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ABSTRACT

Speciation results from the evolution of reproductive isolation between populations. Reproductive barriers can evolve as a direct product of local adaptation, in which individuals discriminate to avoid less fit progeny, or as a by-product of such adaptation. *Drosophila subobscura* possesses fascinating latitudinal clines for several quantitative traits. However the neutral genetic differentiation among populations is low. Therefore, the maintenance of such clines suggests that reproductive barriers exist between populations. The main goal of the present work was to test whether reproductive barriers between populations from two extremes of the cline exist and, if so, if they decrease or increase over time when these populations invade a new common environment. For that we founded two populations of *Drosophila subobscura* from Adraga (Portugal) and Groningen (Netherlands). First, the initial differentiation and early adaptation in life-history traits of both populations were characterized. We found that during the first 11 generations the populations showed differences in several life-history traits. However, in general, the populations of both foundations did not exhibit temporal changes across generations. As for the reproductive barriers we found that at an early (fifth) generation, both populations demonstrated a (marginally significant) preference for assortative mating. However, hybrid breakdown was not detected among populations. Five generations later, assortative mating faded away, indicating a relaxation of the selective pressures in the new environment. This study was important as it suggests that, while pre-zygotic barriers may play a role in the maintenance of a latitudinal cline, they fade away quickly during adaptation in a novel, common environment. The study also revealed the need to add a temporal component to studies of reproductive isolation.

Key-words: Laboratory adaptation; latitudinal cline; reproductive barriers; *Drosophila subobscura*; mating behaviour.

RESUMO

A especiação resulta da evolução de mecanismos de isolamento reprodutor entre populações. Estes mecanismos podem ser classificados como pré-zigóticos ou pós-zigóticos. Os mecanismos pré-zigóticos têm como função impedir cruzamentos interespecíficos, prevenindo o fluxo génico. Uma das barreiras pré-zigóticas mais importantes é o acasalamento preferencial, em que os indivíduos acasalam preferencialmente com indivíduos semelhantes. Por outro lado, os mecanismos pós-zigóticos reduzem o sucesso dos cruzamentos interespecíficos. Como resultado de cruzamentos interespecíficos pode ocorrer 'hybrid breakdown', um fenómeno que consiste numa menor fitness por parte da progenitura de populações diferenciadas em relação às populações parentais.

As barreiras reprodutivas podem evoluir através de diferentes processos. Um deles ocorre quando as barreiras reprodutivas evoluem como resultado da adaptação ao ambiente como, por exemplo, quando populações isoladas desenvolvem diferentes rituais de acasalamento. Portanto, se indivíduos destas populações se encontrarem, a acumulação dessas diferenças impediria o acasalamento. Em populações alopátricas, os indivíduos também se podem adaptar a diferentes ambientes, o que pode levar a barreiras pós-zigóticas. Outro mecanismo é quando a selecção actua directamente sobre os rituais de acasalamento, pois quando as populações estão localmente adaptadas, estas irão beneficiar se não acasalarem com imigrantes. No entanto, é de esperar que a evolução de barreiras pré-zigóticas seja acelerada na segunda situação.

Ao longo de um cline latitudinal, como há uma sugestão de adaptação local devido a uma mudança gradual nas diversas características, barreiras reprodutivas podem surgir, mesmo na presença de fluxo génico. A existência de barreiras reprodutivas é frequentemente estudada num momento no tempo. Além disso, existem poucos estudos que utilizam populações com diferentes backgrounds genéticos para inferir se estas estão reprodutivamente isoladas quando se encontram no mesmo ambiente. Seria interessante estudar se há barreiras reprodutivas entre populações com diferentes backgrounds genéticos e como evoluem ao longo do tempo, quando introduzidas num novo ambiente comum. Ainda há dúvidas sobre os principais tipos e causas de especiação; especificamente, como barreiras reprodutivas evoluem e são mantidas ao longo do tempo, bem como de que modo as barreiras reprodutivas evoluem entre populações expostas às mesmas condições ambientais.

A *Drosophila subobscura* é uma boa espécie para estudar esta temática, pois possui populações parcialmente diferenciadas em extremos de um cline latitudinal. Originalmente uma espécie paleárctica, a *D. subobscura* colonizou a América do Sul e mais tarde a América do Norte. Esta espécie possui um cline latitudinal para várias características, nomeadamente as frequências de inversões cromossómicas e tamanho do corpo. A manutenção desse cline ao longo dos anos e o seu aparecimento independente nos continentes americanos sugerem que existe selecção a operar latitudinalmente ao nível dessas características. Além disso, a diferenciação genética neutral entre as populações ao longo do cline é baixa. Como as populações diferem nas características adaptativas latitudinalmente, algum tipo de barreiras reprodutivas deve existir para manter o cline, ou seja, a diferenciação das populações, ao longo do tempo.

