.2**).

Table 4.4. Summary of the mixed-effects ANCOVAs used to analyze the evolutionary trajectories of the reverse-selected stocks. Data shows the F statistic and respective p-value for the factor *Generation* of each *prior selection* regime and life-history trait analyzed (B_i-corrected data). Significant changes through time are highlighted in bold.

Prior selection regime	*Male starvation resistance*		*Female starvation resistance*		*Early fecundity*	
	F statistic	*p-value*	*F statistic*	*p-value*	*F statistic*	*p-value*
RCPB	1.64754	0.255454	2.18103	0.199540	**8.46003**	**0.033349**
RSPB	**9.63804**	**0.026716**	**8.4163**	**0.033748**	**14.02589**	**0.013322**
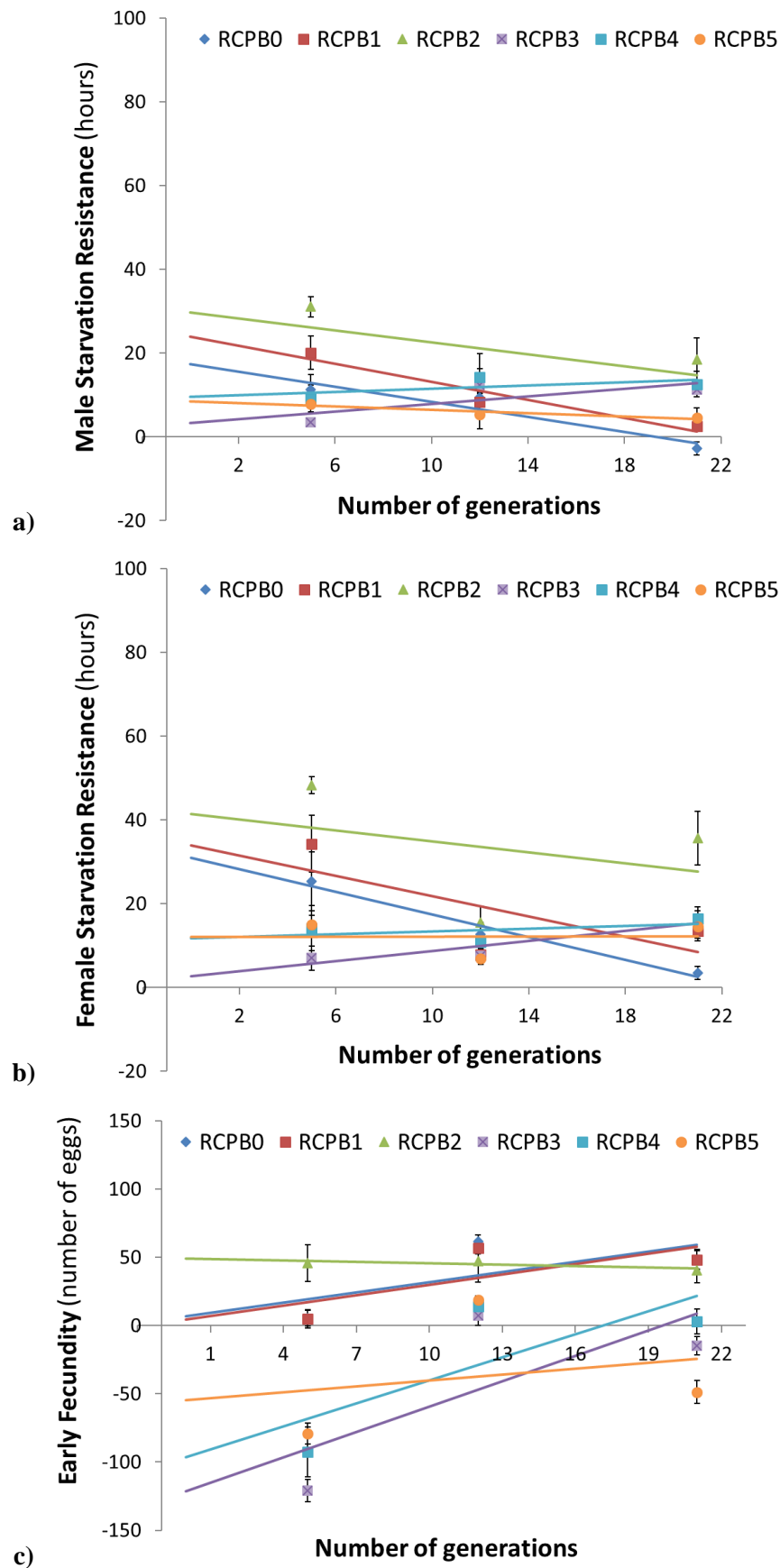
Figure 4.4. Evolutionary trajectories of the RCPB populations for **a)** male, **b)** female starvation resistance, and **c)** early fecundity. Average B_i-corrected values for each B_i ancestor are shown. Error bars denote standard error of mean (computed as differences between replicate populations).
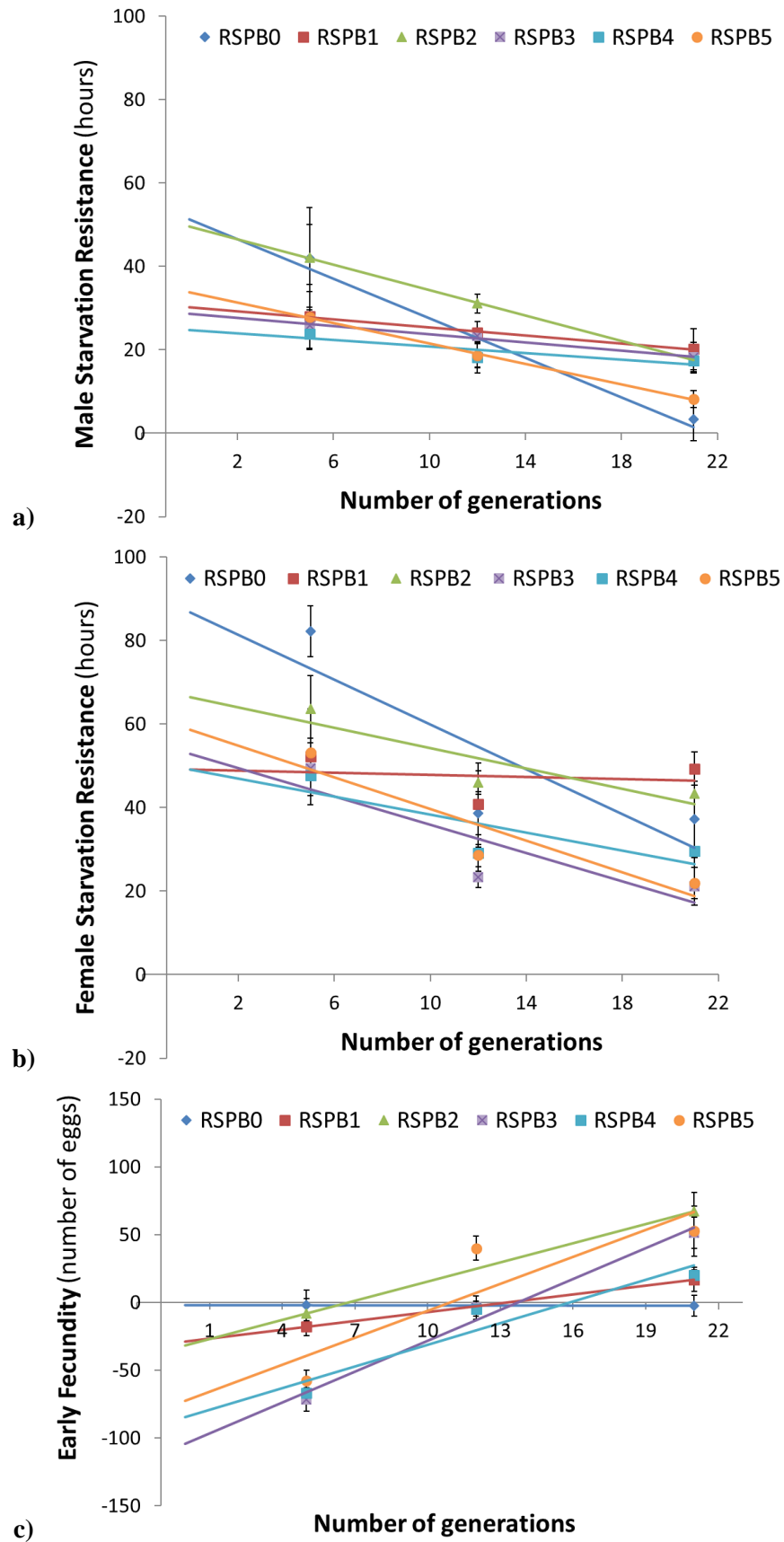
Figure 4.5. Evolutionary trajectories of the RSPB populations for **a)** male, **b)** female starvation resistance, and **c)** early fecundity. Average B_i-corrected values for each B_i ancestor are shown. Error bars denote standard error of mean (computed as differences between replicate populations).

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Prior evolutionary history and reverse evolution

The effect of previous evolutionary history on the process and outcome of reverse selection was tested, comparing RCPB and RSPB in terms of evolutionary trajectories and character states, respectively.

First, the populations were tested at the start of the reverse selection experiment (G5). Initially, when compared with RCPB lines, the RSPB populations showed higher starvation resistance both in males ($p<0.002$) and females ($p<0.002$) but no significant differentiation in early fecundity ($p>0.9$), as detailed in **Table 4.5**. Then, the same test was done after 21 generations of reverse selection (G21). By the end of the experiment, the RCPB and RSPB flies were less differentiated in terms of starvation resistance than at G5, but they were still significantly different in both males ($p\approx0.05$) and females ($p<0.04$). Early fecundity was still similar in both stocks ($p>0.4$). See **Table 4.6** for details.

Table 4.5. Initial differentiation between the reverse-selected lines, RCPB and RSPB. Data are given as the initial (generation 5) mean value ± standard error of mean (as differences between replicates) of each regime, for each life-history trait analyzed. Starvation resistance values are in hours and early fecundity in number of eggs laid per female. Significant differences are highlighted in bold.

Regime	*Male starvation resistance* (hours)	*Female starvation resistance* (hours)	*Early fecundity* (eggs per female)
RCPB	**41.1 ± 2.55**	**67.2 ± 3.72**	170.6 ± 6.56
RSPB	**59.4 ± 2.80**	**102.6 ± 3.36**	172.6 ± 3.77

Table 4.6. Differentiation between the reverse-selected lines, RCPB and RSPB, after 21 generations of reverse selection. Data are given as the final (generation 21) mean value ± standard error of mean (as differences between replicates) of each regime, for each life-history trait analyzed. Starvation resistance values are in hours and early fecundity in number of eggs laid per female. Significant differences are highlighted in bold.

Regime	*Male starvation resistance* (hours)	*Female starvation resistance* (hours)	*Early fecundity* (eggs per female)
RCPB	**29.9 ± 1.38**	**58.2 ± 2.18**	184.8 ± 7.33
RSPB	**36.2 ± 1.54**	**75.5 ± 2.70**	204.8 ± 5.43

The average initial (G5) and end-of-experiment (G21) values for the life-history traits assayed as well as the relevant statistical tests of differentiation to the ancestral stock are summarized in **Figure 4.6**.
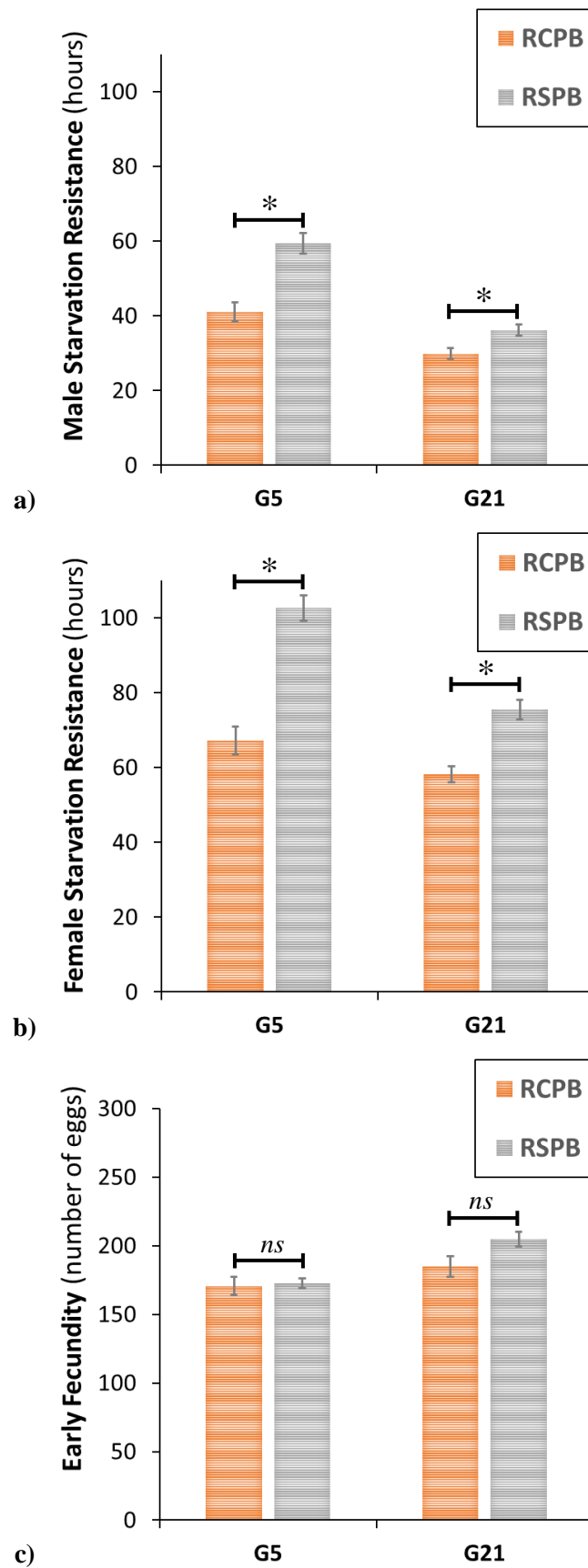
Figure 4.6. Initial (G5) and final (G21) state of RCPB and RSPB for **a)** male, **b)** female starvation resistance, and **c)** early fecundity. Average stock values are shown and error bars denote standard error of mean. p≤0.05 is marked with * and p>0.05 with *ns*.

Finally, the evolutionary trajectories of RCPB and RSPB for the three characters were compared in terms of direction and rate of evolution. The slopes of the evolutionary trajectories of each population were computed (data not shown) and the reverse-selected regimes were compared. Both RCPB and RSPB showed an overall negative slope for starvation resistance (*i.e.* temporal decrease) and an overall positive slope for early fecundity (*i.e.* temporal increase), which means that both regimes responded in the same direction. In terms of the evolutionary rates for starvation resistance, significant differences for both males ($p<0.04$) and females ($p<0.05$) were found; early fecundity rates of the reverse-selected lines showed no significant difference ($p>0.4$). The average evolutionary rates for the life-history traits assayed as well as the relevant statistical tests of differentiation between RCPB and RSPB are summarized in **Figure 4.7**.
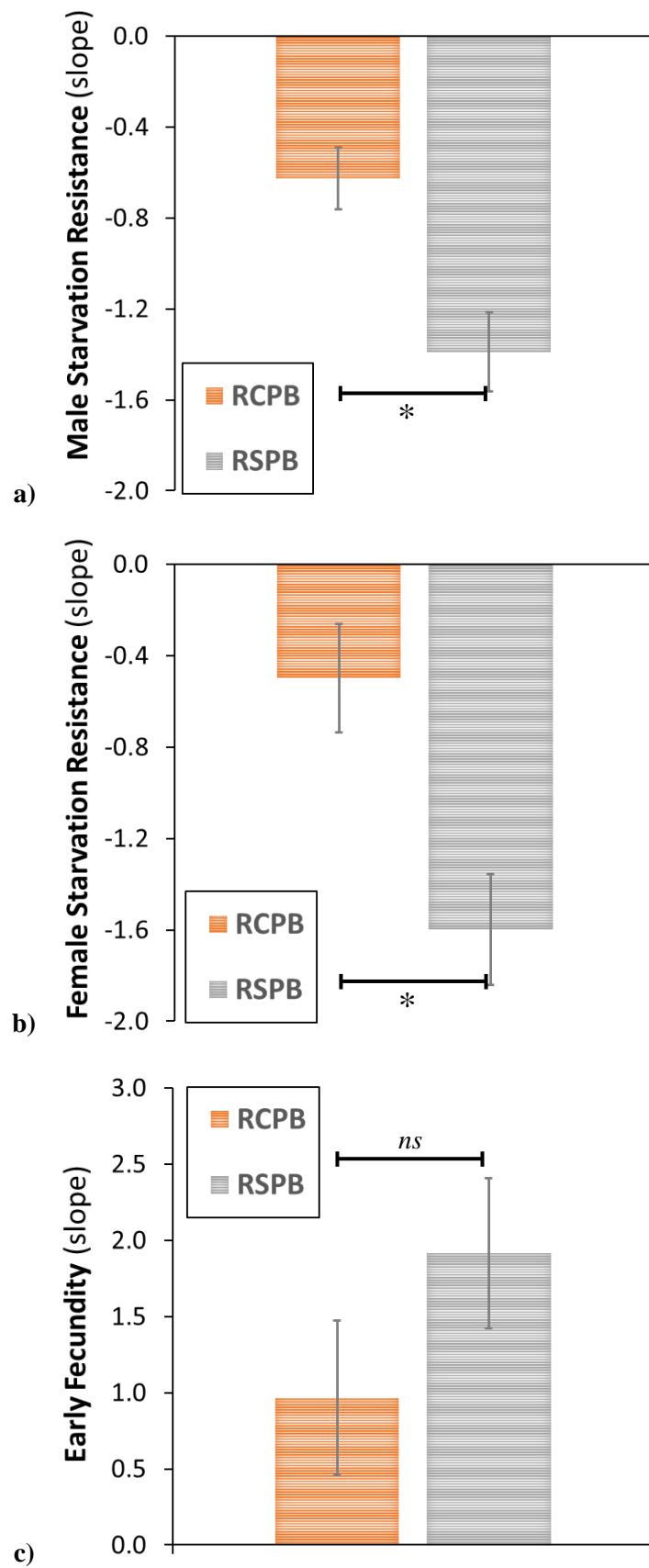
Figure 4.7. Evolutionary rates of RCPB and RSPB for **a)** male, **b)** female starvation resistance, and **c)** early fecundity. Average slopes are shown and error bars denote standard error of mean p≤0.05 is marked with * and p>0.05 with *ns*.

DISCUSSION

Our findings show that Mendelian populations kept in a sustained bottleneck reproduce a general response of reversion to the ancestor, B. Several patterns, trait and/or population-specific were found: (1) a rapid and linear convergence to ancestral values (RCPB male starvation resistance and early fecundity); (2) a rapid decline with partial convergence (female starvation resistance of all populations), and (3) a response that significantly overcame the ancestral state (early fecundity of RSPB populations). We will further analyze these patterns and provide plausible explanations below.

Evolution under reverse selection I: initial divergence and further convergence

Extensive research on experimental evolution of Mendelian populations showed that contrasting selection regimes rapidly cause phenotypic divergence and that populations under the same selection regime quickly converge on common phenotypes (see Garland & Rose 2009; Burke *et al.* 2016). After 15 generations of forward selection, the small populations RSPB and their non-selected counterparts, RCPB, were expected to diverge from the B-ancestor as a consequence of direct response to selection and/or due to the postponed reproduction (see results on SPB and CPB lines in *Chapter Two* of this thesis). This initial differentiation was a *conditio sine qua non* to start the reverse evolution experiment. The divergence expectation was met in both RCPB and RSPB experimental stocks, with higher starvation resistance and lower fecundity (**Table 2.1**, **Figure 4.2**).

In the large-scale reverse evolution experiments by Teotónio & Rose (2000) outbred populations with different past histories of selection, reversion of starvation resistance was clear but contingent to the stock's previous evolutionary history: in some lines starvation resistance converged to the ancestral state but in others reversion stalled (after 12-20 generations). In a different experiment, where selection for starvation resistance was relaxed for 20 generations, a strong decline in the mean trait value was also shown (Passananti *et al.* 2004b). Our results seem to follow this trend of decline of starvation resistance during reverse selection, contingent to the populations' selection history (**Table 2.2**, **Figure 4.2**).

Evolution under reverse selection II: starvation resistance

By generation 21, RCPB flies were less starvation resistant than at the start, but complete reversion to the ancestral state was found only in males (**Table 2.2**). Interestingly,

when analyzing their evolutionary trajectories, the downward trend was not statistically significant, neither in males nor females (**Figure 4.4a,b**; **Table 4.4**). The lack of statistical significance can be explained by the high heterogeneity seen in the rate of the individual population response within each B_i ancestor; this interpopulation variation is most likely due to the diverging effect of genetic drift on the response of the bottlenecked populations. RSPB, the previously forward-selected stock, after 21 generations of reverse selection, showed a significant decline in both male and female starvation resistances, however not reaching full convergence to the ancestor (**Figure 4.5a,b**; **Table 4.4**). This significant linear decrease of the mean character value was confirmed by the RSPB evolutionary trajectories. One possible explanation for the incomplete reversion is that our experimental populations were not given enough time to allow reverse evolution to conclude the process of convergence. The lack of complete reversion to the ancestral state of starvation resistance was previously found in an experiment with over 100 generations of reverse selection (Service *et al.* 1988; Graves *et al.* 1992). Later, a reverse experiment in independent lines selected for starvation resistance (in the same lab), once again revealed a pattern of incomplete reversion with stalling after 12 generations (Teotónio & Rose 2000). On the other hand, parallel selection experiments sustained for more than 100 generations from the Rose laboratory are revealing a greater tendency to convergence (Burke *et al.* 2016; Graves *et al.* 2017), even in populations which failed to converge with 20-100 generations of reverse selection. Even though these later studies do not involve starvation resistance selection, they do show that time may be essential for convergence to occur.

Unfortunately, we were not able to further investigate the genetic mechanisms and find out the causes of partial convergence for starvation resistance in our stocks. It would be very interesting to determine whether (1) the sustained bottleneck these lines had depleted genetic variation, (2) epistasis could have played a role on forestalling reverse evolution, or (3) genotype-by-environment interaction was impeding further return to ancestral state. Given that we did not find an increased response of hybrids in forward-selected small populations (*Chapter Three* of this thesis), we are tempted to say that the lack of genetic variation due to small population size will not be implicated in the partial reversion.