Matos e colaboradores estudam a adaptação ao laboratório da *Drosophila subobscura* há cerca de 20 anos. Os vários estudos têm em comum o facto de as populações, que se estão a adaptar ao laboratório, aumentarem o seu desempenho em relação às características relevantes para a fitness ao longo do tempo. No entanto, tanto o desempenho inicial das populações como a sua taxa de adaptação variou entre

fundações, sendo mais contrastantes entre populações provenientes de diferentes localidades e com diferentes anos de fundação, particularmente para as características menos relevantes para a fitness. Além disso, algumas características não demonstraram padrões consistentes de melhoria ao longo do tempo, nomeadamente resistência à inanição, tempo de desenvolvimento e viabilidade juvenil.

Os principais objectivos deste estudo foram determinar a possível existência de barreiras reprodutivas entre populações de dois extremos do cline latitudinal e, caso existissem, seguir a sua evolução ao longo da adaptação das populações a um novo ambiente comum, o laboratório.

Para tal, foram fundadas duas populações originárias de dois locais com diferentes latitudes, Adraga (Portugal; 38° 47'N, 9° 28'W) e Groningen (Holanda; 53° 21'N, 6° 55'E). Antes de determinar se existiam barreiras reprodutivas entre estas populações, testou-se a diferenciação entre as duas populações no novo ambiente; determinou-se se as populações se estavam a adaptar ao ambiente do laboratório e, se tal se verificasse, se o padrão adaptativo indicava convergência ou contingências evolutivas associadas a um diferente fundo genético inicial. Análise da dinâmica evolutiva de populações oriundas de latitudes contrastantes também em si interesse, de forma a testar a reversão de um cline latitudinal quando as populações se adaptam a um novo ambiente comum. As populações da Adraga (Portugal) e de Groningen (Holanda), sendo originárias de latitudes extremas do cline europeu, apresentam diferenças nas frequências de polimorfismos cromossómicos que podem indicar adaptação a diferentes condições climáticas. Seria, portanto, espectável que as populações apresentassem diferenças em diversas características relacionadas com a fitness, devido à sua adaptação ao ambiente específico. As diferenças genéticas destas populações poderão traduzir-se em diferenças de desempenho num novo ambiente comum. De facto, as populações apresentaram diferenças em várias características da história da vida nas primeiras 11 gerações estudadas. Contudo, as populações de ambas as fundações não exibiram, em geral, mudanças temporais ao longo das gerações. Possivelmente, com futuros ensaios será detectada uma tendência evolutiva.

Após se verificar que havia alguma diferenciação inicial entre populações, testou-se se haviam ou não barreiras reprodutivas entre as populações da Adraga e de Groningen. Para tal, realizaram-se quer ensaios de comportamento de acasalamento quer cruzamentos entre as duas populações, feitos para determinar se havia uma preferência na escolha de parceiros sexuais por fêmeas, e se os híbridos tinham ou não um menor desempenho do que as populações parentais. Verificou-se que, na geração 5, as fêmeas das populações Adraga e Groningen demonstraram uma preferência de acasalamento com machos da sua própria população. Nos ensaios nos quais a fêmea não tinha a hipótese de escolha, houve diferenças entre os dois tipos de machos, tanto na latência da corte como na duração do acasalamento. Os machos de Groningen demoraram menos tempo em média a iniciar a corte do que os machos Adraga. Para além disso, as fêmeas da Adraga acasalaram durante mais tempo com os machos da sua própria população do que com machos de Groningen. Nos ensaios nos quais a fêmea podia escolher o macho com quem acasalar, houve um maior número de acasalamentos homogâmicos que heterogâmicos. Verificou-se ainda que os machos de Groningen acasalaram um maior número de vezes do que os machos da Adraga. No entanto, não foi detectado 'hybrid breakdown' entre as populações. Isto pode dever-se ao facto de estas populações fazerem parte de uma população contínua que atravessa a Europa e que, nestes casos, uma barreira de comportamento de acasalamento evolui primeiro do que a esterilidade e/ou inviabilidade de híbridos. Um segundo conjunto de ensaios de comportamento de acasalamento foi feito após várias gerações de adaptação ao laboratório, para determinar se esta adaptação levaria a mudanças na barreira reprodutiva etológica como, por exemplo, redução devido ao relaxamento das forças selectivas ou aumento devido a

contingências evolutivas. Entre os ensaios realizados nas gerações 5 e 10, o acasalamento preferencial desapareceu, indicando um relaxamento das pressões selectivas no novo ambiente comum.