Evolution under reverse selection III: fecundity

Teotónio & Rose's (2000) reverse-selection results for early fecundity showed generalized convergence to ancestral levels. Our data partially corroborate the previous results,

with rapid and full convergence to control levels in RCPB (**Table 4.2**), confirmed by the stock's evolutionary trajectory (**Figure 4.4c, Table 4.4**). The pattern of increased fecundity with antiparallel decreased starvation resistance is easily expected due to (1) the character's direct relationship with fitness and (2) the extensively reported antagonist pleiotropy between starvation resistance and early fecundity (Service & Rose 1985; Rose *et al.* 1992; Leroi *et al.* 1994a,b; Chippindale *et al.* 1996). Surprisingly, the RSPB response to reverse selection not only equaled the fecundity of the B-controls, but even significantly overcame it (**Table 4.2**), by means of a strongly ascending evolutionary trajectory (**Figure 4.5c**, **Table 4.4**). One possible explanation is that genetic architectural changes undergone during their past evolutionary history, previously inaccessible to the ancestor, pushed the populations to a novel adaptive peak – Wright's *mass selection under changing conditions* (**Figure 4.8**; Wright 1977; Lenski 1988b; Teotónio & Rose 2001). Another explanation lies on the environments at which the populations evolve. It is possible that, during reverse selection, the experimental lines were not subjected to the *exact* same environment as the ancestor. If, in turn, these differences are closer to the assay environment, the experimental populations may be at an advantage, like Leroi *et al.* (1994b) found in one of their experiments. The maintenance regime of the RSPBs involved a 24h egg-laying, whereas the B flies were given two hours only. Early fecundity was, indeed, assessed in conditions more similar to the RSPB lines than the ancestor, with longer egg-laying periods giving them an advantage comparatively to the ancestral Bs (Teotónio *et al.* 2002). Finally, fecundity could also be trading-off with other life-history characters and the visible benefit in fecundity is being achieved at the cost of other unseen, untested traits (given that these flies are still significantly more resistant than the ancestor). The interplay of different trade-offs (*e.g.* fecundity with starvation resistance, egg-to-adult viability, or developmental rate) may implicate non-linear evolutionary patterns. In fact, it is possible that later in time these "super flies" might settle down to fecundity levels comparable to those of the B flies. A combination of these non-exclusive hypotheses can account for the "paradoxal" RSPB *super flies*.

Evolution under reverse selection – prior evolutionary history contingency

The evolutionary rates at which populations respond to reverse selection can be trait-specific: fitness-related traits are expected to converge faster and more consistently than characters more loosely associated with fitness (Travisano *et al.* 1995; Teotónio & Rose 2000; Teotónio *et al.* 2002; Joshi *et al.* 2003; but see Fragata *et al.* 2014b). Thus, a strong history

signature might be harder to erase in morphological traits, for example, compared to life history characters that underlie fitness.

Our experimental design allowed us to test for the effect of previous evolutionary history on the process and outcome of reverse evolution, by comparing the trajectories of one group of populations with a previous history of strong forward selection for starvation resistance (RSPB) with another group which was never subjected to such strong starvation selection (RCPB). These lines were initially very distinct in terms of starvation resistance (**Figure 4.6a,b**; **Table 4.5**); as generations of reverse selection went by they became less differentiated but did not reach the same character state (**Table 4.6**). Their evolutionary trajectories were similar in direction but not in rate, with the RSPB populations showing a much higher rate of convergence than the RCPB (**Figure 4.7**). Therefore, the historical differentiation for starvation resistance was smoothed by reverse evolution but note entirely eliminated. We would expect that for fecundity, a more fitness-related trait, the sign of history might not be seen. But our data on fecundity showed no differentiation between these populations to start with (**Figure 4.6c**; **Table 4.5**), which was of no use in this matter.
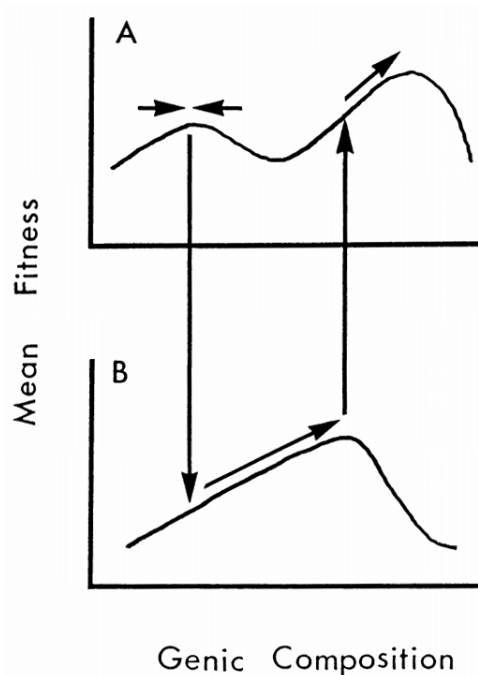
Figure 4.8. Schematics of a shift between two adaptive peaks precipitated by a transient environmental change. In environment A, selection pushed a population to a local adaptive peak, but opposes a shift to another adaptive peak. In environment B, the adaptive topography has changed, causing selection to favor a new genetic composition. Although the environment subsequently reverts to its former state, the population is pushed to a novel adaptive peak. © Lenski (1988b)

Final remarks

The history of a population is extremely important for its subsequent evolution. Namely, in a scenario of reverse colonization, *i.e.* migration back to the prior environment, will populations return to the ancestral state? Here we aimed to understand the consequences of recent divergent selection history in the outcome of life-history reverse evolution. Previous research in the lab has shown clear reversal of fitness-traits when N_e is moderately large (*e.g.* Service *et al.* 1988; Graves *et al.* 1992; Teotónio & Rose 2000; Passananti et al. 2004b). The scientific question addressed here was whether reverse evolution is impeded (or not) by small population sizes. Our major findings show that experimental populations kept in a sustained bottleneck still exhibit reverse evolution albeit with features contingent on character and/or population. Thus, we found (1) rapid and complete reversion, (2) fast response with partial convergence, and (3) steep convergence with a superior outcome to that of the ancestor, three of the possibilities outlined in Teotónio & Rose (2001). Furthermore, we found the signature of evolutionary history to be strong with respect to the *tempo* of reverse evolution.

Chapter Five.

Age-Specific Adaptation to Novel Diets

ABSTRACT

Hamilton's forces of natural selection provide scaling or weighting factors for the impact of selection on each age-specific component of a population's life history. This weighting is heavy at early ages, and then it falls with time, as the force of natural selection declines with chronological age. Applying Hamiltonian reasoning to laboratory experiments conducted in model species raises questions of genotype-by-environment interaction. Therefore, there is large potential for artifacts and confounds that arise from Hamiltonian waves of age-specific adaptation to novel laboratory environments, which can compromise the validity of experiments that use such novel environments. It is, thus, essential to know whether the Hamiltonian waves of age-dependent adaptation to novel environments in fact occur in well-defined laboratory experiments. In this chapter we present the first experimental test of the evolutionary effect of dietary change on the age-dependent adaptation of *Drosophila melanogaster* populations that have been exposed to a specific novel type of food for more than 800 generations. Our results show that surprisingly small changes in diet can have significant effects both on overall longevity and on age-specific mortality rates. Although we could not determine whether adaptation to a novel diet has proceeded to a greater extent at earlier ages compared with later ages, there is some evidence that the longer the flies are given novel diets, the more their mortality rates are increased.

RESUMO

As forças de Hamilton da selecção natural fornecem factores de escala ou ponderação do impacto da selecção em componentes específicos de cada idade na história de vida de uma população. Esta ponderação é maior em idades precoces e decai progressivamente com o tempo, devido à diminuição da força da selecção natural com a idade cronológica. A aplicação da teoria Hamiltoniana a experiências laboratoriais em organismos-modelo levanta problemas de interacção genótipo-ambiente. Existe um grande potencial para artefactos que surgem a partir das ondas Hamiltonianas de adaptação específica da idade para ambientes laboratoriais novos, o que pode comprometer a validade dos ensaios que utilizam tal novidade ambiental. É essencial saber se estas ondas de adaptação dependente da idade, de facto, ocorrem em experiências laboratoriais bem definidas. Neste capítulo apresentamos o primeiro teste experimental do efeito evolutivo da alteração da dieta na adaptação dependente da idade. Para tal, usaram-se populações de *Drosophila melanogaster* que tinham sido expostas a uma determinada dieta por mais de 800 gerações. Os nossos resultados mostram que pequenas mudanças na dieta das populações podem ter efeitos significativos tanto na longevidade média como nas taxas de mortalidade específicas de cada idade. Embora não se tenha podido determinar se houve maior adaptação à nova dieta em idades mais precoces em comparação com idades mais avançadas, os dados sugerem que quanto mais tempo as coortes são expostas às novas dietas, maior é o aumento das suas taxas de mortalidade.

INTRODUCTION

In the last decade we have been struck by a paradox that arises from an ostensible conflict between the biology of human aging and the findings of experimental evolution. Several anthropologists and physicians have drawn attention to the increased incidence of age-associated disorders, like type II diabetes and cardiovascular disease, in populations which have adopted the *agricultural* diet (and lifestyle) within historical times. These authors have claimed that the adoption of a *Paleolithic* or *hunter-gatherer* diet might alleviate many aging diseases, particularly heart conditions (Eaton & Konner 1985; O'Keefe & Cordain 2004; Frassetto *et al.* 2009; Jönsson *et al.* 2009; Lindeberg 2010; Ryberg *et al.* 2013). The rationale behind this idea is that the human population is not sufficiently adapted to the agricultural conditions, dominated by grains and dairy products, but it is well-adapted to the hunter-gatherer diet, a more ancient nutritional environment at which humans were exposed for millions of years. Consequently, the adoption of the agricultural diet is detrimental to human health and some medical anthropologists propose the wholesale adoption of *hunter-gatherer* regimes to fight age-associated diseases (*e.g.* Lindeberg 2010; Ryberg *et al.* 2013).

This statement is apparently in direct contradiction to solid experimental evolutionary research on what is called in our field *evolutionary domestication* (*vid.* Simões *et al.* 2007, 2009). Domestication is an extremely important topic in evolution and may be considered the most ancient evolutionary experiment carried out by humans. Its history dates back about 14,000 years from present, at least in the Middle East and Asia, starting with the domestication of the dog and several livestock species (Mignon-Grasteau *et al.* 2005). Since then, multiple plant species have undergone domestication (**Figure 5.1**). Traditionally, the term *domestication* refers to the genetic changes undergone by our commensal species, from dogs to agricultural animals, from grains to legumes, sometimes with an additional connotation related to behavioral change, especially reduction in *wildness* (Soanes 2003). In evolutionary terms, domestication can be defined as *the population genetic change arising from its transition from nature to deliberate human cultivation* (Simões *et al.* 2007).

Extensive adaptation has been reported in less than 60 generations of experimental evolutionary domestication (Simões *et al.* 2007, 2009). This seemingly implies that the hundreds of generations that most human populations have had to adapt to agricultural conditions should have been more than enough to a full adaptation state to this novel environment (*vid.* Zuk 2013). This apparent contradiction can be mitigated if we bring together

the concepts of evolutionary domestication and *Hamiltonian age-dependent adaptation* to novel environments (Mueller *et al.* 2011, Ch. 11).
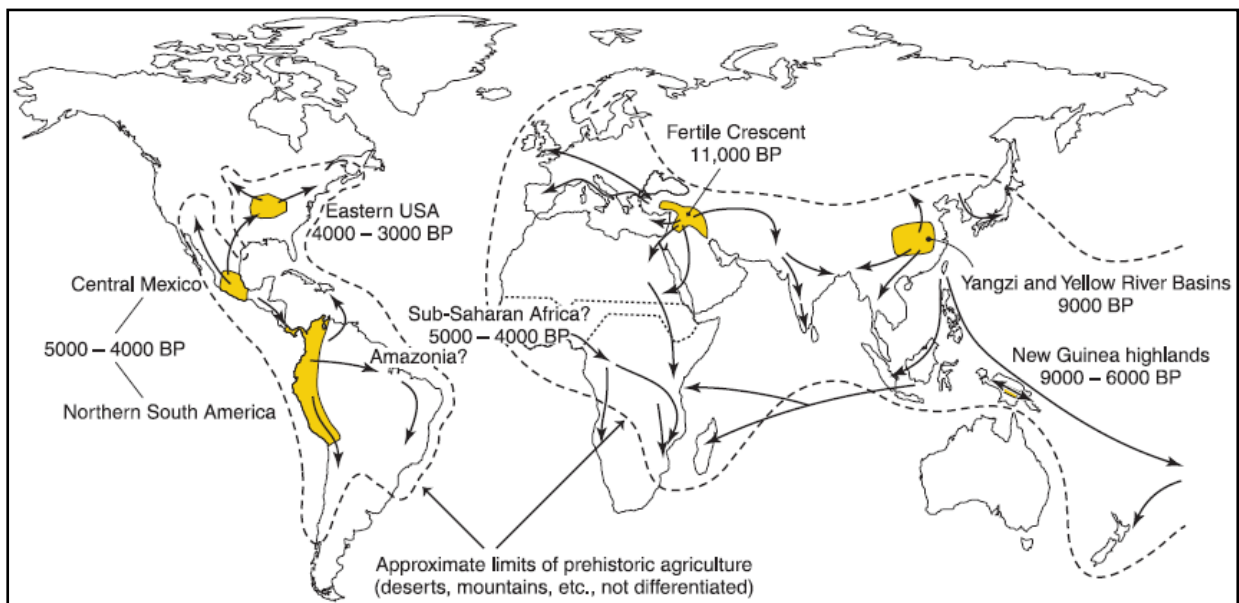
Figure 5.1. Archaeological map of agricultural homelands and the spread of Neolithic/Formative cultures, with approximate radiocarbon dating for the onset of agriculture (Diamond & Bellwood 2003).

Hamilton's (1966) forces of natural selection provide scaling or weighting factors for the impact of selection on each age-specific component of a population's life history. This weighting is heavy at early ages, and then it falls with time, as the force of natural selection declines with chronological age (**Figure 5.2**). This theory can be applied to scenarios in which a population is transferred to a qualitatively novel environment, such as the human transition from hunting-and-gathering to mainly agricultural subsistence. The key point is that the age-specific weighting of the force of natural selection leads to the corollary that selection for domestication will act with full-force *only* at early ages. In this scenario, the evolutionary expectations are that (1) young people from populations with longer agriculture exposure will be well adapted to agricultural diets and (2) older people from the same populations will be less adapted to agricultural than hunter gatherer diets, as the age-specific evolutionary domestication in response to agriculture will progressively decline with age. In effect, individuals from ancestrally agricultural populations will undergo, at later ages, a partial physiological reversion, a kind of *evolutionary time travel,* to a condition of relatively better adaptation to the hunter-gatherer, compared to the agricultural diet.
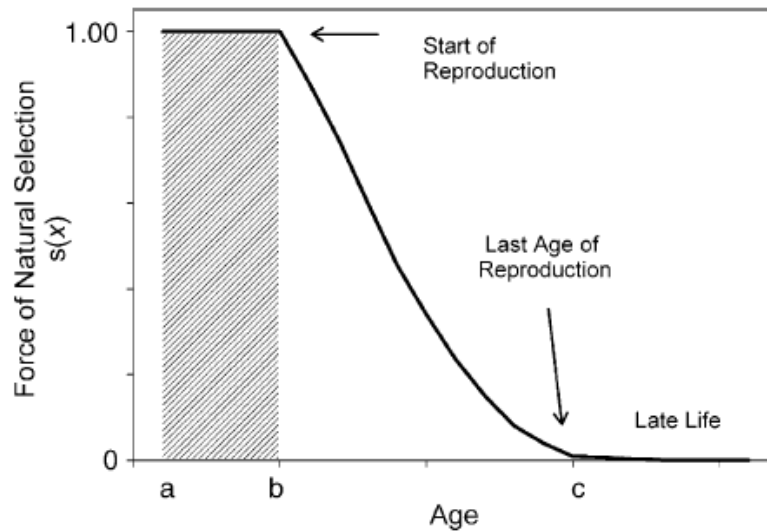
Figure 5.2. The age-specific forces of natural selection acting on mortality. Natural selection is intense at early ages, until the first age of reproduction (from ages *a* to *b*), and then begins to decline rapidly until the last age of reproduction, after which it converges on, and remains at zero. The onset of late-life *plateaus* occurs sometime after age *c*. © Rauser *et al.* 2006.

Thus, far from favoring the universal adoption of a hunter-gatherer regime, this evolutionary reasoning only supports the adoption of such diets later in adult life, perhaps from middle-age onward, at least among individuals with agricultural ancestry. This hypothesis is conveyed graphically in **Figure 5.3**.
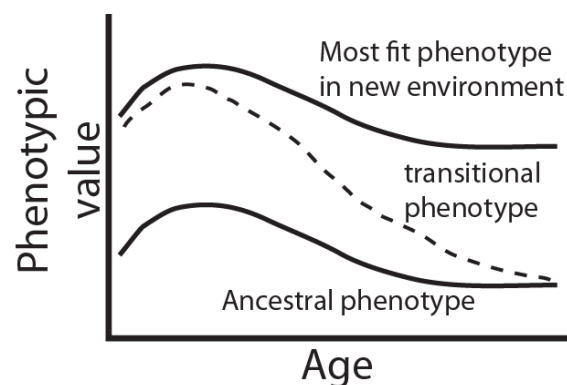
Figure 5.3. Age-specific adaptation to a novel environment. If the most fit phenotype changes in a new environment, and there is sufficient genetic variation, there should be an evolutionary response to the new environment with the population gradually approaching the most fit phenotype in that novel environment. However, the dynamics of this process will depend on the strength of selection. So, we expect the greatest and fastest evolutionary changes to occur at ages where selection is strongest, *i.e.* the early ages. From Mueller *et al.* (2011).

This minor application of Hamiltonian reasoning to one species, however, raises more general questions concerning how experiments on aging are generally conducted on model organisms. These questions revolve around genotype-by-environment (*GxE*) interaction and the amount of laboratory evolution (*i.e.* domestication) that model organisms have undergone

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prior to their use in gerontological experiments. This is a well-known issue in evolutionary biology, where it has been referred to as the *evolutionary no-man's-land* problem (*vid.* Matos *et al.* 2000b; Simões *et al.* 2007). If a laboratory model organism is studied for its aging in an evolutionarily novel environment, there will be difficulties arising from the degree of laboratory-adaptation of the stock under use. **Figure 5.4** illustrates the differences in the evolutionary trajectories between short and long-term studies in *Drosophila subobscura* done by the Matos lab.

Additionally, there will be issues of age-dependent patterns of maladaptation. Age-dependent maladaptation may further increase the problems of *GxE* interaction, which are in any case well-known to trouble experimentation with model organisms in the laboratory (*e.g.* **Figure 5.5**; Leroi *et al.* 1994b). Thus, the potential for artifacts and confounds that arise from Hamiltonian waves of age-specific adaptation to novel laboratory environments will be substantial, and can compromise the validity of experiments that use such novel environments.

This inference is a direct extension of Hamiltonian theory based on the declining forces of natural selection. And, as with Rose and Mueller's previous experimental work testing Hamilton's theory (*cf.* Rose 1991; Mueller *et al.* 2011), what is now needed is to test whether Hamiltonian waves of age-dependent adaptation to novel environments, in fact, occur in well-defined laboratory experiments. Fortunately, there were experimentally evolved populations at University of California, Irvine which allowed an initial, though admittedly crude, test of the Hamiltonian wave hypothesis. We thus present the first experimental test of the effect of dietary change on the age-dependent adaptation of *Drosophila melanogaster* populations that have been exposed to a specific type of food for more than 800 generations. As far as we know, this was the first experimental test of the Hamilton hypothesis, a test which opened doors into a novel and important line of research for evolutionary biology.
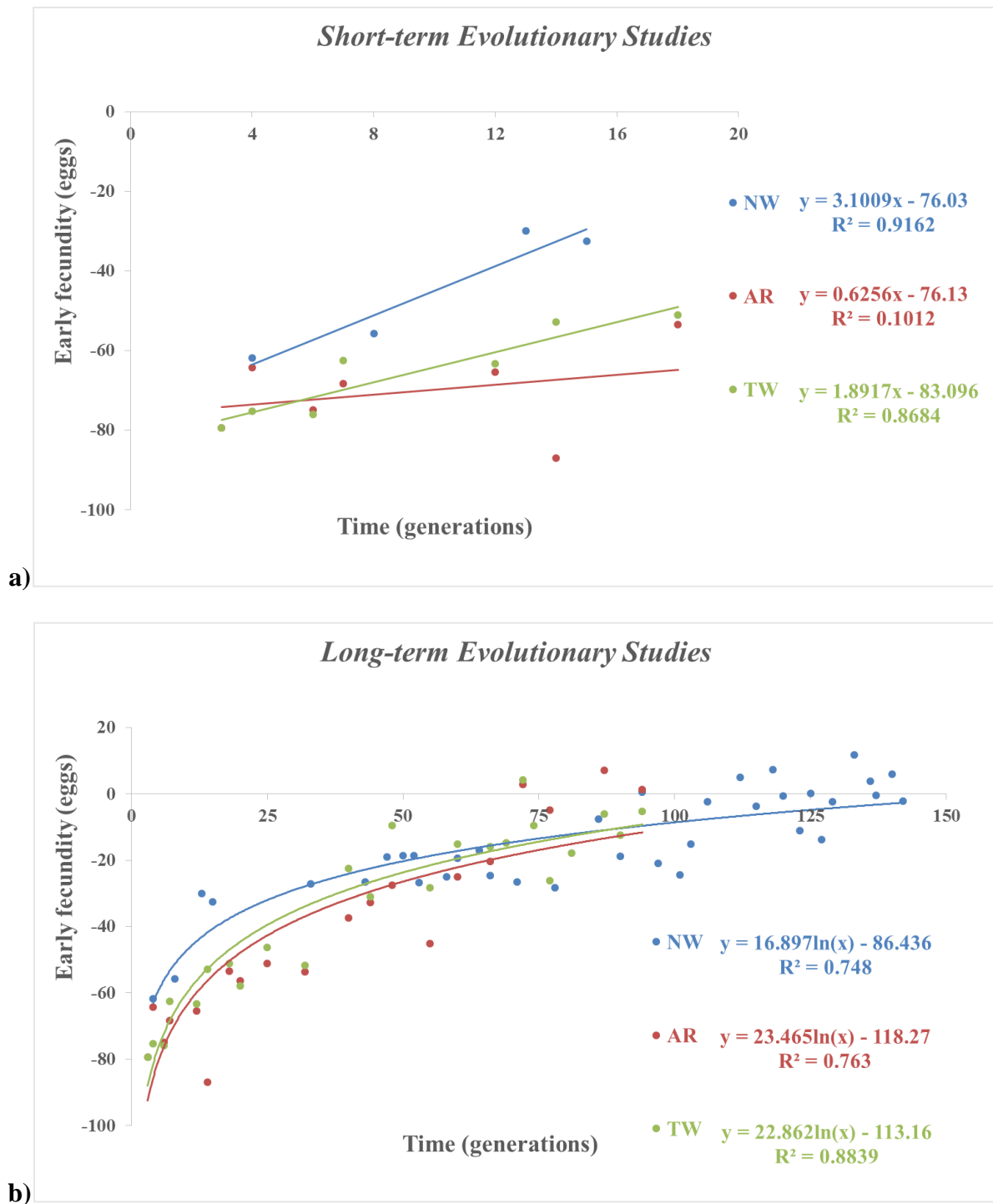
Figure 5.4. Evolutionary trajectories for early fecundity in **(a)** short and **(b)** long-term studies of evolutionary domestication in *Drosophila subobscura*: In the short-term analysis of the adaptive dynamics the results show a positive linear trend, whereas the long-term displays a logarithmic tendency. These results are congruent with an initial high selective pressure and rapid evolutionary rate of domestication, followed by its deceleration (Matos *et al.*, unpublished data).
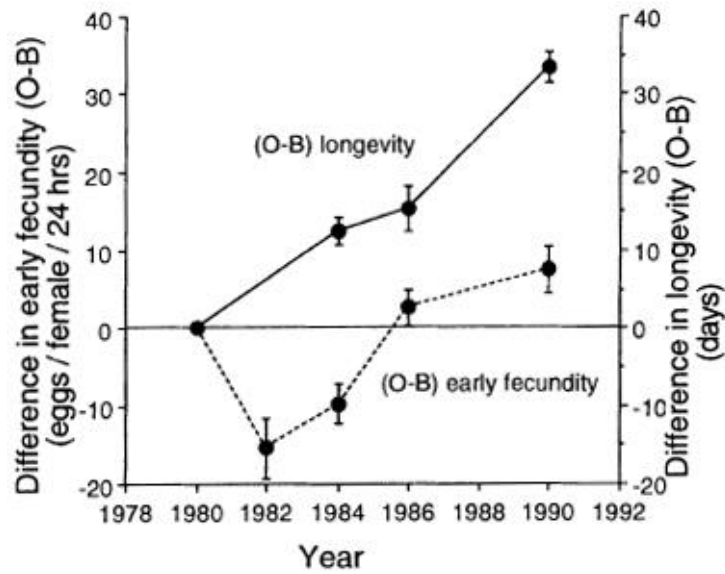
Figure 5.5. Paradoxical evolution of relative B and O fecundity and longevity (bars denote standard errors of means over five pairs of replicate populations). The solid line represents the progress of the difference (O-B) mean longevity: as the O's, which are selected for late-life reproductive success, increase in longevity, the difference becomes increasingly positive. The broken line represents the progress of the difference (O-B) mean early-life fecundity (number of eggs laid by a female in a 24-h period at day 4 after emergence). Initially, the difference is negative, as the O's decline, which is an apparent trade-off. Later the direction of evolution of relative fecundity reverses itself, such that by 1992 the O's have a greater early fecundity than the B's. This differential expression of the trade-off was shown to be due to (1) the O's started to be maintained in a different environment (*e.g.* density and general handling), and (2) the assay conditions were more similar to the O-type environment (Leroi *et al.* 1994b).