Em resumo, este estudo revelou-se pertinente na medida em que estabeleceu que as populações do extremo do cline estão diferenciadas e que existem barreiras reprodutivas pré-zigóticas entre as mesmas. Isto indicia que barreiras reprodutivas comportamentais estarão provavelmente envolvidas na manutenção desse mesmo cline ao longo do tempo. Estas mostraram-se, no entanto, bastante incipientes, sendo que desapareceram ao fim de apenas cinco gerações de selecção. Sendo assim, se neste estudo não estivessem envolvidos ensaios em duas gerações diferentes, não se teria qualquer conhecimento do desaparecimento das barreiras reprodutivas. Isso implica que todos os estudos de isolamento reprodutor têm, à partida, necessidade de ter uma componente temporal, de modo a determinar a presença ou não de barreiras reprodutivas se mantém ao longo do tempo.

Palavras-chave: Adaptação ao laboratório; cline latitudinal; barreiras reprodutivas; *Drosophila subobscura*; comportamento de acasalamento.

CONTENTS

GENERAL INTRODUCTION

Along a latitudinal cline, populations are adapted to their specific environment. Moreover, the differences between populations are gradual along the cline, hence there is probably gene flow between them. Therefore, in order to maintain the latitudinal cline over time, reproductive barriers must exist.

Reproductive barriers can arise through several mechanisms. One of them is when reproductive barriers evolve as a by-product of adaptation to the environment (Dodd, 1989; Rice & Hostert, 1993; Rundle *et al.*, 2000). In this case, populations in allopatry may develop different mating rituals or mate at different timings. Hence, if individuals from these populations were to meet, the build-up of these differences would impede mating. Moreover, in allopatric populations, individuals may also adapt to specific pressures of each environment, which may lead to post-zygotic reproductive barriers. Another mechanism by which reproductive barriers can evolve is by selection acting directly on these traits (Servedio, 2001). Indeed, when populations are locally adapted, they will benefit from not mating with immigrants, hence there may be selection to prevent such mating events. Therefore, pre-zygotic barriers are more likely to evolve in sympatry.

The genus *Drosophila* has many examples of latitudinal clines (James *et al.*, 1995; Pegueroles *et al.*, 1995; Karan *et al.*, 1998; Griffiths *et al.*, 2005; Rako *et al.*, 2007; Arthur *et al.*, 2008), indicating a high differentiation on several traits. However there are not many studies using populations with different genetic backgrounds to test if they are reproductively isolated when inhabiting the same environment (but see Gefen & Brendzel, 2011). Moreover, the existence of reproductive barriers is frequently studied in just one moment in time, as a fixed trait. It would be interesting to study if there are reproductive barriers between populations with different genetic backgrounds and how they evolve over time when in a new common environment.

Drosophila subobscura is a good model to study this thematic due to its latitudinal cline and studies of adaptation to the lab. Its latitudinal cline first evolved in Europe (Prevosti, 1966) and afterwards it evolved independently in South and North America (Prevosti *et al.*, 1990; Gilchrist *et al.*, 2001) (Fig. 1), following a few years of *D. subobscura* colonization. This suggests that selection may be involved and it has to be strong and consistent along the latitudinal gradient. (Balanyà *et al.*, 2003) Moreover, in a study using microsatellites, there are no significant genetic differences in neutral markers between European populations (Pascual *et al.*, 2001). Consequently the maintenance of differences in several traits between populations over time suggests that reproductive barriers may play a role in the maintenance of the cline. Also, there is some indication of post-zygotic isolation between different European populations of *D. subobscura*, namely biased sex-ratio towards females and male sterility in backcrosses due to different chromosomal arrangements (Hauschteck-Jungen, 1990). In previous studies of laboratory adaptation of Matos and collaborators it was observed that populations which are adapting to the laboratory had a performance enhancement in relevant fitness traits (Matos *et al.*, 2002; Simões *et al.*, 2007; Simões *et al.*, 2008; Simões *et al.*, 2009). However, all populations had different initial performances and adaptive rates. Also, these differences were greater between populations from different geographical origins, and year of foundation, particularly for less relevant fitness traits. Moreover some traits did not show consistent patterns of improvement with time, in particular starvation resistance, development time and juvenile viability (Simões *et al.*, 2007; Simões *et al.*, 2008). *Drosophila subobscura* exhibits latitudinal clines in the three continents for some quantitative traits, namely frequencies of many of the chromosome arrangements (Prevosti, 1974; Krimbas & Powell, 1992), wing size (Misra & Reeve, 1964; Prevosti *et al.*, 1990; Gilchrist *et*

al., 2001), body size (Pegueroles *et al.*, 1995) and latitudinal variation in circadian eclosion rhythm (Lankinen, 1993).

With this study we aimed to determine if there were reproductive barriers between two sets of populations from two extremes of the cline. For that purpose, two new populations were founded, one from Adraga (Portugal) and another from Groningen (Netherlands) (Fig. 1), and exposed them to a new common environment: the laboratory.

On the first research article, we determined if the two populations were differentiated in the novel environment after foundation. Moreover, if the populations were adapting to the laboratory environment across generations, and if so whether the adaptive pattern indicated convergence or evolutionary contingencies associated with different initial genetic backgrounds. After we determined that the populations were differentiated, we tested if there were reproductive barriers between these populations through mating behaviour and hybrid breakdown assays (2nd Research Article). And if so, we aimed to monitor the reproductive barriers' evolution during the populations' adaptation to a new common environment: the laboratory.

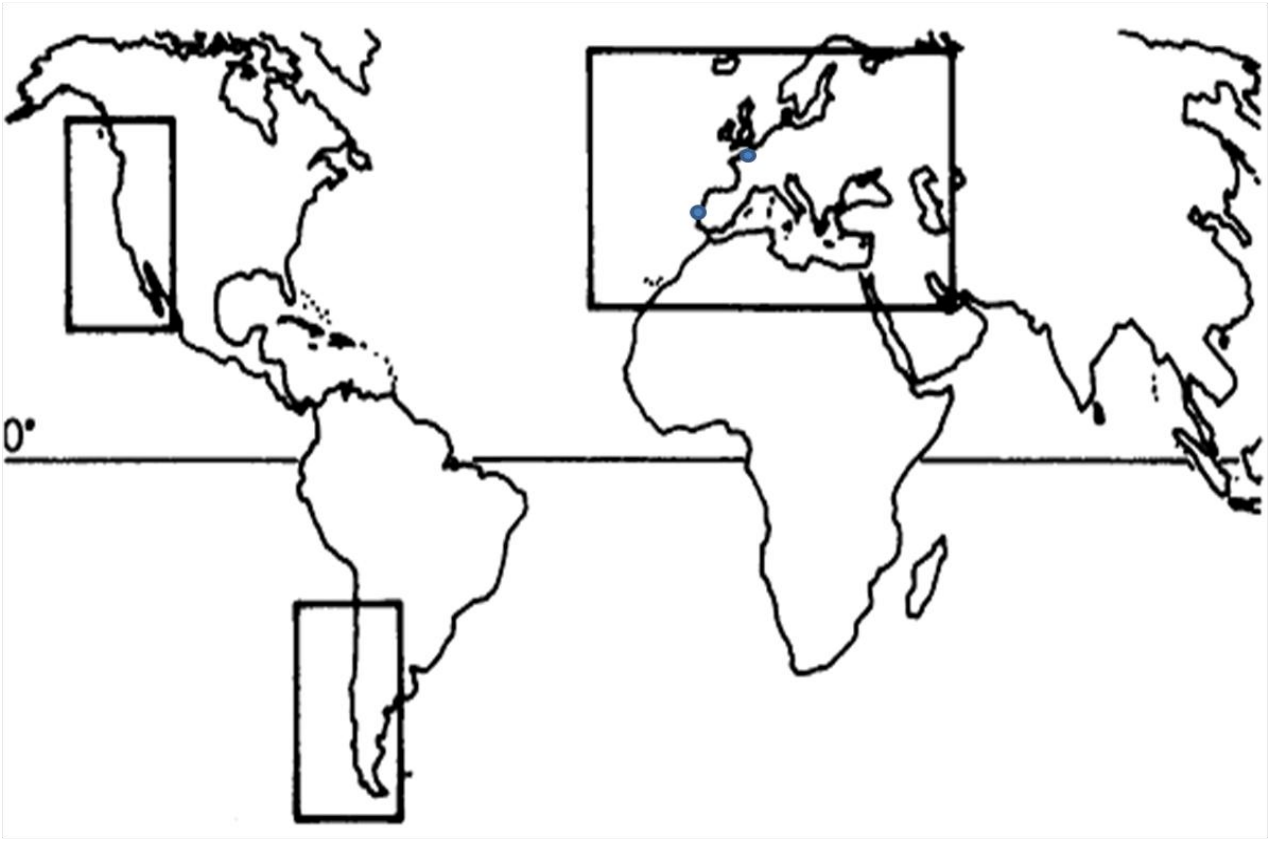

Fig. 1 Distribution area of *D. subobscura* (adapted of Prevosti *et al.* 1988), with the location of Adraga (Portugal) and Groningen (Netherlands).

Evidence of differentiation between of two sets of populations of *Drosophila Subobscura* from two extremes of the European latitudinal cline

Abstract

One of the best examples that populations are adapted to their environment is clinal variation. *Drosophila subobscura* possesses one of the most fascinating latitudinal clines for several quantitative traits. The study of latitudinal clines has been centralized around its identification and its recreation in the laboratory. The one thing that remains to be studied is how populations from the extremes of the cline will evolve when adapting to a new common environment. In order to do this we founded in the laboratory two sets of populations of *Drosophila subobscura* from two extremes of the cline, Adraga (Portugal) and Groningen (Netherlands), and characterized both their initial differentiation and early adaptation in life-history traits. During the first 11 generations studied the populations showed differences in several life-history traits. However, in general, the populations of both foundations did not exhibit temporal changes across generations. Possibly with more assays in the future we will be able to detect an evolutionary trend.