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MATERIALS AND METHODS

Experimental populations

The experiments reported in this chapter involved a five-fold replicated stock of outbred flies of *Drosophila melanogaster*: the ACO_{1-5} populations, derived from the O stock of Rose (1984a) as described in Chippindale *et al.* (1997). These populations have a nine-day-long life cycle and had been adapted to banana-molasses food for more than 800 generations at the time of the experiment.

Food preparation

Regular banana-molasses food was prepared with agar, nipagin, banana, corn syrup, dry active yeast, and barley malt. The experimental foods (orange and avocado) were obtained using the same recipe, in which orange or avocado are substituted for banana, using the same wet weight and no additional syrup provided.

Banana-avocado-orange mortality assay

All populations were reared in vials with regular banana food and were given 9 days to develop. The 30 cohorts were then dumped into transparent acrylic cages (2 x 1000 flies, per population, per treatment) and given the respective diet treatment: banana, orange or avocado (**Table 5.1**). Every 24 hours each cage was given fresh food, assessed for mortality, and individuals were sexed at death. Cohort size was then calculated from complete death records. The cages were kept at room temperature (24ºC ± 1ºC) and their locations were randomized to reduce variation in light distribution.

Table 5.1. Experimental design for the banana-avocado-orange mortality assay. Two cages (α and β) from each population were assessed in each of the three environments (banana, orange and avocado).

Diet / Population	Banana	Orange	Avocado
ACO_i (i = 1, 2, 3, 4, 5)	$ACO_i\ \alpha$ Ba	$ACO_i\ \alpha$ Or	$ACO_i\ \alpha$ Av
	$ACO_i\ \beta$ Ba	$ACO_i\ \beta$ Or	$ACO_i\ \beta$ Av

Banana-orange switch mortality assay

This experiment involved cohorts from the ACO_1 population only. A combination of eight different diet conditions were tested with four cages in each condition (α, β, γ and δ), giving a total of 32 cohorts. Half of the flies were reared in control banana-molasses diet, while the other half were given the experimental orange diet. After 9 days of development, the flies

were dumped into cages with about 1000 individuals per cage and fed daily the same diet in which they were reared. On day 14 of the trial, 8 of the 32 cohorts had their diets switched from banana to orange or vice-versa. The same switch occurred for 8 different cohorts on day 21 and day 28 (**Table 5.2**). After each diet switch, the cohorts were fed that same diet for the remaining of their life cycle. The rest of the protocol was the same as for the banana-orange-avocado mortality assay.

Table 5.2. Experimental design for the banana-orange switch mortality assay. Four cohorts (α, β, γ and δ) from each of the eight treatments were followed. Day (from egg) at which the diet switches started are highlighted in bold.

Day \ Treatment	0-14	15-21	22-28	28-death	Cohorts
BaBaBaBa (1)	Banana	Banana	Banana	Banana	*BaBaBaBa* α, β, γ and δ
BaBaBaOr (2)	Banana	Banana	Banana	**Orange**	*BaBaBaOr* α, β, γ and δ
BaBaOrOr (3)	Banana	Banana	**Orange**	Orange	*BaBaOrOr* α, β, γ and δ
BaOrOrOr (4)	Banana	**Orange**	Orange	Orange	*BaOrOrOr* α, β, γ and δ
OrOrOrOr (5)	Orange	Orange	Orange	Orange	*OrOrOrOr* α, β, γ and δ
OrOrOrBa (6)	Orange	Orange	Orange	**Banana**	*OrOrOrBa* α, β, γ and δ
OrOrBaBa (7)	Orange	Orange	**Banana**	Banana	*OrOrBaBa* α, β, γ and δ
OrBaBaBa (8)	Orange	**Banana**	Banana	Banana	*OrBaBaBa* α, β, γ and δ

Statistical data analysis

In all analyses, the normality and homoscedasticity of data were tested by Shapiro-Wilk (1965) and Brown-Forsythe (1974) tests, respectively. Tukey HSD *post-hoc* tests (1953) were done when appropriate. A significance value of 0.05 (α) was used to test all null hypotheses. The general linear model tests used to analyze mean longevity and age-specific mortality were done in *STATISTICA 13* (Dell 2015). Analyses of age-specific mortality patterns and Gompertz were done in *R* (R Core Team 2013).

Mean longevity statistical analysis for the banana-avocado-orange experiment

The effect of sex and its interaction with diet on mean longevity (average age-specific mortality standardized by the cohort size) was analyzed using the following linear mixed-effects model:

$$Y = \mu + D_i + S_j + Pop + D_i * S_j + D_i * Pop + S_j * Pop + D_i * S_j * Pop + \varepsilon \tag{5.1}$$

where Y is mean longevity, D the diet treatment (fixed factor) applied during the assay ($i =$ banana or $i =$ orange or $i =$ avocado), S the sex of the flies tested ($j =$ males or $j =$ females), and *Pop* the random replicate population.

The effect of diet and selection regime on mean longevity was also analyzed for males and females separately, using the following linear mixed-effects model:

$$Y = \mu + D_i + Pop + D_i * Pop + \varepsilon \tag{5.2}$$

where Y is mean longevity, D the diet treatment applied during the assay ($i =$ banana or $i =$ avocado or $i =$ orange), and *Pop* the replicate population.

Mean longevity statistical analysis for the banana-orange switch experiment

Similarly to the previous experiment, the effect of sex and its interaction with diet on mean longevity was analyzed using the following linear model:

$$Y = \mu + D_i + S_j + D_i * S_j + \varepsilon \tag{5.3}$$

where Y is mean longevity, D the diet treatment applied during the assay ($i =$ BaBaBaBa or $i =$ BaBaBaOr or $i =$ BaBaOrOr or $i =$ BaOrOrOr or $i =$ OrOrOrOr or $i =$ OrOrOrBa or $i =$ OrOrBaBa or $i =$ OrBaBaBa), and S the sex of the flies tested ($j =$ males or $j =$ females).

To analyze the effect of diet and selection regime on mean longevity of males and females (separately) the following linear model was used:

$$Y = \mu + D_i + \varepsilon \tag{5.4}$$

where Y is mean longevity and D the diet treatment as before.

Gompertzian analysis of banana-avocado-orange mortality rates and patterns

Mortality rates and patterns were analyzed by fitting the mortality assay data to a two-stage Gompertz equation (Gompertz 1825), using maximum-likelihood. Let d be the age at

which mortality rates become constant with age or the *break day*. At ages $x < d$, age-specific mortality rates are modeled by the continuous time Gompertz equation and set equal to $A\,e^{(\alpha x)}$, where A is the age-independent mortality rate, and α is the age-dependent parameter. For ages $x > d$, mortality rates are assumed to equal a constant value A_2 (independent of age and different from A).

This is a powerful methodology with which to analyze data from populations that are relatively long-lived and follow Gompertz mortality curves (as these experimental stocks), because (1) it does not assume constant mortality rates, (2) it does not force the data onto a mortality plateau, and (3) it yields more accurate and unbiased estimates of the Gompertz parameters, compared with techniques based on linear and non-linear regression models (*vid.* Mueller *et al.* 1995; Joshi *et al.* 1996a, Drapeau *et al.* 2000). The estimation of A and α from each combination of *population*treatment*sex* was used as data in a mixed-effects ANOVA, which included the following fixed factors: treatment (banana *vs.* orange *vs.* avocado) and sex (female *vs.* male). All these factors were crossed with the five replicate blocks. Additionally, for each *population*treatment*sex* combination, the coefficient of determination, R^2, was calculated, as an indication of the proportion of variation explained by the Gompertz model.

Hamiltonian analysis of mortality rates and patterns

For each combination of *treatment*sex* the data from α and β cages of all 5 populations (*banana-avocado-orange* assay) or from α, β, γ and δ cages (*banana-orange switch* assay) was combined and three-day survivorship intervals were computed. For each interval a new categorical variable was then created, defining the status of each one of the flies (0 = dead or 1 = alive). The counts of each interval were used in a chi-squared test to compare the orange and avocado diets (*banana-avocado-orange* assay) or the switch treatments with all-banana control (*banana-orange switch* assay). The mortality rates in each interval (*i.e.* the age-specific mortality), defined as the logarithm of the total number of deaths to surviving cohort of that age, were plotted and analyzed fitting linear and log-linear least-square curves. The best fitting model was chosen based on the highest proportion of explained variance and the adequate ANCOVA model was used to test the difference between the curves.

RESULTS

General linear model assumptions

Small deviations from normality were accepted, and homoscedasticity was verified by the Brown-Forsythe test. Our distribution tests showed that all populations were homoscedastic and generally normal (data not shown).

Mean longevity analysis of the banana-avocado-orange data

The diet change from banana to orange or avocado caused an overall decrease in mean longevity, an effect which was not significantly different between males and females, though there was a significant interaction between sex and diet treatment (see **Table 5.3** and **Figure 5.6**). The same diet effect was found when sexes were analyzed separately (**Table 5.4**). Furthermore, Tukey HSD *post-hoc* analysis, summarized in **Table 5.5**, showed that orange-fed males (~20.0 days of average adult longevity) were significantly shorter-lived than the avocado-fed males (~21.6 days), and these two had a significantly lower longevity than control males given banana (~27.2). In females, there was no significant difference between orange (~21.5 days) and avocado (22.0 days), which were significantly shorter-lived than the controls (25.5 days).

Table 5.3. Summary of the mixed-effects ANOVA used to analyze the effect of diet change and sex interactions on mean longevity. Data shows the F statistic and respective p-value for the factors *Diet*, *Sex*, and *Diet*Sex* interaction. Significant results are highlighted in bold.

Effect	*F statistic*	*p-value*
Diet	**59.105**	**0.000016**
Sex	0.158	0.711019
Diet*Sex	**16.145**	**0.001554**
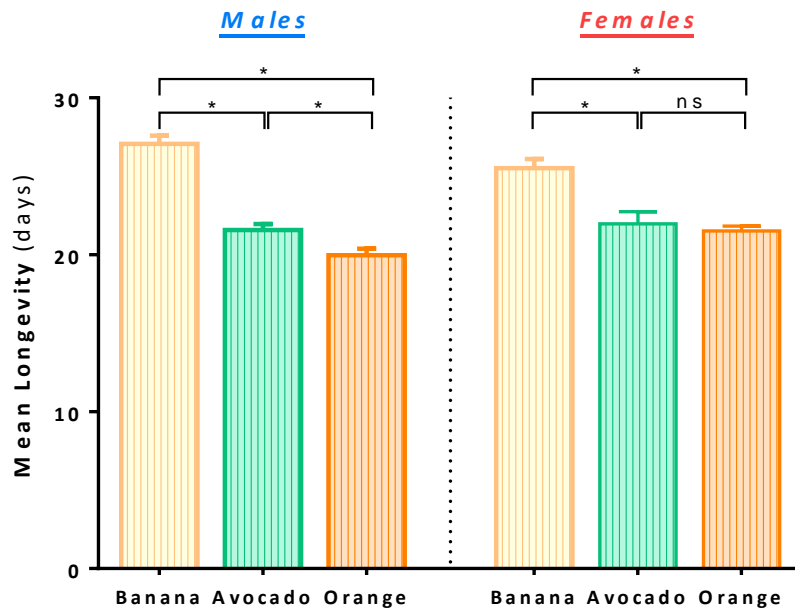
Figure 5.6. Mean longevity (in days as adult) of males and females in banana, avocado and orange. Average stock values are shown and error bars denote standard error of mean. p≤0.05 is marked with * and p>0.05 with *ns*.

Table 5.4. Summary of the mixed-effects ANOVAs used to analyze the effect of diet change on mean longevity of males and females separately. Data shows the F statistic and respective p-value for the factor *Diet*. Significant results are highlighted in bold.

Effect	Males		Females	
	F statistic	p-value	F statistic	p-value
Diet	**106.118**	**0.000002**	**19.97107**	**0.000775**

Table 5.5. Summary of the Tukey HSD test used to analyze the effect of diet change on mean longevity of males and females separately. Data shows p-values for *Diet* tested against the interaction *Diet*Population*. Significant results are highlighted in bold.

Diet	Males			Females		
	Banana	Avocado	Orange	Banana	Avocado	Orange
Banana	------------			------------		
Avocado	**0.000205**	------------		**0.002459**	------------	
Orange	**0.000201**	**0.033041**	------------	**0.001224**	0.793095	------------

Mean longevity analysis of the banana-orange switch data

Switching the food from banana to orange at different points in the cohorts' life cycle caused a general decrease in mean longevity, with a similar effect on males and females (**Tables 5.6**-**7** and **Figure 5.7**). Overall, the longer the flies were subjected to orange, the shorter was their average life span, but the moment at which the food was switched did not influence the cohorts mean longevity (*e.g.* BaBaBaOr ≈ OrBaBaBa; see **Table 5.8** for the multiple comparison results). Furthermore, females lived significantly longer than males (♀=26.096, ♂=24.813, p<0.02) and, when analyzed separately, were found to be less sensitive to diet switching: the only diet switch which was significantly different from the others was *OrOrOrBa* (p<0.02; see **Table 5.9** for the multiple comparison of males and females separately).

Table 5.6. Summary of the mixed-effects ANOVA used to analyze the effect of diet switch and sex interactions on mean longevity. Data shows the F statistic and respective p-value for the factors *Diet*, *Sex*, and *Diet*Sex* interaction. Significant results are highlighted in bold.

Effect	*F statistic*	*p-value*
Diet	**8.22**	**0.000001**
Sex	**6.79**	**0.012190**
Diet*Sex	0.52	0.814638

Table 5.7. Summary of the mixed-effects ANOVAs used to analyze the effect of diet switch on mean longevity of males and females separately. Data shows the F statistic and respective p-value for the factor *Diet*. Significant results are highlighted in bold.

Effect	*Males*		*Females*	
	F statistic	*p-value*	*F statistic*	*p-value*
Diet	**7.490**	**0.000082**	**2.487**	**0.045234**
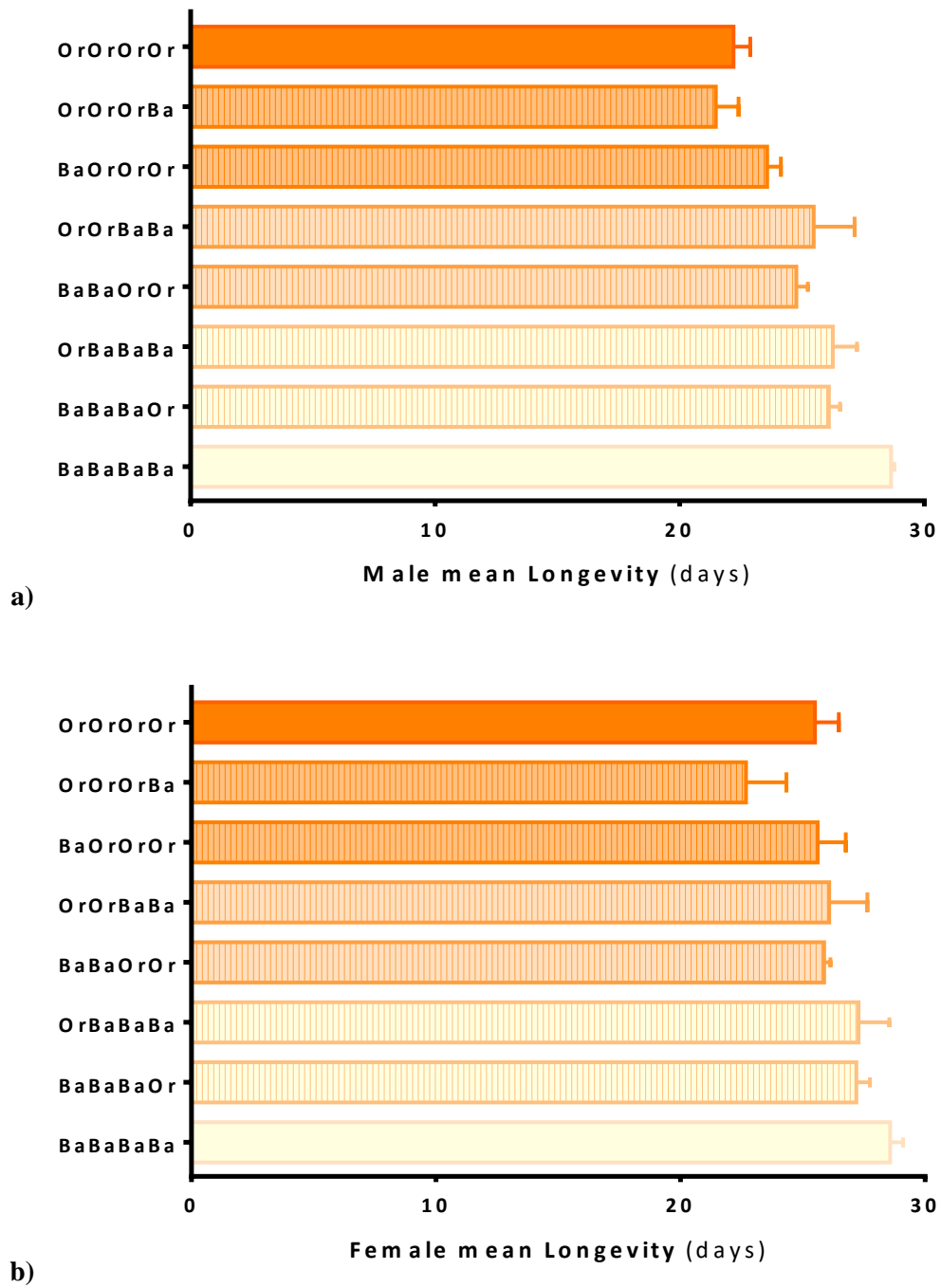
Figure 5.7. Mean longevity (in days as adult) of **a)** males and **b)** females from different diet switch treatments. Average cohort values are shown and error bars denote standard error of mean.

Table 5.8. Summary of the Tukey HSD tests used to analyze the effect of diet switch on mean longevity (males and females together). Data shows p-values for *Diet* tested against the difference between cohorts and least-square means are presented in brackets. Significant results are highlighted in bold.

Diet	*BaBaBaBa* (28.607)	*BaBaBaOr* (26.644)	*OrBaBaBa* (26.771)	*BaBaOrOr* (25.322)	*OrOrBaBa* (25.779)	*BaOrOrOr* (24.591)	*OrOrOrBa* (22.076)	*OrOrOrOr* (23.847)
BaBaBaBa	------------							
BaBaBaOr	0.498127	------------						
OrBaBaBa	0.581878	1.000000	------------					
BaBaOrOr	**0.032834**	0.878023	0.819068	------------				
OrOrBaBa	0.102080	0.986754	0.971314	0.999777	------------			
BaOrOrOr	**0.004049**	0.439981	0.363356	0.995208	0.926462	------------		
OrOrOrBa	**0.000134**	**0.000790**	**0.000555**	**0.036418**	**0.010249**	0.199311	------------	
OrOrOrOr	**0.000470**	0.109345	0.081507	0.805268	0.517980	0.994626	0.625441	------------

Table 5.9. Summary of the Tukey HSD tests used to analyze the effect of diet switch on mean longevity (males and females separately). Data shows p-values for *Diet* tested against the difference between cohorts and least-square means are presented in brackets. The upper triangle refers to males and the lower triangle to females. Significant results are highlighted in bold.

Males / *Females*	*BaBaBaBa* (28.642)	*BaBaBaOr* (26.100)	*OrBaBaBa* (26.261)	*BaBaOrOr* (24.768)	*OrOrBaBa* (25.482)	*BaOrOrOr* (23.572)	*OrOrOrBa* (21.478)	*OrOrOrOr* (22.199)
BaBaBaBa (28.571)	------------	0.442068	0.521514	0.063298	0.199863	**0.006740**	**0.000226**	**0.000534**
BaBaBaOr (27.188)	0.984356	------------	1.000000	0.950389	0.999502	0.448592	**0.016023**	0.060266
OrBaBaBa (27.281)	0.989521	1.000000	------------	0.913546	0.997774	0.373954	**0.011771**	**0.045363**
BaBaOrOr (25.875)	0.667507	0.988444	0.982904	------------	0.998729	0.971808	0.164634	0.428635
OrOrBaBa (26.076)	0.743791	0.995721	0.993039	1.000000	------------	0.757915	0.050338	0.166445
BaOrOrOr (25.610)	0.561886	0.967710	0.956429	1.000000	0.999986	------------	0.668351	0.942084
OrOrOrBa (22.675)	**0.017225**	0.116850	0.103923	0.467961	0.393785	0.572203	------------	0.998644
OrOrOrOr (25.495)	0.516132	0.953292	0.938950	0.999997	0.999938	1.000000	0.618333	------------

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Data analysis of life-long diet regimes based on Gompertz model fitting

The Gompertz model used to analyze the mortality patterns in the *banana-avocado-orange* experiment revealed significant differences between the diets to which the flies were exposed during adulthood. The age-independent mortality rate (*A*) was significantly larger in *orange* compared with the other treatments, which were not shown to differ significantly from each other. In terms of the age-dependent mortality parameter (α), *banana* and *orange* estimates were not significantly different from each other but significantly smaller than that for *avocado*. **Table 5.10** shows the estimated Gompertz parameters (*A* and α) for the *banana-avocado-orange* comparisons, in which there was no diet switching throughout the adult phase, and **Table 5.11** presents the results of their statistical comparison. Although females had a significantly smaller *A* ($p<0.01$) and a significantly larger α ($p<0.002$), no *sex*diet* interactions were found ($p>0.1$). **Figure 5.8** illustrates the log-transformed age-specific mortality rates observed for males and females in the banana-avocado-orange experiment.