Key-words: Laboratory **a**daptation; evolutionary trajectory; convergence; novel environment; latitudinal cline.

Introduction

Endler (1977) defined a geographical cline as a 'gradient of a measurable trait' and its slope as a measure of the degree of differentiation between populations. 'Latitudinal clines have fascinated evolutionary biologists for years, because they provide evidence for the role of local adaptation in the differentiation of populations (Kirkpatrick & Barton, 2006). In spite of this interest much debate is still involved on the specific evolutionary mechanisms involved in clinal evolution (Santos *et al.*, 2005).

The genus *Drosophila* has many species that exhibit latitudinal clines for several traits, including body size, frequencies of inversion polymorphisms and heat and cold resistance (ref. Hoffmann *et al.*, 2003). Some of these studies have concluded that inversion polymorphisms may be responsible for the latitudinal variation of other traits, namely body and wing size. This clinal variation is probably due to climatic differences along the cline (Rako *et al.*, 2007). Most of the previous studies about clines only confirmed the existence of clinal variation for certain traits (e.g. Storz *et al.*, 2001; Arthur *et al.*, 2008) or tried to recreate the cline under laboratory conditions (e.g. Anderson, 1966; Cavicchi *et al.*, 1985; Santos *et al.*, 2005).

An issue that remains to be studied is how populations from different regions of a cline will behave when adapting to a new common environment. When different populations are introduced in a new environment they may converge, diverge or maintain their differences over time, as a function of their specific histories (Cohan & Hoffmann, 1989; Travisano *et al.*, 1995). In particular, the introduction of wild populations to the laboratory environment generally leads them to adapt to this new environment (Simões *et al.*, 2009). Also, if costs are involved in the adaptive process, this may lead to decreased fitness in their ancestral (natural) environment. Since the maintenance of differentiated populations of extremes of a cline in the lab may make them lose some of the local adaptation patterns they possessed (e.g. the clinal pattern), their adaptive

rate may thus shed light on the evolution of the cline. However, extrapolations between the lab and nature deserve caution (James *et al.*, 1997; Griffiths, *et al.*, 2005). The study of the reversion of a latitudinal cline under laboratory conditions, is thus of high interest both in evolutionary, methodological and conservation terms.

Both clinal evolution (e.g. Pascual *et al.*, 2001; Balanyà *et al.*, 2003; Fragata *et al.*, 2010) and adaptation (Simões *et al.*, 2009) to the laboratory environment have been extensively studied in *Drosophila subobscura* Originally a Paleartic species, *Drosophila subobscura* has colonized South America and later North America about three decades ago (Prevosti *et al.*, 1989). This species displays well documented latitudinal clines in the three continents in the frequency of chromosome arrangements (Prevosti, 1974; Krimbas & Powell, 1992), wing size (Misra & Reeve, 1964; Prevosti *et al.*, 1990; Gilchrist *et al.*, 2001) , body size (Pegueroles *et al.*, 1995) and latitudinal variation in circadian eclosion rhythm (Lankinen, 1993). Despite the fact that developmental time and viability of *D. subobscura* doesn't demonstrate a latitudinal cline, it is known to vary geographically (Budnik *et al.*, 1991). Moreover, the capacity to tolerate extreme temperatures is more important to maintain clinal patterns than the thermoregulation behaviours that occur locally (Rego *et al.*, 2010). The fact that over time the genotypes characteristic of low latitudes increased in populations of higher latitudes, where the temperature rose, makes this species a good tool to monitor global climate changes (Balanyà *et al.*, 2006). Considering all these studies, temperature seems a good candidate as selective factor driving clinal evolution. Nevertheless, Santos *et al.* (2005) have found, in a study of thermal selection in this species, that temperature is not sufficient to generate a cline in the laboratory.

Matos and collaborators have studied the adaptation to the laboratory of *Drosophila subobscura* for 20 years (Matos *et al.*, 2000a; Matos *et al.*, 2002; Matos *et al.*, 2004; Simões *et al.*, 2007; Simões *et al.*, 2008). A common observation in several studies was an increase in performance of relevant fitness traits of the populations that are adapting to the laboratory (Matos *et al.*, 2002; Simões *et al.*, 2007; Simões *et al.*, 2008; Simões *et al.*, 2009). Nevertheless, both the initial performance of populations and the adaptive rate varied between foundations, contrasting between locations as well as year of foundation, particularly for less relevant fitness traits. Moreover some traits did not show consistent patterns of improvement with time, in particular starvation resistance, development time and juvenile viability (Simões *et al.*, 2007; Simões *et al.*, 2008). Given the close-by nature of the locations of foundation, the differences observed might be due not only to differences in the genetic background of the natural populations, but also to sampling effects that arise from founder effects plus early drift effects in the first generations after foundation. Studies involving foundations from contrasting geographical locations are worth doing to extend these observations. In particular, the analysis of the evolutionary dynamics of populations derived from contrasting latitudes will allow to test for reversion of a latitudinal cline when populations adapt to a novel, common environment. Will populations derived from contrasting geographical locations converge to similar values during adaptation to a common environment? How much will these populations differ both at foundation and during the early steps of adaptation?

In this present work we propose to expose collected populations from the two extremes of the latitudinal cline in Europe to the same common environment: the laboratory. The main goals are a) to test if the populations are differentiated in the novel environment; b) to determine if the populations are adapting to the laboratory environment, and if so whether the adaptive pattern indicates convergence or evolutionary contingencies associated with different initial genetic backgrounds.

Materials and Methods

Foundation and Maintenance of Populations

Three regimes were used in this study: two sets of experimental populations, Gro and Ad, and a control regime, TA. The control regime, already adapted to the laboratory environment, was founded in 2001 and had 115 generations when the new populations were founded. The experimental populations were collected in August of 2010 from two localities with different latitudes: Groningen, Netherlands (53° 21' N, 6° 55' E) and Adraga, Portugal (38° 47' N, 9° 28' W). The collected individuals underwent a triage with CO_2 anaesthesia and a stereoscope after which Groningen had 180 females and 20 males and Adraga had 246 females and 73 males. Each founding female was placed in a vial with a male from the corresponding locality. In the first egg laying, death of 2nd instar larvae was observed. As it had happened before in this laboratory, this mortality was once again attributed to a bacterial contamination. Therefore the populations were treated with tetracycline for one generation and with ceftriaxone and spectinomycin in the following generation. Due to the antibiotic treatment all assays were postponed for two generations and the populations were maintained in isolated lines until the fourth generation. In order to prevent inbreeding depression, in the 1st generation each n female line was crossed with a n+1 male line and in the 2nd generation each female line was mated to a male retrieved from the mixture of males from every line. In the third generation all the lines were mixed, using 5 females and males from each line. In the fourth generation the two experimental populations were three-folded replicated.

All populations were maintained at standard laboratory conditions that consists in discrete generations of 28 days; temperature of 18C with a photoperiod of 12L/12D. Culture medium was composed of agar, corn meal, dead brewer's yeast, charcoal colouring and nipagine. The adult and larval densities were around 50 individuals and 70-80 eggs per vial, respectively. Population sizes were maintained as much as possible around 1000-1200 individuals (for details see Matos *et al.* 2002).

Fecundity and Starvation Assays

Fecundity was assayed at three generations: generations 4 (with no replicas), 6 and 11 (with replicas) after foundation. At generations 6 and 11, the three replicate populations of each species were used, pairing individuals according to replicate number. The mated pairs were formed using virgin individuals that were sexed using CO_2 anaesthesia less than 6 hours after adult emergence. The pairs were transferred daily to new vials and the eggs counted. In these assays, the traits analyzed are age of first reproduction (A1R – number of days until the female laid her first egg), early fecundity (F1-7 – number of eggs laid in the first week of life) and peak fecundity (F8-12 – number of eggs laid on the last five days of the assay). After the fecundity assay, the pairs were transferred to a medium with no nutrients to test starvation resistance. Around 24 mated pairs were assayed per population per assay.

Juvenile Traits Assay

To determine developmental time and juvenile viability, we collected 6-8 vials with 60 eggs from each population using eggs laid over a period of 6h. The assay was performed at the normal laboratory conditions. Around eighteen days after the harvest, the imagoes started to emerge. We checked for imagoes every 5

hours of the light period and collected them. After freezing the imagoes, we counted and sexed them up at the stereoscope. We waited for 48 hours without imagoes to end the assay. In this assay development time of each sex and juvenile viability were calculated for every population. The development time was estimated as the number of hours since the egg being laid until the emergence of the imago. The viability was determined as the total number of adults collected per vial divided by 60. The assay of juvenile traits was performed at generation 5.

Statistical Analysis

All statistical analyses were performed using Microsoft Excel and Statsoft Statistica.

Fecundity and Starvation assays
In these analyses we used the individual data. For generation 4, because there were no replicates, an ANOVA was used to test whether there were differences between regimes in the traits analyzed according with the following model:

$$Y = \mu + R + \epsilon$$

where R is the fixed factor Regime with three categories (Ad, Gro, TA). To test for significant differences between pairs of regimes, orthogonal contrasts were performed. For generations 6 and 11, a two-way mixed ANOVA was used to test whether there were differences between regimes in the traits analyzed according to the model:

$$Y = \mu + R + P\{R\} + \epsilon$$

where R is the fixed factor Regime with three categories (Ad, Gro, TA) and P is the random factor Population nested within each regime ($Ad_{1\text{-}3}$, $Gro_{1\text{-}3}$ and $TA_{1\text{-}3}$). Orthogonal contrasts were done to test for specific differences.