The two-stage Gompertz analysis could not be done, because the best fit *break day* for avocado and orange treatments was greater than the age at death of the oldest fly, *i.e.* after the last fly died. Thus, the flies treated with the novel diets lacked a distinctive break day, and the populations did not show a well-defined late-life plateau.

Table 5.10. Gompertz parameters for *banana*, *avocado* and *orange* diets, estimated from the non-linear mixed-effects model fitted by maximum likelihood, for: **a)** males and **b)** females.

a)

Par. ♂ \ Diet	*Banana*	*Avocado*	*Orange*
A	0.019	0.017	0.029
α	0.068	0.100	0.068

b)

Par. ♀ \ Diet	*Banana*	*Avocado*	*Orange*
A	0.012	0.010	0.022
α	0.084	0.116	0.084

Table 5.11. *p-values* from the non-linear mixed-effects model for all paired diet treatments for: **a)** the age-independent parameter, *A* and **b)** the age-dependent parameter, α. Significant results are highlighted in bold.

a)

Comp of A	Banana	Avocado	Orange
Banana	----------	0.493	**0.045**
Avocado	0.493	----------	**0.006**
Orange	**0.045**	**0.006**	----------

b)

Comp of α	Banana	Avocado	Orange
Banana	----------	**0.001**	0.999
Avocado	**0.001**	----------	**0.001**
Orange	0.999	**0.001**	----------
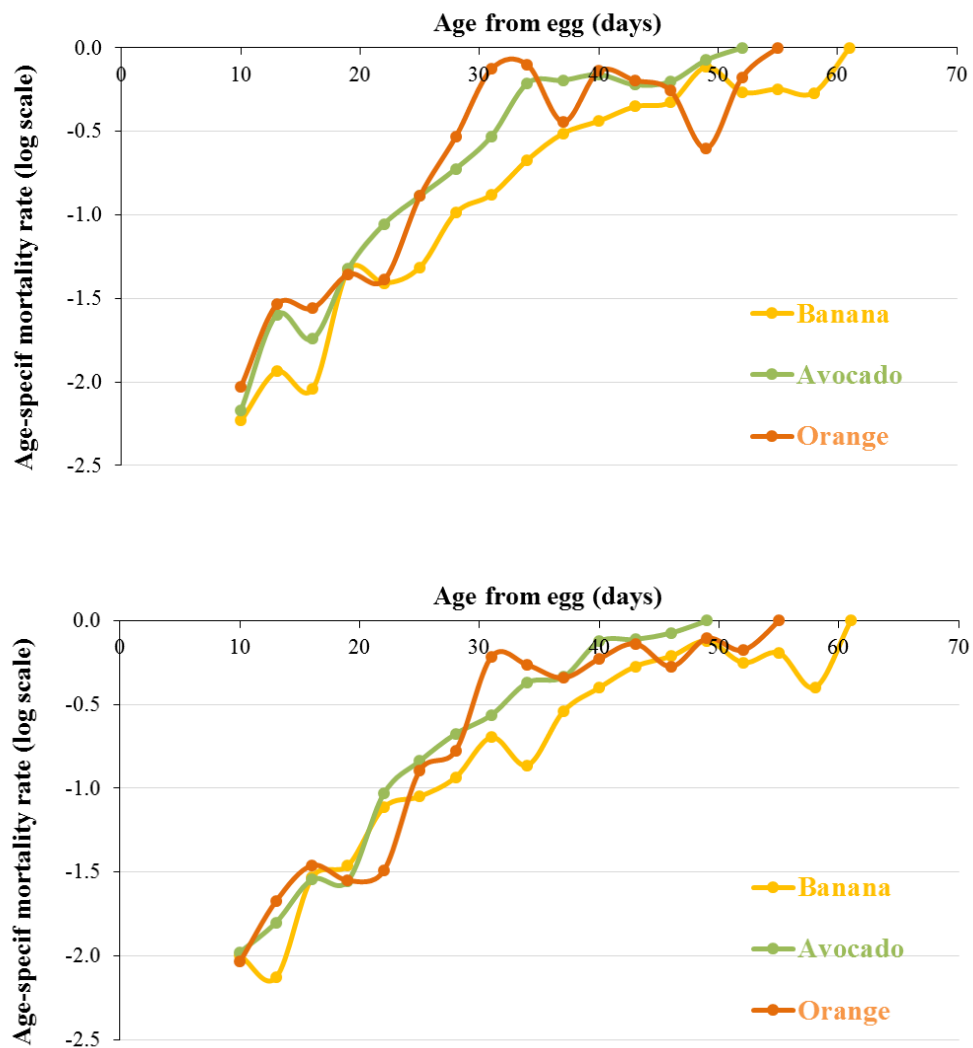
Figure 5.8. Age-specific mortality rates for **a)** males and **b)** females in banana, avocado, and orange food. Data shows the average log-transformed two-day age-specific mortality computed as the fraction of deaths over the remaining cohort.

Disclaimer on Gompertz analysis of diet switch experiment

Because of the nature of the experiment, the *banana-orange* switch data was not analyzed using the Gompertz modelling. Switching the diet during the cohorts' life cycle is expected to cause changes in their age-specific mortality rates that will most likely compromise the Gompertz goodness of fit.

Hamiltonian analysis of mortality rates for the banana-avocado-orange experiment

The analysis of age-specific mortality rates with life-long maintenance of specific dietary regimes (*banana-avocado-orange*) characteristically showed most significant differentiation at later adult ages, compared with early adult ages, for both males and females (**Figures 5.9** and **5.10**). There was no significant differentiation at the most advanced ages most likely due to the small numbers of surviving flies in all cohorts.

The age-specific mortality curves of each treatment were analyzed by fitting linear and log-linear models. The best fit, chosen based on the coefficient of determination (R^2), was the log-linear model (**Table 5.12**) and a significant age-dependence of the mortality rates in each environment was found (p<0.001), although there was no significant effect of *diet* or *diet*age* interaction (p>0.3). **Table 5.13** summarizes the ANCOVA results for males and females.

Table 5.12. Coefficients of determination (R^2) computed for linear and log-linear model fitting for: **a)** males and **b)** females, in the *banana-avocado-orange experiment*. The best fit for each *sex*diet* interaction is highlighted in bold.

R^2 for ♂ / Diet	Linear	Log-linear
Banana	0.937	**0.965**
Avocado	0.890	**0.963**
Orange	0.729	**0.831**

a)

R^2 for ♀ / Diet	Linear	Log-linear
Banana	0.946	**0.964**
Avocado	0.944	**0.976**
Orange	0.874	**0.912**

b)

Table 5.13. Summary of the fixed-effects ANCOVA used to test the age-dependence effect of diet change on age-specific mortality of males and females separately. Data shows the F statistic and respective p-value for the factors *Diet*, *Age* (log scale) and *diet*age* interaction. Significant results are highlighted in bold.

Effect	Males		Females	
	F statistic	p-value	F statistic	p-value
Diet	1.4233	0.253154	0.0771	0.925920
Log (age)	**468.0763**	**0.000000**	**687.1272**	**0.000000**
Diet*Log(age)	0.9431	0.398109	0.3109	0.734748
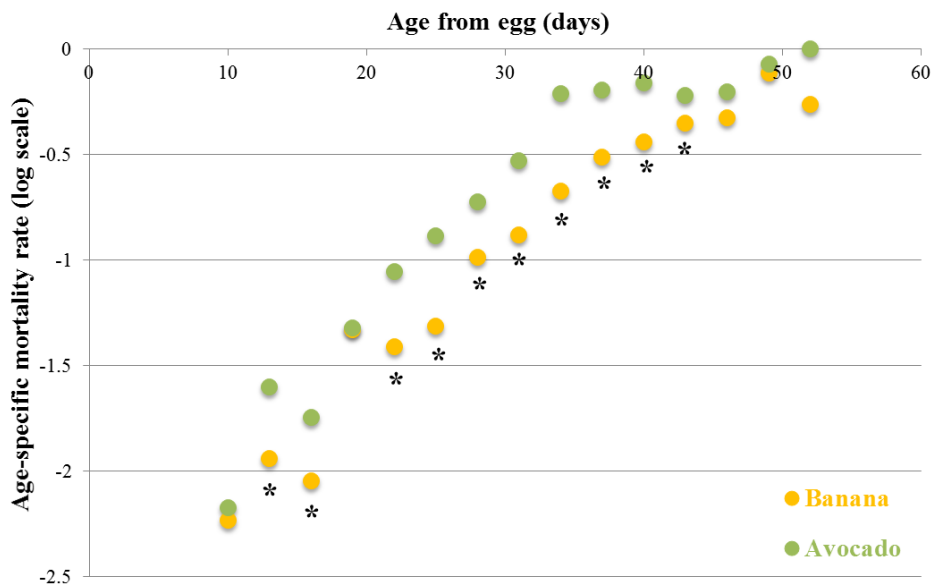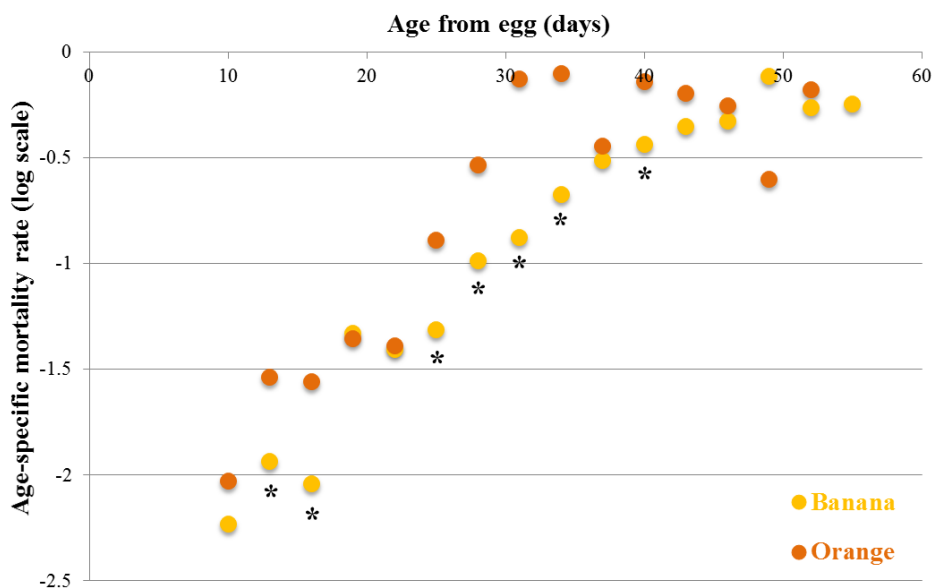
Figure 5.9. Age-specific mortality rates for males in **(a)** banana *vs.* avocado and **(b)** banana *vs.* orange. Data shows the average log-transformed three-day age-specific mortality computed as the fraction of deaths over the remaining cohort. Significant differentiation between each pair of compared diets is marked with an asterisk.
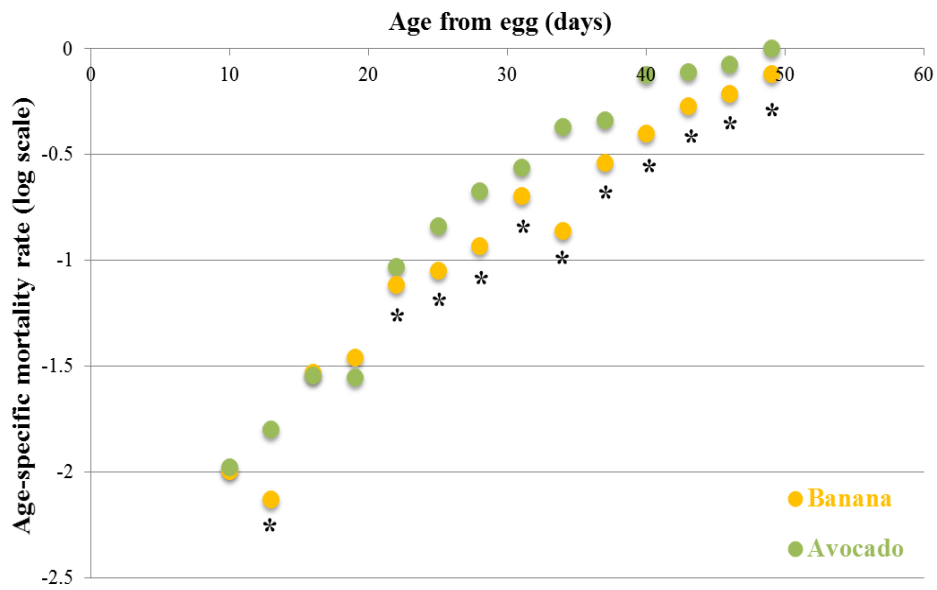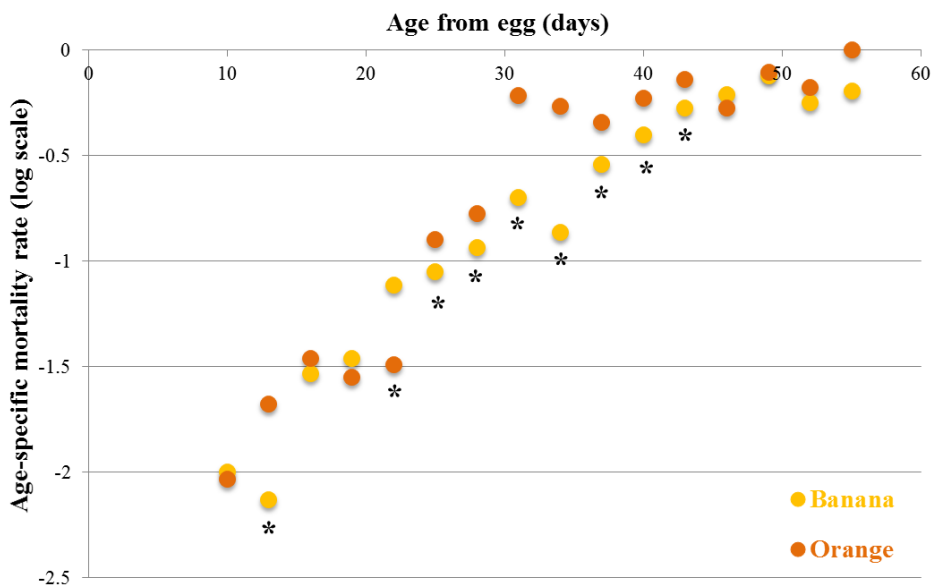
Figure 5.10. Age-specific mortality rates for females in **(a)** banana *vs.* avocado and **(b)** banana *vs.* orange. Data shows the average log-transformed three-day age-specific mortality computed as the fraction of deaths over the remaining cohort. Significant differentiation between each pair of compared diets is marked with an asterisk.

Hamiltonian analysis of the mortality rates for the banana-orange switch experiment

The analysis of age-specific mortality rates with the *banana-orange switch* regimes showed that the longer the duration of orange-based medium exposure during the life of a cohort, as well as the earlier the exposure starts, the greater the statistical differentiation from the control always-banana cohort (**Figures 5.11** and **5.12**). However, this pattern was not absolutely consistent, perhaps reflecting the small number of cohorts undergoing comparison.

The age-specific mortality curves of each *banana-orange switch* treatment were analyzed by fitting linear and log-linear models. The best fit, chosen based on the coefficient of determination (R^2), was in most cases the log-linear model (**Table 5.14**). Here we found not only a significant age-dependence of the mortality rates in each environment, but also a significant effect of *diet* and *diet*age* interaction ($p<0.001$), *i.e.* a significant age-dependence effect on the timing of diet switching. **Tables 5.15** summarizes the ANCOVA results for males and females.

Table 5.14. Coefficients of determination (R^2) computed for linear and log-linear model fitting for: **a)** males and **b)** females, in the *banana-orange switch experiment*. The best fit for each *sex*diet* interaction is highlighted in bold.

R^2 for ♂ / Diet	Linear	Log-linear
BaBaBaBa	0.955	**0.977**
BaBaBaOr	0.814	**0.842**
BaBaOrOr	0.900	**0.957**
BaOrOrOr	0.881	**0.955**
OrOrOrOr	0.755	**0.890**
OrOrOrBa	0.752	**0.883**
OrOrBaBa	**0.951**	0.950
OrBaBaBa	0.899	**0.910**

a)

R^2 for ♀ / Diet	Linear	Log-linear
BaBaBaBa	0.951	**0.967**
BaBaBaOr	**0.962**	0.947
BaBaOrOr	0.926	**0.978**
BaOrOrOr	0.945	**0.964**
OrOrOrOr	0.909	**0.960**
OrOrOrBa	0.900	**0.968**
OrOrBaBa	0.921	**0.974**
OrBaBaBa	**0.934**	0.912

b)

Table 5.15. Summary of the fixed-effects ANCOVA used to test the age-dependence effect of diet switch on age-specific mortality of males and females separately. Data shows the F statistic and respective p-value for the factors *Diet*, *Age* (log scale) and *diet*age* interaction. Significant results are highlighted in bold.

Effect	Males		Females	
	F statistic	p-value	F statistic	p-value
Diet	**6.485**	**0.000002**	**6.505**	**0.000002**
Log (age)	**1459.103**	**0.000001**	**2735.366**	**0.000001**
Diet*Log(age)	**5.795**	**0.000008**	**5.685**	**0.000011**

Age-specific mortality of males in BaBaBaBa vs. BaBaBaOr
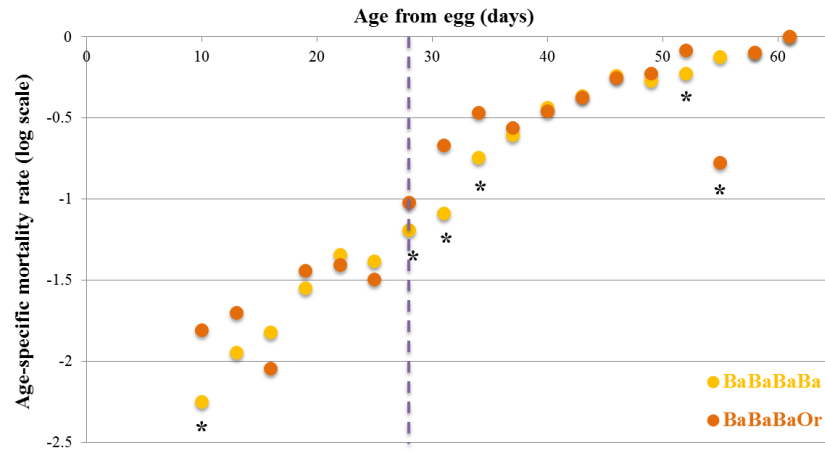
a)

Age-specific mortality of males in BaBaBaBa vs. OrBaBaBa

b)

Age-specific mortality of males in BaBaBaBa vs. BaBaOrOr

c)

Age-specific mortality of males in BaBaBaBa vs. OrOrBaBa

d)

152
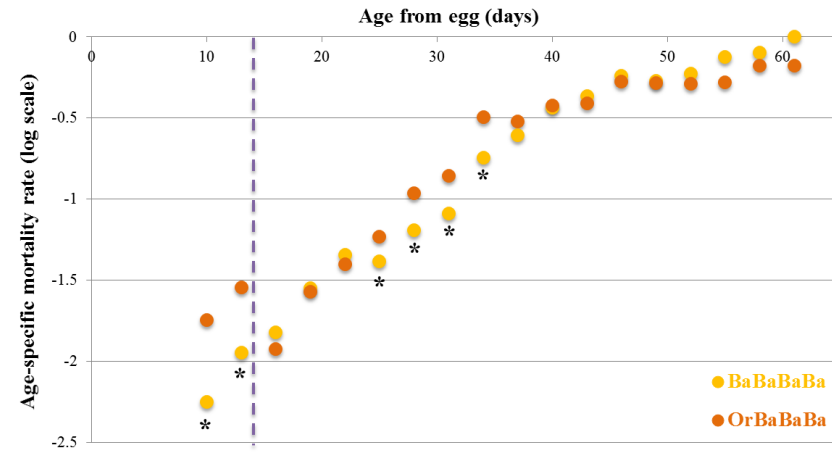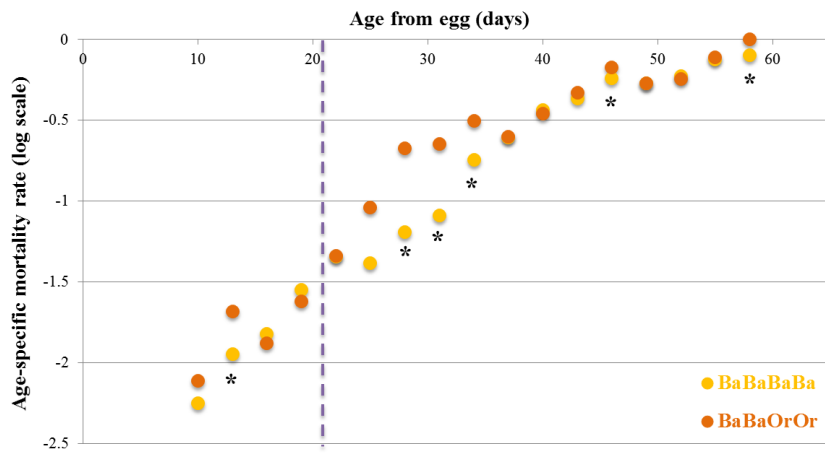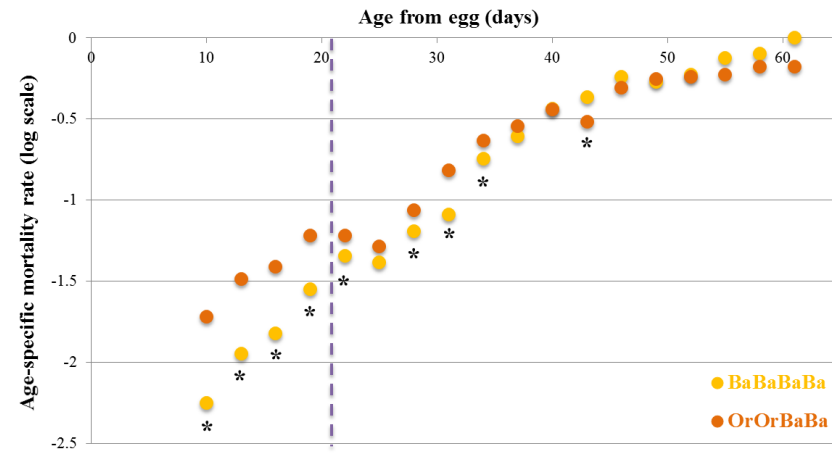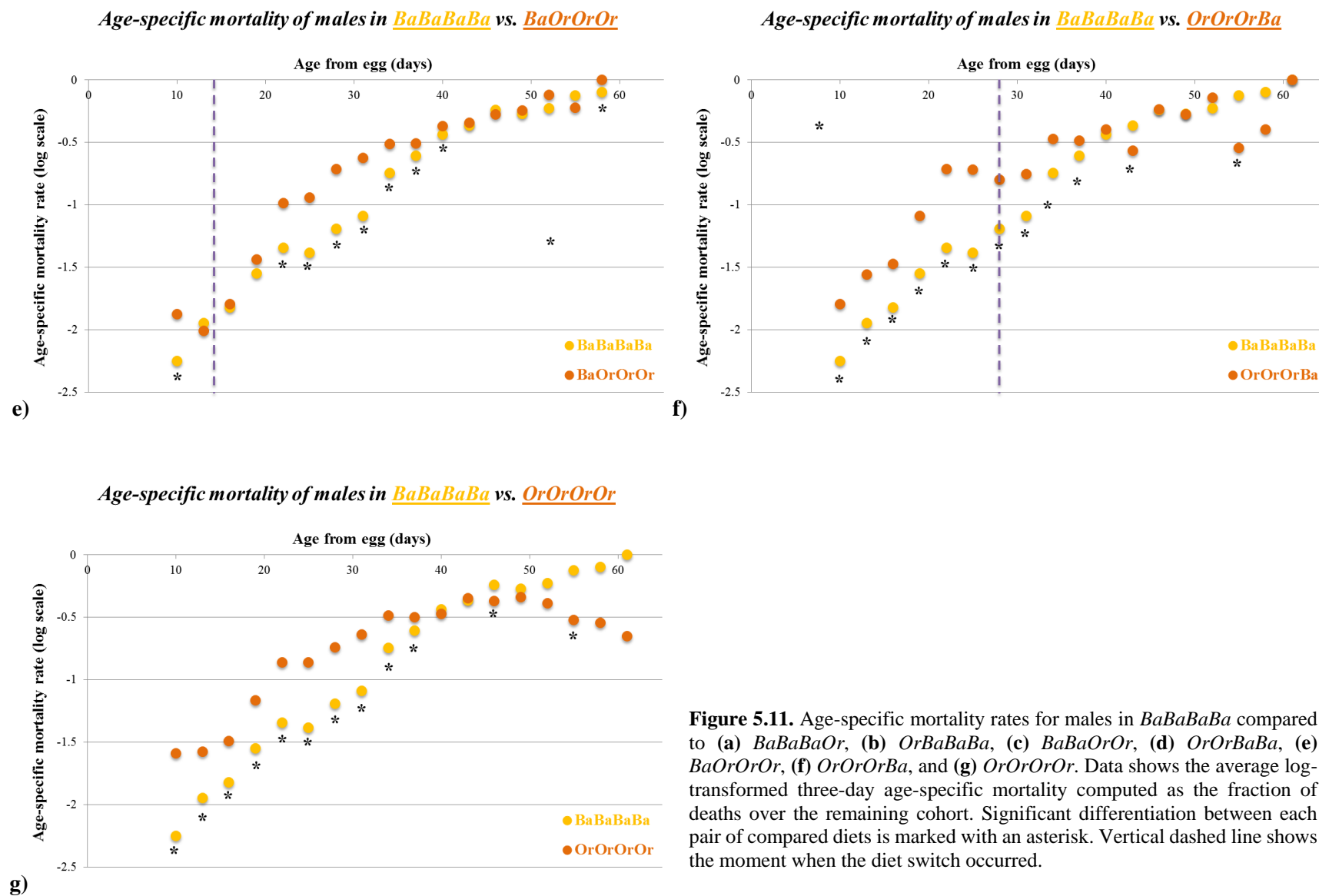
Figure 5.11. Age-specific mortality rates for males in *BaBaBaBa* compared to **(a)** *BaBaBaOr*, **(b)** *OrBaBaBa*, **(c)** *BaBaOrOr*, **(d)** *OrOrBaBa*, **(e)** *BaOrOrOr*, **(f)** *OrOrOrBa*, and **(g)** *OrOrOrOr*. Data shows the average log-transformed three-day age-specific mortality computed as the fraction of deaths over the remaining cohort. Significant differentiation between each pair of compared diets is marked with an asterisk. Vertical dashed line shows the moment when the diet switch occurred.

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Age-specific mortality of females in *BaBaBaBa* vs. *BaBaBaOr*

a)

Age-specific mortality of females in *BaBaBaBa* vs. *OrBaBaBa*

b)

Age-specific mortality of females in *BaBaBaBa* vs. *BaBaOrOr*

c)

Age-specific mortality of females in *BaBaBaBa* vs. *OrOrBaBa*

d)

Age-specific mortality of females in BaBaBaBa vs. BaOrOrOr

e)
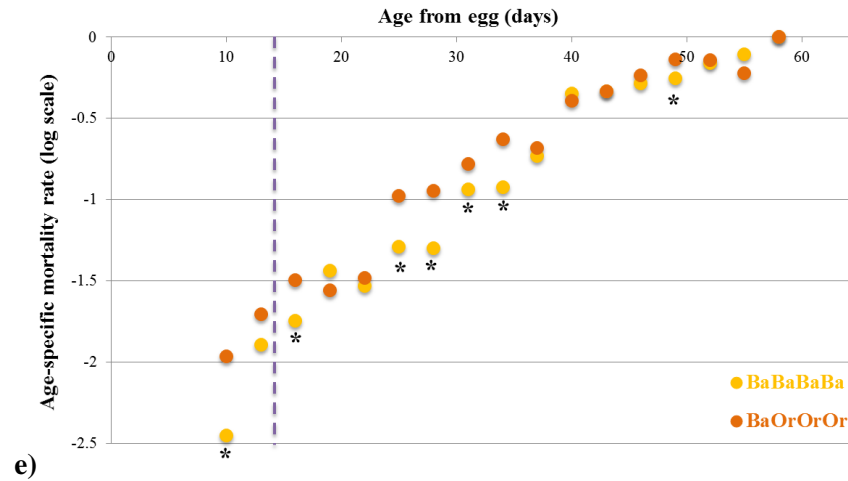
Age-specific mortality of females in BaBaBaBa vs. OrOrOrBa

f)
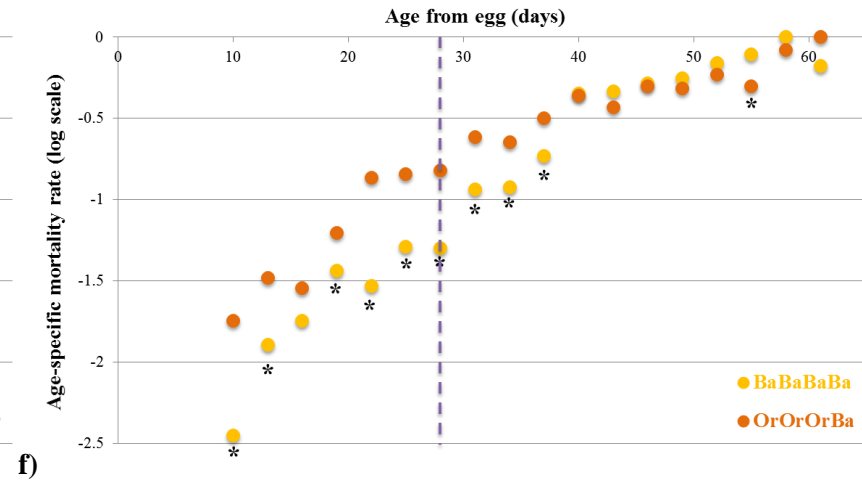
Age-specific mortality of females in BaBaBaBa vs. OrOrOrOr

g)
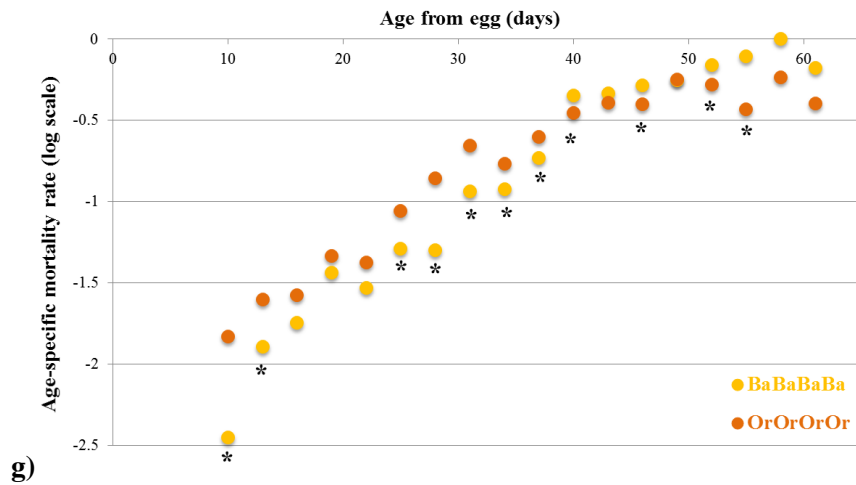
Figure 5.12. Age-specific mortality rates for females in *BaBaBaBa* compared to **(a)** *BaBaBaOr*, **(b)** *OrBaBaBa*, **(c)** *BaBaOrOr*, **(d)** *OrOrBaBa*, **(e)** *BaOrOrOr*, **(f)** *OrOrOrBa*, and **(g)** *OrOrOrOr*. Data shows the average log-transformed three-day age-specific mortality computed as the fraction of deaths over the remaining cohort. Significant differentiation between each pair of compared diets is marked with an asterisk. Vertical dashed line shows the moment when the diet switch occurred.

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DISCUSSION

Lindeberg (2010) contributed a comprehensive discussion of the relationship between human diet and chronic disease, particularly those chronic non-infectious diseases that could be considered age-associated. A key conclusion of his review is that many features of the age-dependent pathophysiology of chronic human diseases, such as cardiovascular disease and metabolic syndrome, arise from the agricultural diet. Evidently, this mode of reasoning is based on the concept of inadequate adaptation to the agricultural diet, with significant benefits to be achieved by switching back to a diet that resembles that of a hunter-gatherer. Regardless of the prominence of views like those of Lindeberg (2010) and others (*e.g.* Eaton & Konner 1985; Ryberg *et al.* 2013) concerning the effects of qualitative human dietary change in human evolution, the adoption of an agricultural way of life must have radically changed the demographic patterns of human survival, at least initially.

The results of our dietary experiments in *Drosophila* corroborate this hypothesis: very small changes in diet (like the ground fruit) can have significant effects on mortality rates, both overall **(Figure 5.8)** and over specific age-intervals in which these changes are imposed (**Figures 5.11** and **5.12**). Specifically, mortality rates are characteristically lower when the experimental cohorts are provided with the banana medium to which they have been adapted for more than 800 generations.

A more subtle point that arises from these experiments is that the longer the flies are given moderately novel diets, the more their mortality rates are increased in most cases – there seems to be a quantitative effect of diet novelty, independent of when it is introduced (**Figures 5.11** and **5.12**). This is not as striking or as consistent of an effect in these data, perhaps because of a lack of statistical power. But again, it supports the view that genotype-by-environment effects may arise from diet. In other words, we would suggest that, if these fruit flies had evolved for 800 generations on avocado or orange-based medium rather than banana, then the experimental cohorts that were given avocado or orange food would have had lower mortality rates and higher longevity than those given banana.

Given Hamilton's forces of natural selection (1966), adaptation to a novel environment will scale according to age when there is age specificity to (at least some of) the genetic variation that underlies such adaptation to the novel environment. That is, Hamilton's forces scale the intensity of natural selection that, qualitatively, we can expect adaptation to a novel diet to proceed very effectively at early ages. But at later adult ages, we should expect to see a

quantitative and progressive reduction in the extent of adaptation to the novel diet. In part, the present study suffers from the lack of a *more ancestral* food regime than banana (which is in fact *relatively recent*), that might allow a test of the evolutionary expectation of the Hamiltonian scenario directly.

In this study we compared mortality patterns in populations long-adapted to banana under banana food and under moderately novel diets. According to Hamiltonian theory (Mueller *et al.* 2011), a change of diet should have a *stronger effect* if made at an *earlier age*. This was not observed in our data, where the amount of time with the new nutrient was the main factor affecting survival. However, in our experiments, there was an association between how early the new nutrient was introduced and how long it was imposed, as after the switch the diet remained the same for the rest of the cohort's life. This did not allow us to accurately test whether age, *per se*, affects how flies survive on a novel diet. A better comparison would have been to make a larger set of age-dependent diet-switch combinations, with diets differing not only with in the age of the change but also the amount of time under different nutrients.

Final remarks

Despite the experimental design issues, our dietary change experiments confirm the need for caution in interpreting experimental data, because of the considerable potential for confounds that arise from *genotype-by-environment* interactions and lack of adaptation to novel laboratory environments. Future studies involving more populations evolving under different diets, with diet switching between novel and ancestral environments, as well as life-long characterization of several life-history traits would provide better tests of the application of Hamiltonian theory to human aging and health.

Chapter Six.

Aging and Mortality Patterns in Urea-adapted Populations

ABSTRACT

The problem of adaptation to nitrogenous waste has been an ongoing project of Mueller and collaborators for over 20 years, who created and maintained outbred lines of *Drosophila melanogaster* selected for increased urea tolerance and their life-cycle matched controls. This experimental system enabled us to test the hypotheses that (1) dietary adaptation is age-specific and (2) long-term adaptation to urea might lead to loss of fitness in the ancestral environment. Here we studied the adult mortality patterns of Mueller's replicated stocks of *Drosophila* adapted to the presence of urea during their larval and early adult stages for more than 250 generations, and their controls. Our results confirm the previous findings that exposure to environmental urea increases mean longevity, despite the population's evolutionary history, and that the increase in longevity is achieved by lowering the age-independent mortality rates. Our data did not verify the hypothesis (1) of age-dependence of dietary adaptation – cohorts exposed to urea did not show a decrease in differentiation of mortality rates with age. Furthermore, the urea-adapted populations showed lower mortality rates than their matched controls in the ancestral banana food – the hypothesis (2) of loss of adaptation to the ancestral environment was also not corroborated. On the one hand, the present results do not support the Hamiltonian expectations that greater adaptation should occur at early ages which feature stronger forces of selection. On the other hand, we have not adequately characterized the range of life-history characters that might be impinged on by urea, or adaptation to urea for that matter (*e.g.* reduced fecundity and the consequent benefits to longevity).

RESUMO

A adaptação a resíduos metabólicos como os produtos azotados tem sido amplamente estudada por Mueller e colaboradores nos últimos 20 anos, equipa que criou e manteve em laboratório populações de *Drosophila melanogaster* seleccionadas para a tolerância à ureia e respectivos controlos. Este sistema experimental permitiu-nos testar as hipóteses de que (1) a adaptação uma nova dieta é específica da idade e (2) a adaptação a longo prazo à ureia pode levar à perda de *fitness* no ambiente ancestral. Neste capítulo, estudámos os padrões de mortalidade adulta em populações de *Drosophila* adaptadas à presença de ureia durante as fases larvar e de jovem adulto durante mais de 250 gerações, e respectivos controlos. Os nossos resultados confirmaram os obtidos anteriormente de que a exposição ambiental a ureia aumenta a longevidade das populações, independentemente da sua história evolutiva. Confirmou-se também que este aumento de longevidade é conseguido através da redução das taxas de mortalidade independentes da idade. Os dados obtidos não verificaram a hipótese (1) da dependência da idade na adaptação uma nova dieta – não houve diminuição da diferenciação das taxas de mortalidade com a idade em coortes expostas a ureia. Adicionalmente, as populações adaptadas a ureia mostraram taxas de mortalidade mais baixas do que os controlos no ambiente ancestral – a hipótese (2) de perda de adaptação ao ambiente ancestral também não foi corroborada. Por um lado, estes resultados não apoiam a expectativa Hamiltoniana de maior adaptação em idades mais jovens, onde a força da selecção natural é maior. Por outro lado, não foram estudadas adequadamente as características da história de vida que podem estar a ser negativamente afectadas pela ureia ou adaptação à ureia (*e.g.* redução da fecundidade e os consequentes benefícios para a longevidade).

INTRODUCTION

The problem of adaptation to nitrogenous wastes has been an ongoing project of the Mueller laboratory at UC Irvine (*e.g.* Joshi *et al.* 1996a,b; Joshi *et al.* 1998, Pierce *et al.* 1999, Borash *et al.* 2000). This work, in turn, arose from the long-standing interest of Mueller in density-dependent selection and evolution of larval adaptation to crowded conditions (*e.g.* Mueller & Sweet 1986, Mueller 1988a,b; Joshi & Mueller 1988, Mueller *et al.* 1993). Since the work of MacArthur & Wilson (1967), among the first researchers to explore systematically the evolutionary consequences of extreme population densities, much progress has been made in understanding how density-dependent selection shapes life-history evolution. Most of the close dissection of its biological details has come from empirical studies with *Drosophila* (reviewed by Mueller 1995). Initially, such studies focused on understanding the biology of traits thought to be advantageous under conditions of extreme larval crowding. These traits ranged from primarily behavioral characters, like pupation height, larval feeding rate and locomotor activity, to predominantly physiological, as larval efficiency in biomass production and tolerance to nitrogenous metabolic waste (Joshi *et al.* 1996b). But more importantly, the relevance of these traits to the density-dependent evolution of life histories was unequivocally demonstrated by showing that they did, in fact, evolve in laboratory populations of *Drosophila melanogaster* maintained at high larval densities for several generations (Mueller & Sweet 1986; Joshi & Mueller 1988, 1993, 1996; Mueller *et al.* 1991, 1993).

The connection Mueller found between density-dependent selection and adaptation to nitrogenous waste was first established when his laboratory *K* populations were found to have lower feeding rates (Joshi & Mueller 1988). Mueller then hypothesized that the decrease in feeding rates might have been related to increased levels of nitrogenous waste in the media in which the *K* populations had evolved. Therefore, the Mueller lab deliberately created outbred lines of *Drosophila melanogaster* cultured with two different kinds of nitrogenous waste: *ammonia* (AX flies, *vid* Borash *et al.* 2000) which larval fruit flies excrete, and *urea* (MX and UX flies, *vid* Joshi *et al.* 1996a,b, 1998; Pierce *et al.* 1999; Borash *et al.* 2000), which arises only at low levels in fruit fly culture. Both of these nitrogenous compounds had been reported to accumulate in the food of crowded *Drosophila* cultures (Botella *et al.* 1985, Borash *et al.* 1998). These substances do not appear to target any specific biochemical processes solely, but instead have general cytotoxic effects. Urea is a protein denaturant (Somero & Yancey, 1997), and larvae reared on urea-containing media have increased levels of proteins containing isoaspartyl residues, a form of protein damage (David *et al.* 1999). Ammonia's effects are less

well understood, but it appears to be neurotoxic from vertebrate research (Cooper & Plum 1987) and may affect pH regulation. We were particularly interested in the lines that had been cultured with exposure to high levels of urea generation after generation, precisely because urea is a relatively rare toxic substance in the lives of *Drosophila* species.

The most relevant results from the urea-exposure experiments performed by Mueller and collaborators for the present purpose were (1) the increase of larval tolerance to urea as a direct response to selection, (2) the greater survivorship and development time of selected lines when urea was present, (3) the greater longevity and lower fecundity when the cohorts were in urea-supplemented food, regardless of their evolutionary history, and (4) the general increase in development time in urea-laden environments, showing the detrimental effect of this nitrogenous waste (Joshi *et al.* 1996a,b; 1998). Adding urea to the food improved mean longevity of all populations in Mueller's laboratory, regardless of their past selection history – evolved larval tolerance doesn't seem to affect this effect on adults. This increase in longevity seemed to involve lowering the age-independent mortality rates rather than altering the rate of aging. Moreover, urea-selected lines (MX) had higher mean longevities on urea-supplemented food, when compared to MC, their matched controls (**Figure 6.1**, Joshi *et al.* 1996b).
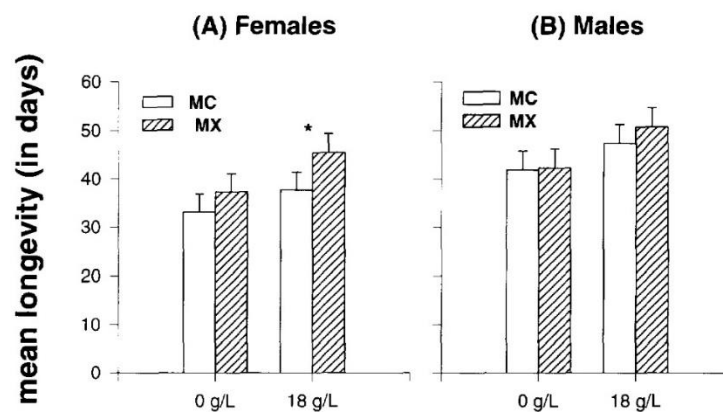
Figure 6.1. Mean longevity of (A) female and (B) male flies from the urea-adapted populations (MX) and their matched life-cycle controls (MC) in banana (0g/L) and urea-supplemented food (18 g/L). The error bars denote 95% confidence intervals about the mean of the five replicate populations of each regime. The asterisk marks a significant difference in mean longevity of MX females compared to MC females. All other differences between MX and MC are not significant (Joshi *et al.* 1996b).

Long-term exposure to urea led reduced fecundity when compared to fecundity on regular banana food, and this inhibition was accentuated with increasing exposure to urea. The Joshi *et al.* experiments on fecundity (1996b, 1998) also show that selection for increased larval tolerance to this nitrogenous waste does not seem to affect the response of adult components of fitness to urea-supplemented food (**Figure 6.2**). These results provide another illustration of the ubiquitous trade-off between reproduction and survival involved in an environmental effect on longevity.

Figure 6.2. Mean fecundity of MX and MC flies (urea-adapted populations and their controls, respectively) assayed in banana (0g/L) and urea-supplemented food (18 g/L), after being maintained as adults in (A) regular food, or (B) urea food. The error bars denote 95% confidence intervals about the mean of the ten MX and MC replicate populations. Data for MX and MC populations were pooled because there was no significant effect of selection regime nor any significant regime interactions (Joshi *et al.* 1998 adapted).

Another very valuable characteristic of these urea-banana stock systems is that the urea selection regime involved urea exposure *only at young ages*, rather than throughout a long life-cycle. As such, these populations together with their life-cycle matched controls, provided an accidental experiment in early-life adaptation to a relatively toxic diet. Given the perspective offered in *Chapter 5*, then, the differences between urea-adapted lines and their matched controls with respect to their tolerance for urea-laden environments provide a simple test of the degree to which dietary adaptation is age-specific.