In order to test whether there were differences between generations 6 and 11, the following model was used:

$$Y = \mu + R + P\{R\} + G + R^{*}G + P\{R\}^{*}G + \epsilon$$

where R and G are the fixed factors Regime and Generation, respectively, and P is the random factor Population. The Generation has two categories (6, 11).

Juvenile Traits Assay – In these analyses we used vials as individual data. A three-way mixed ANOVA was used to test whether the development time was different between regimes and sexes according with the model:

$$Y = \mu + R + P\{R\} + S + R^{*}S + P\{R\}^{*}S + \epsilon$$

where R and S are the fixed factors Regime and Sex, respectively, and P is the random factor Population. The

factors Regime, Sex and Population include three (Ad, Gro, TA), two (female and male) and 3 categories nested within each regime ($Ad_{1\text{-}3}$, $Gro_{1\text{-}3}$ and $TA_{1\text{-}3}$) respectively. To test if there were differences in viability for the different regimes, a two-way mixed ANOVA was done according to the model:

$$Y = \mu + R + P\{R\} + \epsilon$$

where R is the fixed factor Regime and P is the random factor Population.

Evolutionary Trajectories

All analyses of trajectories were done using linear regressions, with the difference between the mean values of traits for each replicate population and its control population (e.g. Ad_1-TA_1). Since the assay of generation 4 was done with no replicas, it was given the same value to the three replicas for that generation. To assess if there were significant temporal dynamics for each regime, an ANCOVA was performed using the model:

$$Y = \mu + P + G + P^*G + \epsilon$$

where P is the random factor Population with three categories ($Ad_{1\text{-}3}$ or $Gro_{1\text{-}3}$) and G is the covariate Generation with three values (4,6, 11).

To test if there were differences between Ad and Gro trajectories across generations an ANCOVA was performed using the following model:

$$Y = \mu + R + P\{Regime\} + G + R^*G + G^*P\{Regime\} + \epsilon$$

where R is the fixed factor Regime, P is the random factor Population and G is the covariate Generation.

To test whether differences between AD and Gro evolved through time we also did an ANCOVA using the differences between Ad and Gro at each assayed generation using the model:

$$Y = \mu + PD + G + PD^*G + \epsilon$$

where PD is the random factor Population Difference with three categories ($Ad_{1\text{-}3}$ – $Gro_{1\text{-}3}$) and G is the covariate Generation.

Results

Adult Traits

At generation 4 Gro had a better performance than Ad for all traits (Fig. 1). Nevertheless the differences between Ad and Gro were not significant, except for the male starvation resistance (Table 1). There were in general significant differences between each one of the experimental regimes and the control, the latter presenting better performance in all fecundity traits (Fig. 1). Significant differences across the three regimes were also observed (Table S1).

At generation 6, Ad and Gro showed significant differences for all traits (Table 1). Also, Ad and Gro were globally significantly different from the control. There were significant differences across regimes for age of first reproduction, early and peak fecundities and marginally significant differences in female starvation

resistance (Table S1, Appendix I). The populations within each regime were significantly different for peak fecundity and starvation resistance, which indicates the heterogeneity of the replicates for these characteristics.