In this chapter we study the adult mortality patterns of populations of *Drosophila melanogaster* adapted to the presence of urea during their larval and early adult stages for more than 250 generations (UX), together with their matched controls, not adapted to urea (AUC). The UX-AUC stock system was created by Mueller and collaborators in the Fall of 1996, similarly to the MX-MC populations (*vid* Borash *et al.* 2000 for more details). This study system allowed us to experimentally test the expectations of the *Hamiltonian wave* hypothesis discussed in *Chapter 5*. In particular, (1) whether or not urea tolerance fades with adult age, implicating an age-dependent pattern of evolutionary domestication, and (2) the effect on aging and late-life of returning populations long-selected (to urea) to their ancestral environment (absence of urea).

MATERIALS AND METHODS

Experimental populations

The experiments in this chapter involved two five-fold replicated stocks of outbred flies of *Drosophila melanogaster*: (1) the UX_{1-5} populations, adapted to urea exposure as larvae and young adults for more than 250 generations; and (2) the AUC_{1-5} populations, cultured in regular banana-molasses food from egg to adult, without urea (control conditions). Both sets of populations were derived from the UU stock from Joshi & Mueller (1996), as described in Borash *et al.* (2000). Each B population from Rose (1984a) was used to originate one UU population, which in turn, originated one UX and one AUC population ($B_i \rightarrow UU_i \rightarrow UX_i$, AUC_i, $i = 1 - 5$). Consequently, the UX and AUC populations bearing the same numerical subscript are more closely related to each other, compared to other populations subjected to the same selection regime (*vid.* Joshi *et al*. 1996a,b; 1998). It is important to bear in mind that both the UX and AUC stocks are given a reproductive window at 3 weeks of age from eggs, due to the stretching of the developmental period caused by urea in the UX stocks. The AUC stocks have always been handled to match such life-history shifts, in order to separate qualitative medium adaptation (with urea *vs.* without urea) from selection that arises from shifts in the timing of reproduction.

Food preparation

Regular banana-molasses food was prepared with agar, nipagin, banana, corn syrup, dry active yeast, and barley malt. The urea food was obtained by adding 16g of crystallized urea to each liter of banana food.

Mortality assay

All populations were reared in vials with regular banana food for two generations of common garden and were given 14 days to develop. The cohorts were then dumped into transparent acrylic cages (2 x 1000 flies, per population, per treatment) and given the respective diet treatment: banana or urea (**Table 6.1**). Every 24 hours each cage was given fresh food, assessed for mortality, and individuals were sexed at death. Cohort size was then calculated from complete death records. The cages were kept at room temperature (24ºC ± 1ºC) and their locations were randomized to reduce variation in light distribution.

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Table 6.1. Experimental design for the mortality assay. Two cages (α and β) from each population were assayed in both environments (banana and urea).

Diet \ Population	Banana	Urea
UX $_i$ (i = 1, 2, 3, 4, 5)	UX $_i$ α Ba	UX $_i$ α Ur
	UX $_i$ β Ba	UX $_i$ β Ur
AUC $_i$ (i = 1, 2, 3, 4, 5)	AUC $_i$ α Ba	AUC $_i$ α Ur
	AUC $_i$ β Ba	AUC $_i$ β Ur

Statistical data analysis

In all analyses, the normality and homoscedasticity of data were tested by Shapiro-Wilk (1965) and Brown-Forsythe (1974) tests, respectively. A significance value of 0.05 (α) was used to test all null hypotheses. The general linear model tests used to analyze mean longevity and age-specific mortality were done in *STATISTICA 13* (Dell 2015). Analyses of age-specific mortality patterns and Gompertz were done in *R* (R Core Team 2013).

Mean longevity statistical analysis

The effect of sex and its interaction with diet on mean longevity (average age-specific mortality standardized by the cohort size) was analyzed using the following linear mixed-effects model:

$$Y = \mu + R_i + D_j + S_k + Pop\{R_i\} + R_i * D_j + R_i * S_k + D_j * S_k + R_i * D_j * S_k + D_j * Pop\{R_i\} \\ + S_k * Pop\{R_i\} + D_j * S_k * Pop\{R_i\} + \varepsilon \tag{6.1}$$

where *Y* is mean longevity, *R* represents the selection regime (i = UX or i = AUC), *D* the diet treatment applied during the assay (j = urea or j = banana), S the sex of the flies tested (k = males or k = females), and *Pop* the random replicate population nested in the regime.

The effect of diet and selection regime on mean longevity was also analyzed for males and females separately, using the following linear mixed-effects model:

$$Y = \mu + R_i + D_j + Pop\{R_i\} + R_i * D_j + D_j * Pop\{R_i\} + \varepsilon \tag{6.2}$$

where *Y* is mean longevity, *R* represents the selection regime (i = UX or i = AUC), *D* the diet treatment applied during the assay (j = urea or j = banana), and *Pop* the random replicate population nested in the regime.

Gompertzian analysis of mortality rates and patterns

The mortality rates and patterns were analyzed by fitting the mortality assay data to a two-stage Gompertz equation (Gompertz 1825), using maximum-likelihood. The estimates of **A** and **α** from each combination of *population*diet*sex* were used as data in a mixed-effects ANOVA, which included the following fixed factors: diet (banana *vs.* urea) and sex (female *vs.* male). All these factors were crossed with the five replicate blocks. Additionally, for each *population*diet*sex* combination, the coefficient of determination, R^2, was calculated, as an indication of the proportion of variation explained by the Gompertz model. See the *Materials and Methods* section of *Chapter Five* for more details on this analysis.

Hamiltonian analysis of mortality rates and patterns

For each combination of *diet*regime*sex* the data from α and β cages from all 5 populations were combined and three-day survivorship intervals were computed. For each interval, a new categorical variable was then created, defining the status of each one of the flies (0 = dead or 1 = alive). The counts of each interval were used in a chi-squared test to compare all combinations. The mortality rates in each age-interval, defined as the logarithm of the total number of deaths over the number of the cohort surviving to that age, were plotted and analyzed by fitting linear and log-linear least-square curves. The best fitting model was chosen based on the highest proportion of explained variance and the adequate ANCOVA models were used to test the differences between the curves.

RESULTS

General linear model assumptions

Small deviations from normality were accepted, and homoscedasticity was verified by the Brown-Forsythe test. Our distribution tests showed that all populations were homoscedastic and generally normal (data not shown).

Mean longevity analysis

The addition of urea to the medium fed to the flies caused a general increase in mean longevity, regardless of the cohorts' regime or the sex of the flies. Furthermore, the urea-adapted cohorts (UX) showed a generally higher longevity across the environments where they were tested and no significant interactions between sex, diet, and regime were found (see **Table 6.2** and **Figure 6.3**). Similar effects of diet and selection regime were found when sexes were analyzed separately: the presence of urea led to a longevity increase of ~7.6 days in males and ~8.2 days in females; also, UX flies lived longer than their matched AUC controls by ~6.9 days, in males, and 8.5 days, in females (**Table 6.3**).

Table 6.2. Summary of the mixed-effects ANOVA used to analyze the effect of diet, regime, and sex interactions on mean longevity. Data shows the F statistic and respective p-value for the factors *Diet*, *Sex*, *Regime*, and their interactions. Significant results are highlighted in bold.

Effect	*F statistic*	*p-value*
Diet	**60.7743**	**0.000053**
Regime	**9.3521**	**0.015627**
Sex	**13.1791**	**0.006682**
Diet*Regime	0.0123	0.914384
Diet*Sex	0.8568	0.381710
Regime*Sex	1.2143	0.302525
Diet*Regime*Sex	1.0709	0.331003
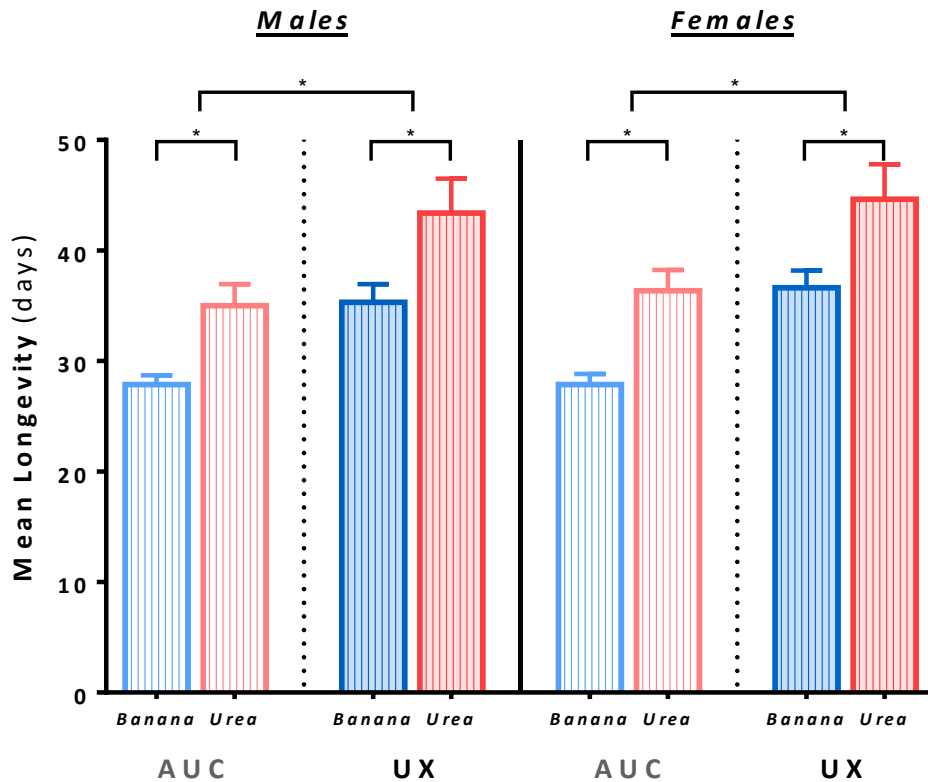
Figure 6.3. Mean longevity (in days as adult) of males and females of AUC and UX, in banana and urea. Average stock values are shown and error bars denote standard error of mean. Significant differences are marked with *.

Table 6.3. Summary of the mixed-effects ANOVAs used to analyze the effect of diet and regime on mean longevity of males and females separately. Data shows the F statistic and respective p-value for the factors *Diet*, *Regime*, and *Diet*Regime*. Significant results are highlighted in bold.

Effect	*Males*		*Females*	
	F statistic	*p-value*	*F statistic*	*p-value*
Diet	**64.3460**	**0.000043**	**48.5626**	**0.000116**
Regime	**8.3163**	**0.020392**	**10.2935**	**0.012455**
Diet*Regime	0.2399	0.637402	0.0409	0.844783

Two-stage Gompertz analysis of mortality rates and patterns

The Gompertz model fitting provided us with estimates of the mortality rates (A and α) and late-life parameters (*plateau height* and *break day*), which are shown in **Table 6.4** for males and females separately. **Figures 6.4** and **6.5** show the age-specific mortality of each *regime*diet* interaction for males and females, respectively.

The mortality data analysis showed that females have a significantly lower age-independent mortality, A ($p<0.02$) and higher age-dependent mortality, α ($p<0.01$), but no significant interaction between sex, regime and diet was found ($p>0.2$). The presence of urea significantly lowered A in both UX ($p<0.01$) and AUC ($p<0.03$) regimes, and the effect on AUC was significantly greater ($p<0.05$). Other than the difference between males and females, the analysis of age-dependent mortality (α) showed only significant differences due to the interaction of selection regime and diet ($p<0.03$), *i.e.* urea increased the rate of aging in UX but decrease it in AUC.

In terms of late-life, although without a significant difference, females entered late-life earlier ($p>0.3$) with higher plateau heights ($p>0.1$), across all regimes and food treatments. In both environments, UX males entered late-life at significantly later ages ($p<0.04$) than AUC with insignificantly lower plateau heights ($p>0.07$). The same pattern was observed in females.

Table 6.4. Age-independent (A) and age-dependent (α) mortality rates, plateau height (*ph*) and break day (*bd*) estimated from the two-stage Gompertz analysis for all *diet*regime* combinations in **a)** males and **b)** females. All A, α and *ph* estimations were significantly different from zero, and all *bd* were significantly different from infinity, indicating the existence of late-life plateaus.

MALES	UX		AUC	
Diet \ *Param.*	Banana	Urea	Banana	Urea
A	0.007	0.001	0.012	0.005
α	0.067	0.087	0.076	0.071
ph	0.236	0.263	0.151	0.321
bd	57.0	64.5	45.2	57.0

a)

FEMALES	UX		AUC	
Diet \ *Param.*	Banana	Urea	Banana	Urea
A	0.005	0.002	0.007	0.004
α	0.070	0.073	0.093	0.074
ph	0.274	0.290	0.301	0.329
bd	56.0	57.5	40.4	52.7

b)
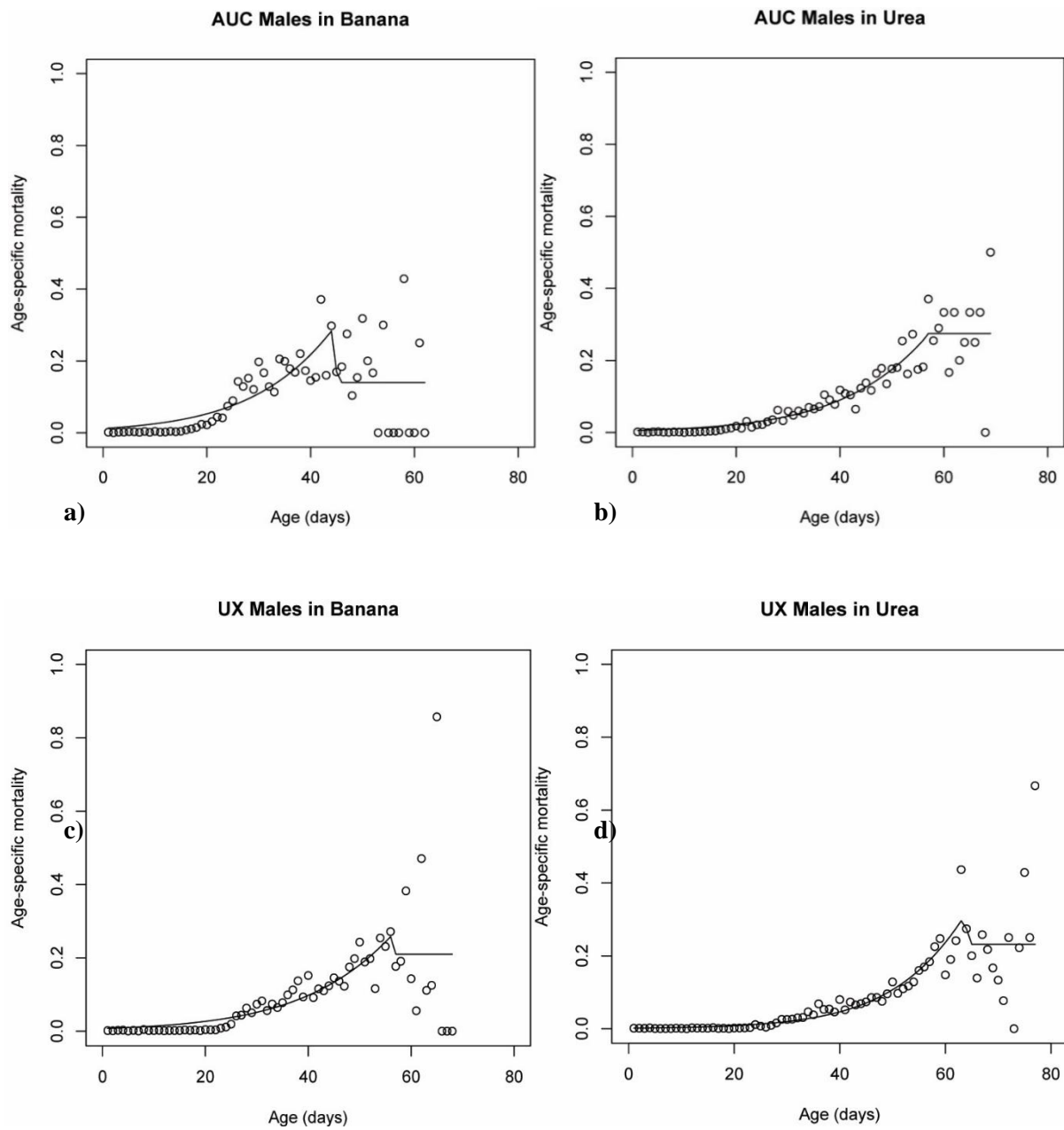
Figure 6.4. Age-specific mortality for males of each *regime*diet* interaction: **a)** AUC in banana, **b)** AUC in urea, **c)** UX in banana, and **d)** UX in urea.
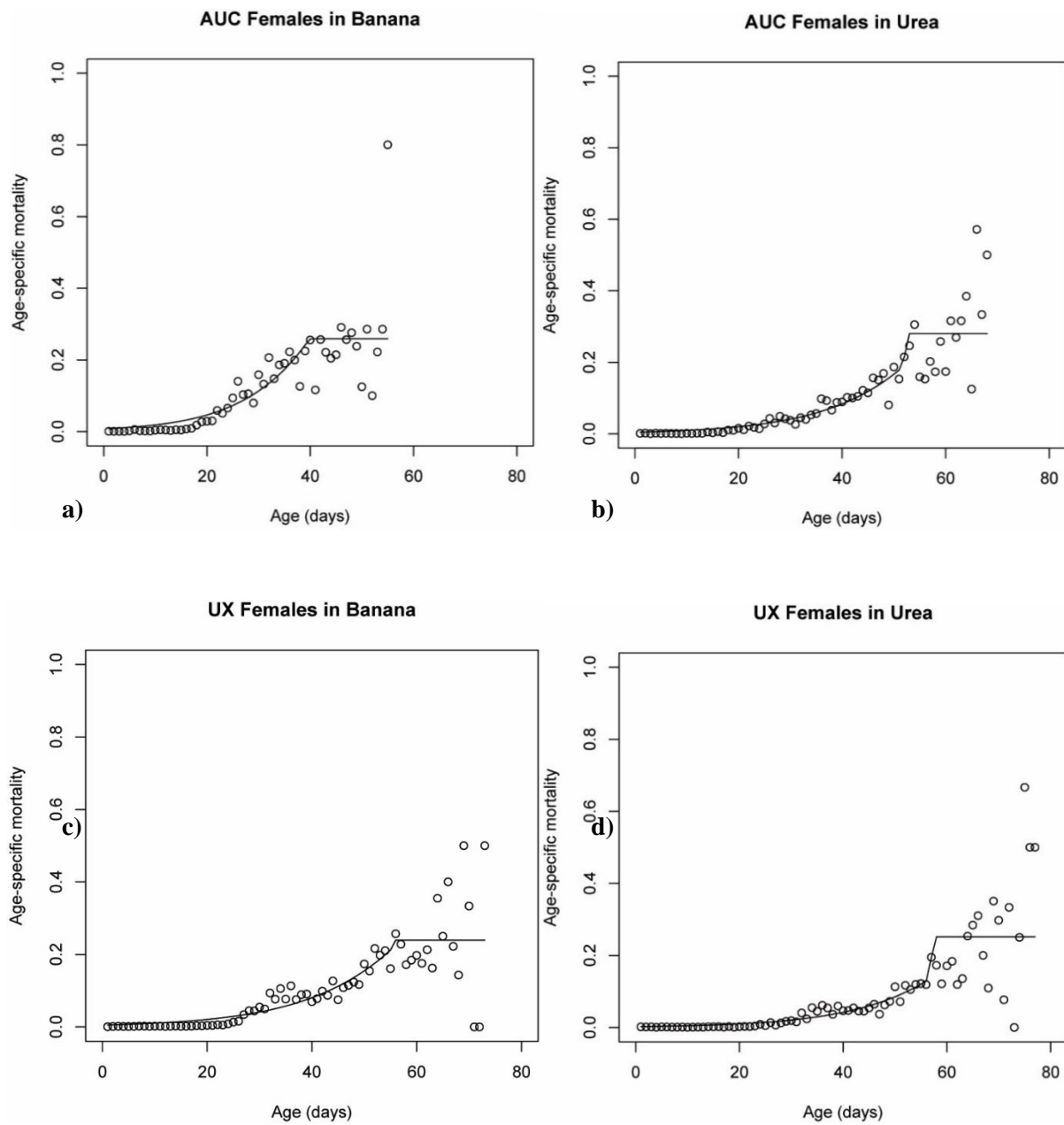
Figure 6.5. Age-specific mortality for females of each *regime*diet* interaction: **a)** AUC in banana, **b)** AUC in urea, **c)** UX in banana, and **d)** UX in urea.

Hamiltonian analysis of mortality rates

The analysis of the age-specific mortality rates of the UX and AUC male cohorts, showed in general more significant differentiation at later compared with earlier adult ages and the differentiation pattern observed for females was more consistent throughout the cohorts' life (**Figures 6.6** and **6.7**). Adding urea to the medium fed to the adults caused a significant decline in mortality across all ages, in both UX and AUC cohorts. This reduction in mortality was more evident on female flies of the controls (80% of the age classes were differentiated between environments). Furthermore, the UX cohorts always showed a significantly lower mortality than AUC cohorts, when tested in the same environment.

The age-specific mortality curves of each *regime*diet* combination were analyzed by fitting linear and log-linear models. The best fit, chosen based on the coefficient of determination (R^2), was in all cases, except one, the log-linear model (**Table 6.5**), and in that one case the coefficient was only 0.4% units apart. The ANCOVA model used to analyze the age-specific mortality data showed a significant age-dependence of the mortality rates ($p<0.001$), but no other significant interactions were found ($p>0.5$). **Table 6.6** summarizes the ANCOVA results for males and females.

Table 6.5. Coefficients of determination (R^2) computed for linear and log-linear model fitting for: **a)** males and **b)** females, of UX and AUC cohorts, in both banana and urea environments. The best fit for each *sex*regime*diet* interaction is highlighted in bold.

Regime	UX		AUC	
Diet / R^2 for ♂	Banana	Urea	Banana	Urea
Linear	0.885	**0.921**	0.828	0.896
Log-linear	**0.905**	0.917	**0.908**	**0.945**

a)

Regime	UX		AUC	
Diet / R^2 for ♀	Banana	Urea	Banana	Urea
Linear	0.832	0.921	0.849	0.908
Log-linear	**0.919**	**0.926**	**0.929**	**0.960**

b)

We, then, tested whether urea adaptation fades with adult age, *i.e.* if UX flies were less different from AUC flies at older ages, by comparing the age-specific mortality rates in urea only. The results showed no significant differences for both males and females (interaction *age*regime*, **Table 6.7**). Finally, we checked if the adaptation to urea caused loss of adaptation in the ancestral environment, and if so if it was age-dependent, by confronting UX and AUC in banana medium. The mortality rates showed no significant differences between the two regimes (factor *regime*, **Table 6.8**) nor age-dependence of those differences (interaction *age*regime*, **Table 6.8**).

Table 6.6. Summary of the fixed-effects ANCOVA used to test the age-dependence effect of diet and regime on age-specific mortality of males and females separately. Data shows the F statistic and respective p-value for the factors *Diet*, *Age* (log scale), *Regime*, and interactions. Significant results are highlighted in bold.

Effect	Males		Females	
	F statistic	p-value	F statistic	p-value
Diet	0.432	0.512776	0.540	0.464577
Regime	0.057	0.811497	0.170	0.681384
Log (age)	**845.707**	**0.000001**	**1128.954**	**0.000001**
Diet*Regime	0.323	0.571509	0.242	0.623828
Diet*Log (age)	0.006	0.941069	0.003	0.957718
Regime*Log (age)	0.153	0.696515	0.254	0.615422
Diet*Regime*Log (age)	0.354	0.553523	0.433	0.512089

Table 6.7. Summary of the fixed-effects ANCOVA used to test the age-dependence effect of regime on age-specific mortality of males and females in urea. Data shows the F statistic and respective p-value for the factors *Age* (log scale), *Regime*, and interaction *Age*Regime*. Significant results are highlighted in bold.

Effect	Males		Females	
	F statistic	p-value	F statistic	p-value
Regime	0.3940	0.533775	0.4951	0.485368
Log (age)	**522.8898**	**0.000001**	**701.8543**	**0.000001**
Regime*Log (age)	0.0255	0.873928	0.0147	0.903959

Table 6.8. Summary of the fixed-effects ANCOVA used to test the age-dependence effect of regime on age-specific mortality of males and females in banana. Data shows the F statistic and respective p-value for the factors *Age* (log scale), *Regime*, and interaction *Age*Regime*. Significant results are highlighted in bold.

Effect	Males		Females	
	F statistic	p-value	F statistic	p-value
Regime	0.0452	0.832817	0.0027	0.958979
Log (age)	**345.9620**	**0.000001**	**459.2631**	**0.000001**
Regime*Log (age)	0.4002	0.530866	0.5516	0.462000
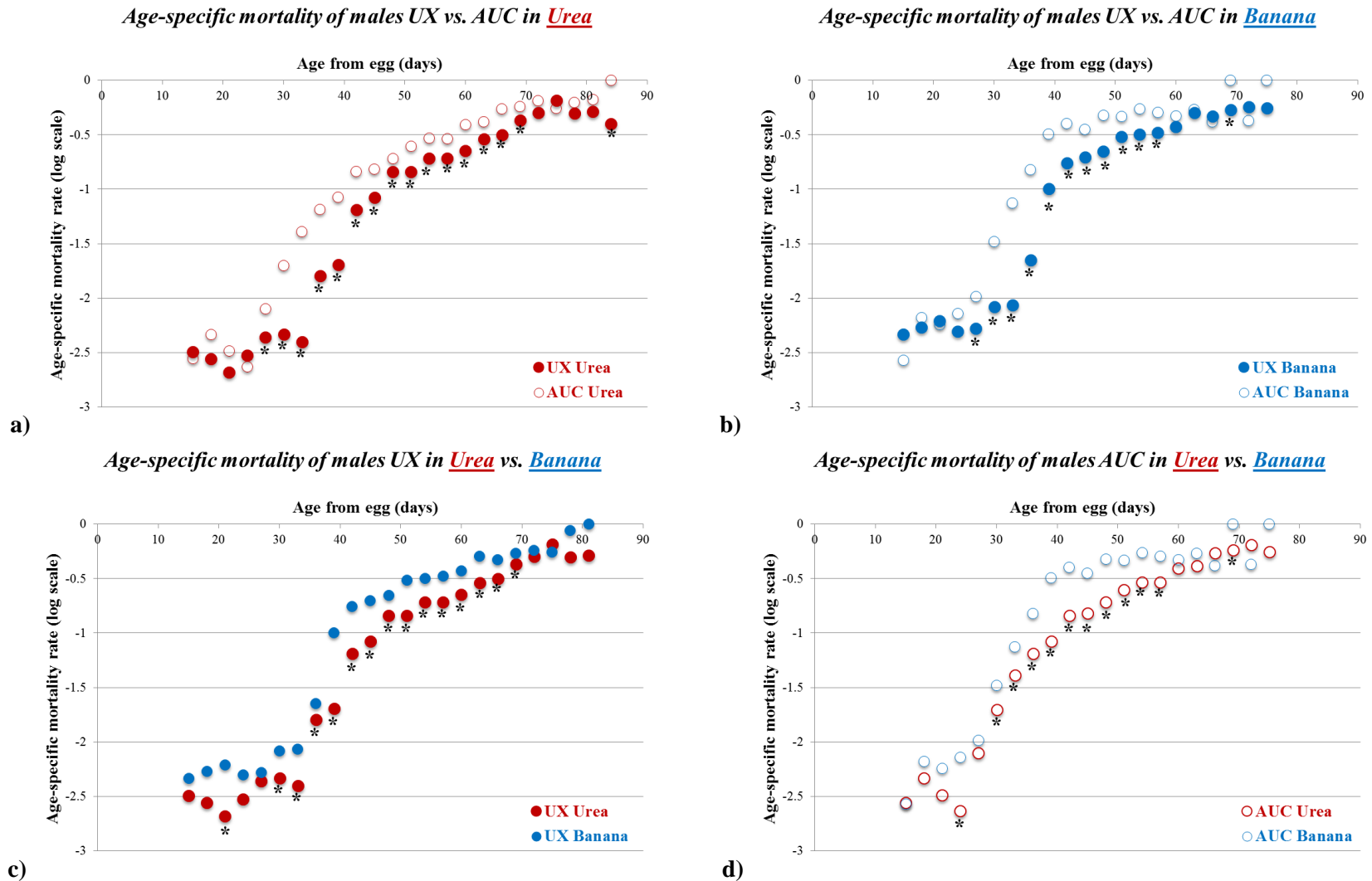
Figure 6.6. Age-specific mortality rates for males of **(a)** UX and AUC in urea, **(b)** UX and AUC in banana, **(c)** UX in urea and banana, and **(d)** AUC in urea and banana. Data shows the average log-transformed three-day age-specific mortality computed as the fraction of deaths over the remaining cohort. Significant differentiation in age-specific mortality between each pair of *regime*d*iet* is marked with an asterisk.

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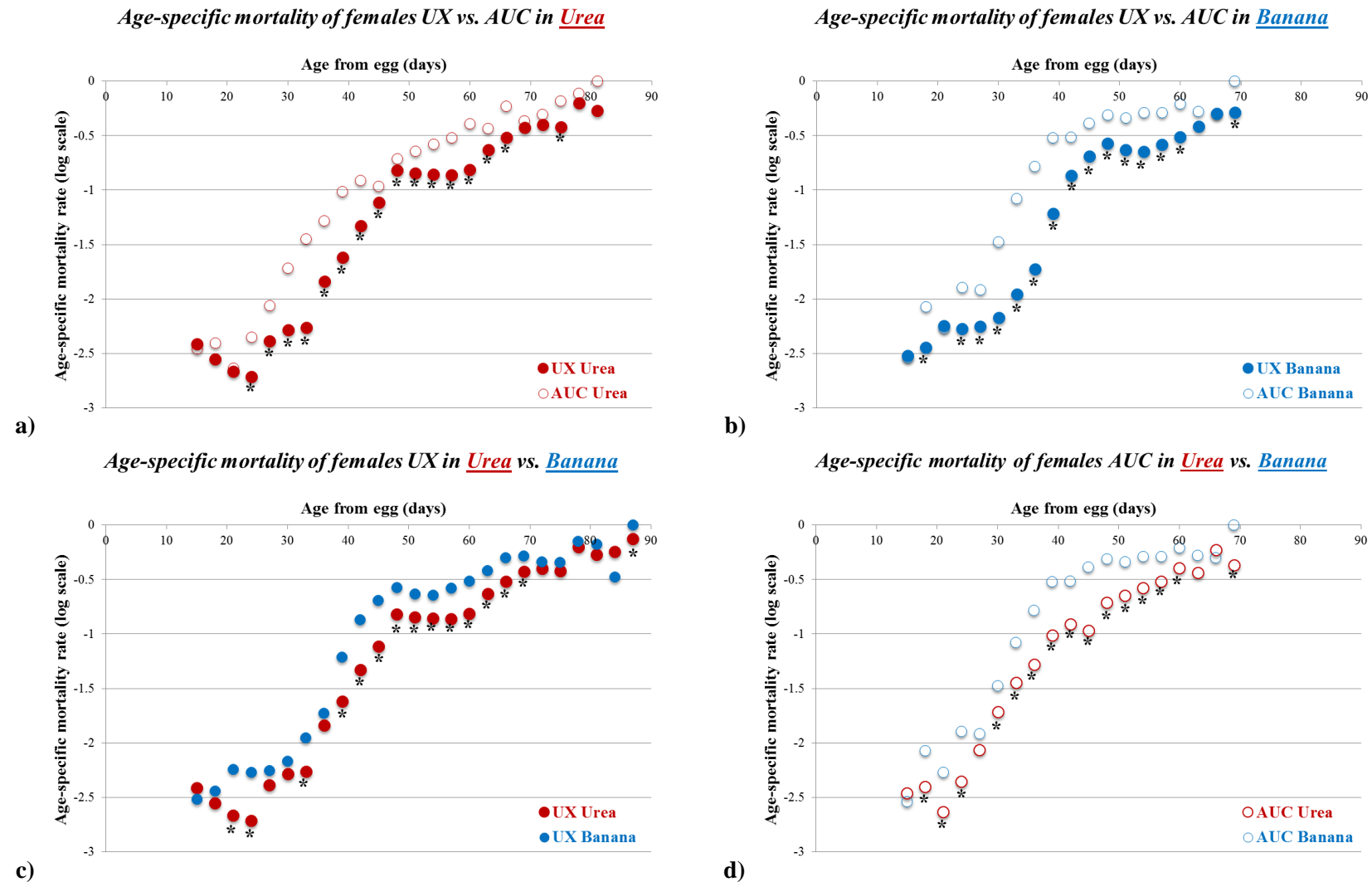
Figure 6.7. Age-specific mortality rates for females of **(a)** UX and AUC in urea, **(b)** UX and AUC in banana, **(c)** UX in urea and banana, and **(d)** AUC in urea and banana. Data shows the average log-transformed three-day age-specific mortality computed as the fraction of deaths over the remaining cohort. Significant differentiation in age-specific mortality between each pair of *regime*diet* is marked with an asterisk.

DISCUSSION

The experiments reported in this chapter came as an attempt to test whether Hamiltonian waves of age-dependent adaptation to novel conditions occur in a well-defined laboratory environment. To do so, we resorted to a replicated system of urea-adapted populations created by Mueller and collaborators two decades ago.

Consistent with what has been previously seen by Joshi *et al.* (1996b), exposure to environmental urea increases longevity in experimental cohorts of *Drosophila melanogaster*, regardless of the evolutionary history of the populations (**Figures 6.1** and **6.3**). Once again, the increase in longevity seems to be achieved by lowering the age-independent mortality rates rather than altering the rate of aging (**Table 6.4**, **Figures 6.4** and **6.5**, Joshi *et al.* 1996b). Of greater interest is that the urea-adapted UX populations have reduced mortality levels when exposed to urea, compared to their matched AUC controls (**Figures 6.6a** and **6.7a**), again confirming previous independent findings by Joshi *et al.* (1996b, 1998).

According to Hamilton's (1966) forces of natural selection, the weight of selection on life-history is heavy at early ages, falling with time, as the force of natural selection declines with chronological age. In these experiments, we did not find the fading of adaptation to urea with adult age, as the differentiation of mortality rates did not decrease with age in urea-exposed cohorts (**Figures 6.6a** and **6.7a**); interaction *age*regime* **Table 6.7**). Hence, our first hypothesis of age-dependence of dietary adaptation is not corroborated with respect to age-specific mortality rates. Nonetheless, this finding does not preclude the validation of this hypothesis for other fitness-related traits, such as age-specific fecundity, not measured in this study. Furthermore, our second hypothesis concerning the effect on aging and late-life of returning long-selected populations to their ancestral environment also lacked verification from these experimental data. On the ancestral banana medium, the urea-adapted populations had overall reduced mortality rates compared to the banana-adapted control populations (**Figures 6.6b** and **6.7b**; factor *regime* **Table 6.8**), and that feature was irrespective of age (interaction *age*regime* **Table 6.8**). If costs of adaptation were involved (Kassen 2002, Kawecki & Ebert 2004) we would expect that urea adaptation would lead to reduced performance on the ancestral banana medium, in particular at young ages, while at older ages the performance might improve when returning to the ancestral environment. Yet again, the fact that this was not observed for mortality rates does not preclude the possibility that other life-history characters behave in accordance with our second hypothesis.

Final remarks

The present results provide no support for Hamiltonian expectations with respect to age-specificity of adaptation, where greater adaptation should occur at early ages due to stronger forces of selection. There is also no evidence that adapting to urea involves any cost for functional performance under ancestral conditions. Indeed, it seems that adaptation to the novel environment enhanced performance on the ancestral environment, a wholly unexpected result.

It is important to note that we have not adequately characterized the range of life-history characters that might be involved in the adaptation to urea. Specifically, this system has been well-characterized for a striking benefit to longevity from reduced fertility (Joshi *et al.* 1996b; 1998). What we may be seeing are the benefits to longevity that arise from impaired fecundity, among other measures of adult reproductive activity. At this point, we have insufficient information to decide between several alternatives. More experiments involving other characters (and possibly embarking on the metabolomics train) are needed to clarify the scientific importance of the results presented here, shedding brighter light on the evolutionary features of age-specific adaptation.

Chapter Seven.

General Discussion

Understanding the several factors that constrain evolution is one of the long-standing questions in evolutionary biology, ever since the establishment of the evolutionary genetic paradigm in the twentieth century (Fisher 1930; Wright 1931). When a population colonizes a new environment, it faces several challenges that will limit and eventually impede the success of the colonization event: *(i)* the imposition of entirely novel environmental conditions to which the colonizers are not adapted, *(ii)* the effective population size (N_e) of the colonizing population, *(iii)* the age-specific demography, such as the age-distribution of the colonizers, and *(iv)* the hybridization with residents and/or subsequent migrants from the source population. Later on, if the successful colonizers return to their ancestral environment, similar issues may again impinge on their success (Carson & Templeton 1984; Wade & McCauley 1988; Allendorf *et al.* 2013; Santos *et al.* 2013). The main focus of this thesis was to study the constraints of adaptation on colonization success, by means of experimental evolution with a rigorous and highly-replicated design (see **Figure 7.1**), using *Drosophila melanogaster* stocks with a well-known evolutionary history.

This thesis, divided in five experimental chapters, aimed to answer ten evolutionary questions relevant for colonization of a new habitat: *(i)* How do populations respond to novel demographic conditions? *(ii)* How will life-history evolve in response to new, harsh conditions? *(iii)* How will the N_e affect the evolutionary response to selection? *(iv)* Will populations from the same ancestral source population evolve the same way? *(v)* What are the relative roles of history, chance, and selection in shaping the populations' evolution? *(vi)* How does hybridization at different N_e affect the evolutionary dynamics of populations under directional selection? *(vii)* Will sustained small N_e hinder the process and outcome of reverse colonization? *(viii)* What is the impact of novel challenges, like diet change, during a colonization event? *(ix)* Is dietary adaptation age-specific? And, finally, *(x)* Will long-term adaptation to novel, harsh conditions lead to fitness loss in the ancestral environment?
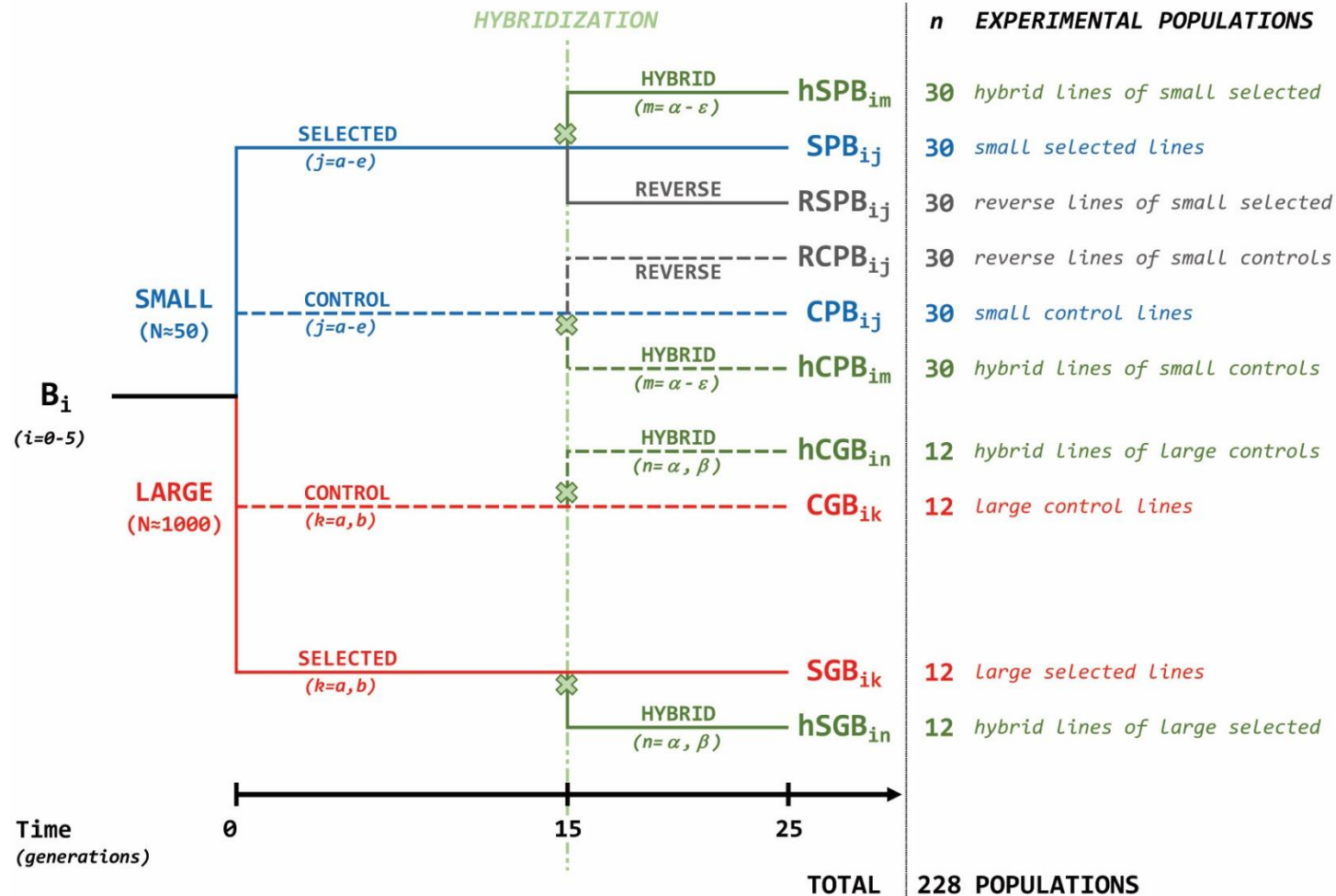
Figure 7.1. Phylogeny of the experimental populations. Each B_i ancestor gave origin to ten small-sized (P; N ≈ 50) and four large-sized populations (G; N ≈ 1000), half of which were selected for starvation resistance (S) while the other half were used as controls (C). After 15 generations of forward selection, all populations were hybridized (h) within each combination of selection, size, and B_i ancestor; starvation resistance selection was then resumed among all the S lines, both hybridized and unhybridized. Also after 15 generations of forward selection, derivatives of the small populations (SPB_{ij} and CPB_{ij}) were created and a reverse-selection experiment (R) was started. N: census size of each line; n: number of populations (lines).

It looks like size really matters…

The set of experiments reported in *Chapter Two*, the central study of this thesis, responds to the first five questions through the largest-scale and highest-replication experiment ever done in Mendelian populations. The results are summarized in **Figure 7.2.**

First, the idiosyncrasies of the forward selection protocol caused demographic differences between the B_i ancestors and the experimental populations, with a longer life cycle in the latter. These differences led to a secular increase in starvation resistance and an antiparallel decline in fecundity in the control lines (**Figure 7.2a**), a pattern previously observed in other experiments in the Rose lab (*e.g.* Service & Rose 1985; Hutchinson *et al.* 1991; Rose *et al.* 1992).

Second, the life-history evolution of our populations corroborated theoretical predictions and previous findings (*e.g.* Rose *et al.* 2004): a clear temporal increase of starvation resistance as a response to strong directional selection with a concomitant decline of early fecundity (**Figure 7.2b**).

Third, and on one hand, in the absence of strong directional selection a drastic reduction in population size resulted in a significant decline in fecundity, showing clearly the loss of function caused by small N_e (**Figure 7.2c**). On the other hand, the direct response to said selection was significantly reduced in magnitude in the smaller lines (**Figure 7.2d**). These results matched the general expectations of lower fitness due to depressed N_e, as smaller populations are subjected to more severe inbreeding and loss of heterozygosity due to stronger drift, with consequently less effective selection (Woodworth *et al.* 2002; Willi et *al.* 2006; Hartl & Clark 2007; Hare *et al.* 2011; Hoffmann *et al.* 2017). The secular decline of early fecundity might be less pronounced in small lines due to the starvation-fecundity trade-off, but sharper due to inbreeding depression. These opposite evolutionary forces lead to unclear expectations. The smaller lines showed a steep decline in the trait value, due to the combination of inbreeding and the previously described trade-off between starvation resistance and fecundity (**Figure 7.2e**). Nevertheless, the large lines raise a paradox that we are not able to disentangle. Despite the absolute drop in fecundity between initial and last generations (**Figure 7.2e**), the analysis of the control-corrected data showed quite inconsistent trends across replicates, which raises the question: if the trade-off is manifested in small lines, why is it not consistently present in the large lines that clearly responded with increased starvation resistance (see heterogeneity of the evolutionary trajectories in **Figure 2.8c**)?

Fourth, we started this experiment with multiple source populations, the six B populations, and we derived several replicate lines from each. At a minimum, variation among these B populations is expected to occur due to the secular drift among them, with a loss of genetic variation from individual populations. It is also expected that through time there will be an increase in differentiation between populations derived from the same B ancestor thanks to genetic drift (*e.g.* Hänfling & Brandl 1998). At the end of the experiment, the greater the strength of drift, the greater the expected divergence between populations. Our results corroborated these predictions, with a significantly higher variance among smaller populations compared to that among larger populations. Under strong directional selection, the classic expectation is a reduction of between-population variance, as higher fitness alleles increase in frequency while the least-adapted are counter selected (Lynch & Walsh 1998). Nonetheless, uniform selection combined with genetic drift may result in the fixation of different alleles causing higher divergence than drift by itself (Cohan 1984; Cohan & Hoffmann 1986). This is more evident when selection acts on rare alleles and/or the underlying genetic basis is polygenic (Cohan & Hoffman 1989). While selection increased the variance among large populations, as predicted by Cohan (1984), that was not observed in small populations where such effect should have been strengthened by drift (**Figure 7.2f**). It is an open question what the causes of such contrasting patterns among populations of different sizes are. Future studies including genomic analysis of the underlying genetic basis of adaptation and diversification may help clarify this issue.

Finally, the relative roles of history, chance, and selection in shaping evolution is a long-standing topic for debate among evolutionary biologists. Previous experimental work has shown that these factors depend on how close a trait is to Darwinian fitness, as well as the biological level under analysis (Travisano *et al.* 1995; Teotónio & Rose 2000; Teotónio *et al.* 2002; Joshi *et al.* 2003; Blount *et al.* 2008; Bedhomme *et al.* 2013; Fragata *et al.* 2014a,b; Spor *et al.* 2014; Seabra *et al.* 2018). Besides corroborating these findings, our experiments combining strong selection and high replication show that history and chance play preponderant roles in smaller populations, but are quickly overrun by selection, particularly in larger populations.
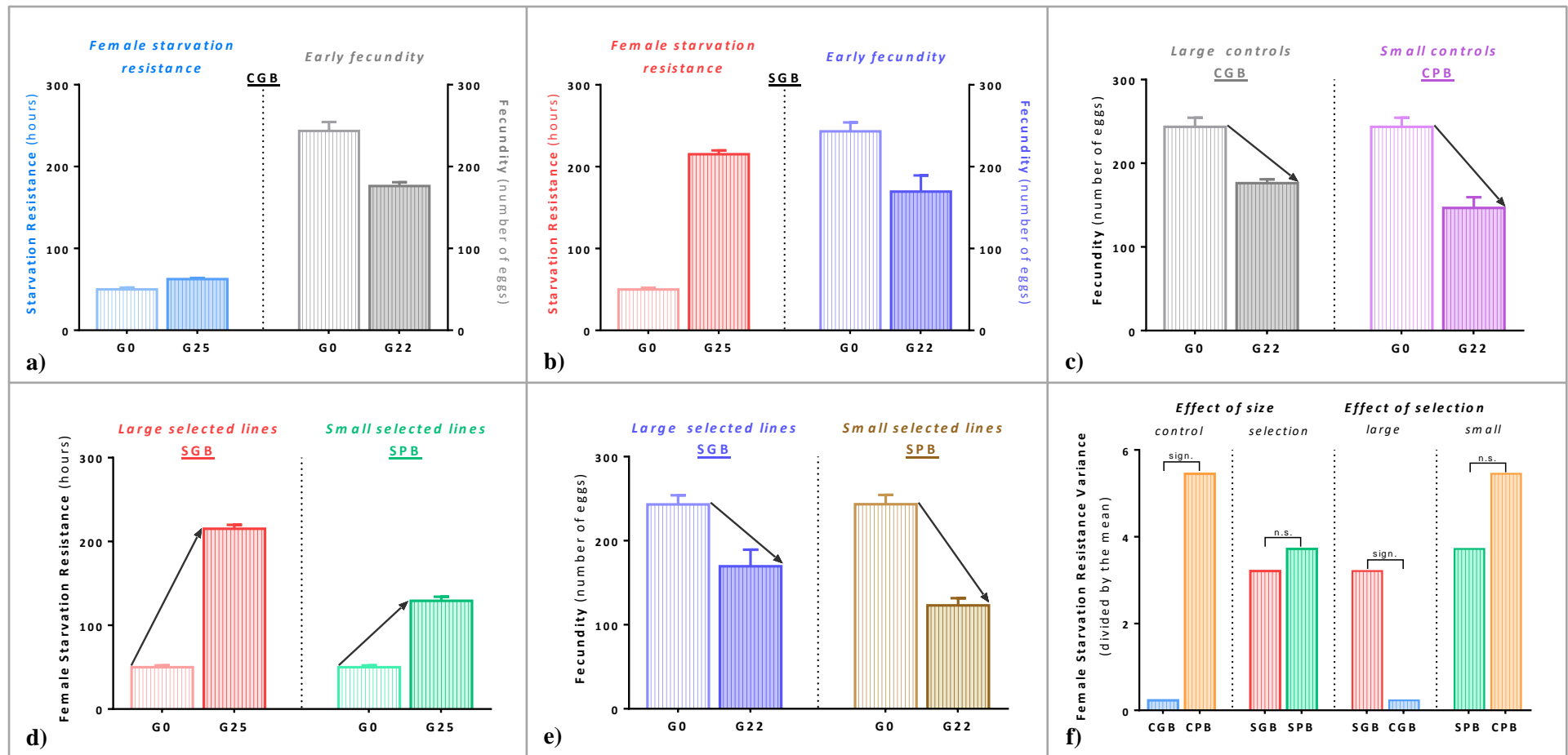
Figure 7.2. Summary of Chapter Two main results. **a)** Secular increase in starvation resistance and decline in early fecundity of CGB controls; **b)** Temporal increase of starvation resistance and decline of early fecundity of SGB lines, as response to strong directional selection; **c)** Fitness loss in CPB lines due to small N_e (sharper temporal decline than CGB controls); **d)** Impaired response of SPB to strong directional selection due to small N_e (lower rate than SGB); **e)** Steep decline in early fecundity of SPB and paradoxical non-significant trend of SGB; and **f)** Diverging effect of size in control (but not in selected lines) and diverging effect of strong selection in large (but not small lines). In **a-e)** data shows average regime values for females and error bars denote standard error of mean computed as differences between history; in **f)** data shows variance of each regime standardized by the mean. *sign.* – significant; *n.s.* – not significant.

Hybridization: the good, the bad, and the unpredictable.

Interpopulation hybridization has been offered as a strategy to ameliorate the effects of inbreeding depression, thereby increasing the fitness of declining populations, and decreasing extinction risk (Tallmon *et al.* 2004; Hedrick 2005; Edmands 2007; Frankham *et al.* 2011; Hedrick *et al.* 2011). Nevertheless, the complex genetic architecture of fitness-related traits, and the multiple factors that can interfere with the outcome of a hybridization event, make it extremely difficult to predict the evolutionary consequences of an interpopulation cross in any given scenario (Allendorf *et al.* 2013). The highly-replicated experimental design used in *Chapter Three* experiments was an attempt to understand the evolution of populations under different combinations of directional selection, population size, and gene flow. In particular, we were interested in analyzing how hybridization after sustained small N_e impacted the response to continued strong selection.

Fifteen generations of the combined effects of drift and selection were expected to cause an increase in homozygosity by descent and population differentiation, with a much stronger effect in the small lines. Hybridization was expected to restore some lost heterozygosity, causing a general increase in the values of functional traits – *heterosis* (Falconer & Mackay 1996; Hartl & Clark 2007). In general, most populations (except the large controls) slightly improved their resistance to starvation and worsened their early fecundity immediately after an interpopulation cross. If pervasive inbreeding depression was affecting the experimental populations, we would expect the general improvement of all traits. Our observations fit a model where a fecundity-starvation trade-off is allied to the heterosis dependence on directional dominance (Falconer & Mackay 1996; Roff 1997; Lynch & Walsh 1998), where the genes responsible for the trade-off have dominance effects *in the same direction* (see **Figure 3.8**). In contrast, hybrid females of the large controls showed a slight decrease in starvation resistance. It remains unknown whether this can be explained by means of *outbreeding depression*. The hybridization event could conceivably have broken up *coadapted gene complexes*, resulting in lower fitness of the resulting hybrids (Templeton 1986; Lynch 1991; Fenster *et al.* 1997; Lynch & Walsh 1998).

The subsequent evolutionary consequences of the interpopulation cross were even less straightforward. Hybridization caused little to no effect on the large experimental lines under control conditions, which is not surprising due to the inverse relationship between heterozygosity loss, as well as population differentiation, and N_e (Wright 1951; Hartl & Clark 2007). But it is somewhat unexpected in small lines, also with no apparent effect of

hybridization on starvation resistance. In contrast to starvation resistance, these small hybrid controls showed an immediate drop in early fecundity followed by a sharp temporal increase, reaching the parental stock value. This could be explained by means of outbreeding depression and further temporal alleviation of the negative effects of hybridization. Nonetheless, outbreeding depression is not a classical expectation when populations of small sizes under similar selection regimes are considered (Ralls *et al.* 2013).

The contribution of hybridization to the direct response to selection in different N_e conditions was the subject we were most interested in. Our findings were a little puzzling: although all experimental populations showed the expected temporal increase in starvation resistance, no significant effect of hybridization was found in either large or small lines (**Figure 7.3**). The loss of genetic variants for starvation resistance could have been consistent across populations (*e.g.* rare alleles), making them very similar and, thus, insensitive to hybridization. However, the small populations showed temporal differentiation during the forward selection experiment (*Chapter Two*), which contradicts the hypothesis stated above. Perhaps small populations lost *environmental homeostasis* due to inbreeding, increasing population environmental variance (Fox & Reed 2010). Such an effect might have led to higher interpopulation variance in small compared to large lines, without necessarily increasing additive variance after hybridization. Another hypothesis depends on the genomic foundations of life-history traits (Flatt & Heyland 2011). In particular, it is commonly found that experimental evolution among outbreeding Mendelian populations does not commonly lead to allelic fixation in genome-wide analyses (Chevin & Hospital 2008; Burke *et al.* 2010; Phillips *et al.* 2016; Graves *et al.* 2017; Seabra *et al.* 2018). This new paradigm of evolutionary genetics suggests that small N_e have less effect on evolutionary outcomes than expected in conventional population genetic theories. Genomic analysis on the experimental populations presented here could further inform us on this matter.
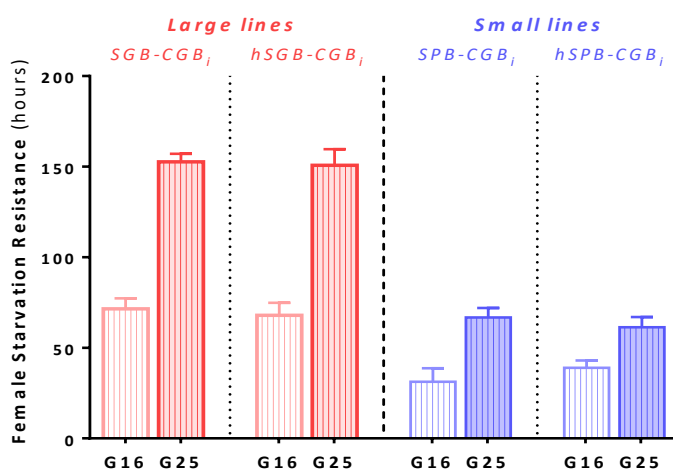
Figure 7.3. Evolutionary response to starvation resistance selection of parental and hybrid lines under different N_e. Data shows average CGBi-corrected values for each regime and error bars denote standard error of mean computed as differences between history.

As far as we know, no comparably scaled experiments have been done by others under such carefully controlled conditions. We thus hypothesize that conventional theory (*e.g.* Falconer & Mackay 1996; Hartl & Clark 2007) concerning the effects of hybridization and population size may have survived up to this point mainly due to a lack of experiments of sufficiently powerful design to test its predictions. Whether a stronger bottleneck, a longer-term experiment, or a post-cross population expansion would change the impact of hybridization that we report here remains unknown. More experiments may give us further insight on this matter.

Sustained bottlenecks may shape the ability to reverse-colonize.

Chapter Four replicated a scenario of reverse colonization, where populations with different selection history migrate back to their prior environment. Experiments using moderately large N_e previously done in the Rose lab showed that relaxing selection on starvation resistance leads to a clear evolutionary reversion of the character (*e.g.* Service *et al.* 1988; Graves *et al.* 1992; Teotónio & Rose 2000; Passananti *et al.* 2004a). We wanted to analyze the consequences of, after forward selection, sustaining the small N_e during the first 21 generations of reverse selection. We found that reverse evolution to B-ancestors was not impeded by the sustained bottleneck, but featured contingencies on character and/or population. We found cases where we saw: (1) rapid and complete reversion; (2) fast response with partial convergence; and (3) steep convergence, followed by divergence, with a superior outcome to that of the ancestor, three of the possibilities outlined in Teotónio & Rose (2001). Our major findings are summarized in **Figure 7.4.**

First, the rapid and complete reversion of starvation resistance was achieved by males (though not females) of the previously weakly selected stock, RCPB (**Figure 7.4a**). Nevertheless, there was considerable heterogeneity in the rate of the individual population response. This between-population variation was probably due to genetic drift fostering the divergence of small-sized lines, as predicted by classical population genetic theory (Falconer & Mackay 1996). The same pattern was seen in RCPB populations' early fecundity, which can be explained by (1) the character's direct relationship with fitness and (2) the extensively documented antagonistic pleiotropy between starvation resistance and early fecundity (Hutchinson *et al.* 1991; Rose *et al.* 1992; Leroi *et al.* 1994a,b; Chippindale *et al.* 1996; Passananti *et al.* 2004a).

Second, rapid reversion without reaching full convergence to the ancestor was seen in the starvation resistance of the previously forward-selected stock, RSPB (**Figure 7.4b**). One explanation might be that our experimental populations were not given enough time to allow reverse evolution to conclude the process of convergence. In fact, other experiments in the Rose lab, sustained for more than 100 generations, are revealing a general tendency to convergence (Burke *et al.* 2016; Graves *et al.* 2017), although involving other traits. Nevertheless, Teotónio & Rose (2000) found incomplete reversion of starvation resistance in other large-sized lines that were first selected for starvation resistance, and then reverse-selected for 50 generations. The fact that, in that paper, the response of starvation-selected lines stalled after a dozen generations suggests that the evolutionary reversion process changes slope and eventually allows later convergence. We did not detect such plateauing in our study covering 21 generations of reverse evolution, which might be a consequence of a slower evolutionary process due to the small size of our populations.

Finally, the fecundity of the RSPB group of populations not only equaled that of its B ancestors, but even significantly overcame it (**Figure 7.4c**; see also **Figure 4.5**). This *super fly* phenotype may be due to several non-exclusive hypotheses. On the one hand, the genetic architectural changes undergone during the populations' past evolutionary history, previously inaccessible to the ancestor, pushed the populations to a novel adaptive peak (Wright 1977; Lenski 1988b; Teotónio & Rose 2001). On the other hand, the experimental lines during reverse selection were not subjected to the *exact* same environment as the ancestor, but in closer conditions to the assay environment, giving them an advantage comparatively to the ancestral B flies. Such an evolutionary impact of a genotype-by-environment interaction has been previously found in the Rose lab, explaining the apparent paradoxical disappearance of a trade-off involving selection for aging (Leroi *et al.* 1994a,b; Rose *et al.* 1996). Moreover, fecundity could also be trading-off with other life-history characters and the visible benefit in fecundity is being achieved at the cost of other unseen, untested traits.

We were not able to investigate the genetic mechanisms underlying these reverse evolution patterns. Further experiments involving different traits, more generations under small and large N_e, and genomic analysis will allow us to deepen our knowledge on the *tempo* and *mode* of reverse evolution.
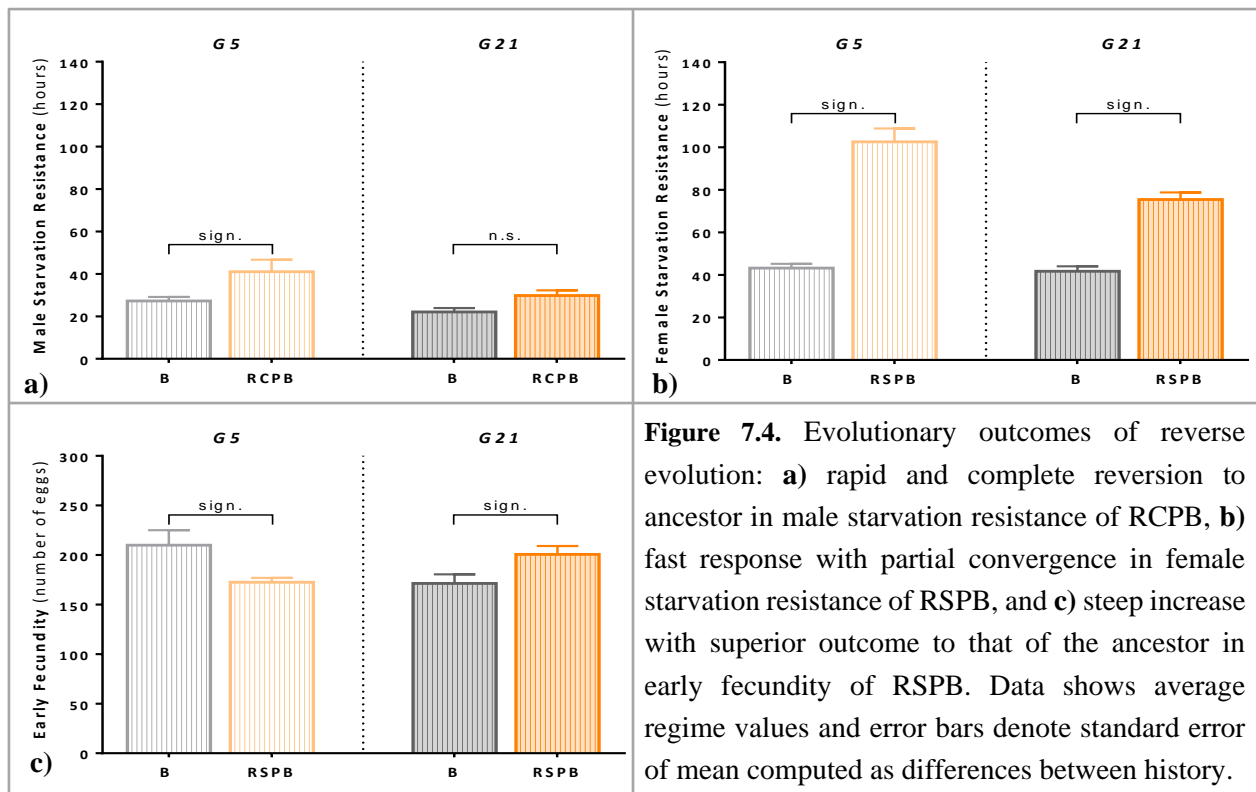
Figure 7.4. Evolutionary outcomes of reverse evolution: **a)** rapid and complete reversion to ancestor in male starvation resistance of RCPB, **b)** fast response with partial convergence in female starvation resistance of RSPB, and **c)** steep increase with superior outcome to that of the ancestor in early fecundity of RSPB. Data shows average regime values and error bars denote standard error of mean computed as differences between history.

Dietary challenges during colonization and reverse colonization

According to Hamilton's analysis of the forces of natural selection (1966), the impact of selection is heavy at early ages, and then falls with time, as the force of natural selection declines with chronological age. We can, thus, expect adaptation to a novel environment – like a different diet during a colonization event – to proceed very effectively at early ages, but to be progressively reduced at later adult ages. The *Chapter Five* and *Chapter Six* experiments provide an attempt to test the evolutionary effect of dietary change on age-dependent adaptation to a novel environment. As far as we know these were the first experimental tests of the *"Hamiltonian wave"* hypothesis for adaptation, which opened doors into a novel and important line of research for evolutionary biology.

First, our results show that very small changes in diet, like switching the fruit in which the food is based to orange or avocado, can have significant effects both on overall longevity and age-specific mortality rates, which is not surprising due to the lack of adaptation to these novel environments (see **Figure 5.3**).

Second, and following the Hamiltonian reasoning, a change of diet should have a stronger effect the earlier the switch occurred. Our results did not corroborate this expectation but indicated that the amount of time with the new nutrient was the main factor increasing

mortality rates (*e.g.* see **Figure 5.12a** and **f**). In this experiment, there was a direct association between *how early* the switch of nutrient occurred and *how long* it was imposed, because after the switch the diet remained the same for the rest of the cohort's life. This feature prevented us from accurately testing whether age, *per se*, affects how flies survive on a novel diet. To do so we would have needed more age-dependent diet-switch combinations, with diets differing in the age of the change and the amount of time under different nutrients.

Third, and to further investigate the Hamiltonian age-specificity of diet adaptation, we took advantage of a *Drosophila* stock system created by Mueller and collaborators which consisted of a replicated stock of flies that were exposed to urea (UX) for hundreds of generations, but only at young ages, and their matched controls (AUC), never exposed to the nitrogenous waste. The establishment of these stocks provided an accidental experiment in early-life adaptation to a relatively toxic diet. Our results showed that exposure to environmental urea increased longevity, in an age-independent manner and regardless of the evolutionary history of the populations (see **Figure 6.3**). We further showed that the urea-adapted populations, when exposed to urea, had lower mortality levels than their matched controls. Both findings were consistent with those of Joshi *et al.* (1996a, 1998). On the hypothesis of age-specific Hamiltonian waves of natural selection, we would expect a loss of differentiation of mortality rates with age between urea-adapted and non-adapted populations, when exposed to urea. Although we did not find the predicted age-specific decrease in differentiation (**Figures 6.6a** and **6.7a**), this doesn't mean this hypothesis is not verified for some other type of life-history character, such as age-specific fecundity (*e.g.* Burke *et al.* 2016), that we were not able to adequately characterize.

Finally, our last question concerned another possible scenario of reverse colonization, where large populations long-selected for more than 200 generations to novel stressful conditions (presence of urea) returned to their ancestral nutritional environment (absence of urea). Here the UX-AUC system has proven very useful, considering urea-supplemented food the novel diet and the regular banana medium the ancestral one. In a Hamiltonian scenario, and if costs of adaptation are involved (Kassen 2002, Kawecki & Ebert 2004), we would expect that urea adaptation would lead to reduced fitness of the UX populations in the ancestral environment, particularly at early life. Conversely, at older ages, the performance may improve when returning to the ancestral environment. Our results showed that, irrespective of dietary environment and age, urea-adapted populations had reduced mortality rates when compared to the banana-adapted controls (**Figures 6.6a,b** and **6.7a,b**), providing no support for Hamilton's

(1966) expectations (or for costs of adaptation, for that matter). Once again, this does not preclude the possibility that other life-history characters behave in accordance with the age-specificity of adaptation, where greater adaptation should occur at early ages due to stronger forces of selection.

Future research endeavors

The large and complex set of experiments presented in this thesis were an attempt to respond to some fundamental evolutionary questions that are relevant in a colonization scenario, particularly targeting the impact of population size. Despite the high replication and intricate experimental design, many questions remain unanswered and are worthy of further research. The natural next step will be to use high-throughput next-generation sequencing to analyze frozen samples collected from the laboratory populations subjected to starvation selection. Analyzing the genomic changes that underlie forward and reverse evolution, with and without gene flow, will give us a better understanding of how history, selection, and effective population size shape evolution during colonization. Finally, we are aware that the experimental tests of the Hamiltonian wave of adaptation presented here were undeniably basic. Experiments involving more traits, other diets, allowing food switches every few hundreds of generations, and going deep on the metabolomics will, most likely, provide crucial knowledge on the *tempo* and *mode* of age-specific adaptation.

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