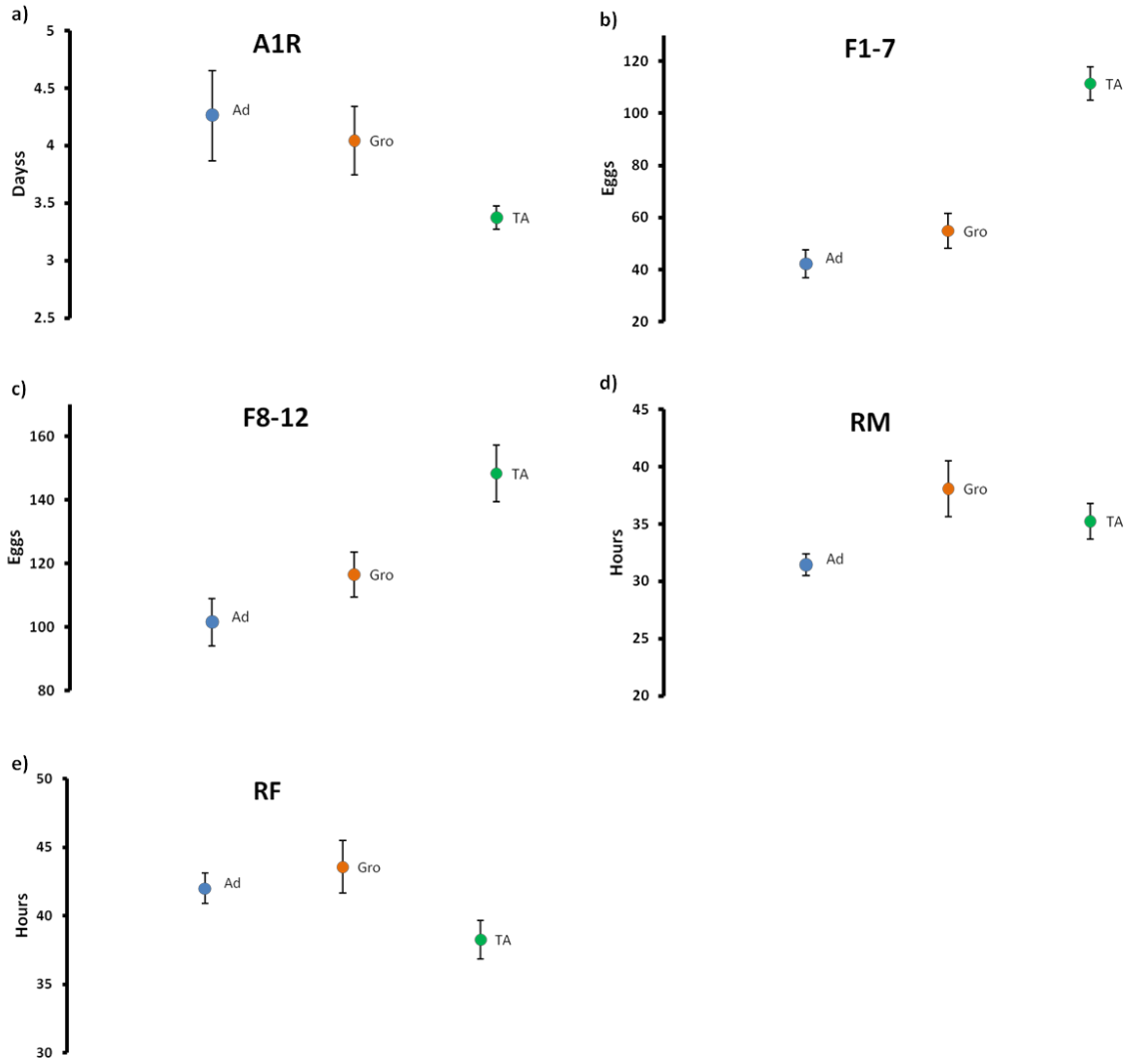

Fig. 2 Means of the five traits analysed of populations Ad and Gro (at generation 4) and TA. a) A1R – age of first reproduction; b) F1-7 – early fecundity; c) F8-12 – peak fecundity; d) RM – male starvation resistance; e) RF – female starvation resistance. Standard error bars were given by individual data.

At generation 11, Ad and Gro remained significantly different in early and peak fecundities, but not in the other traits (Table 1). Additionally, Ad and Gro differ significantly from the control in both age of first reproduction and the two fecundity traits, though no longer in starvation resistance traits. Early and peak fecundities remained significantly different across regimes (Table S1). The population factor was significant for age of first reproduction and early fecundity and marginally significant for peak fecundity, which indicates the heterogeneity of the replicas for these characteristics.

In Table 2 the results of the comparison between generations 6 and 11 are shown. There were substantial differences between regimes in the age of first reproduction, early and peak fecundities across the two generations. Of all the traits considered, only peak fecundity had significant differences (p = 0.001) between the two generations across the two regimes. No significant interaction between generation and regime was detected in all traits, indicating that differences between regimes were maintained between generations.

Table 1 Contrasts between the pairs of regimes from de ANOVA at generations 4, 6 and 11 of Ad and Gro. A1R – age of first reproduction; F1-7 – early fecundity; F8-12 – peak fecundity; RM – male starvation resistance; and RF– female starvation resistance. DF = 1 for each comparison.

	Comparison	A1R	F1-7	F8-12	RM	RF
G4	Ad vs. Gro	0.6129	0.2244	0.2101	0.0072*	0.4755
	Ad vs. TA	0.0363*	0.0000***	0.0001**	0.1004	0.0870^{MS}
	Gro vs. TA	0.1084	0.0000***	0.0056*	0.2544	0.0153*
G6	Ad vs. Gro	0.0068*	0.0013*	0.0001**	0.0000***	0.0000***
	Ad vs. TA	0.0000***	0.0000***	0.0000***	0.0000***	0.7812
	Gro vs. TA	0.0671^{MS}	0.0000***	0.0000***	0.0758^{MS}	0.0000***
G11	Ad vs. Gro	0.6720	0.0000***	0.0000***	0.9084	0.1378
	Ad vs. TA	0.0000***	0.0245*	0.0398*	0.7015	0.6995
	Gro vs. TA	0.0001**	0.0000***	0.0000***	0.7846	0.2498

^{MS} 0.10 > p > 0.05; *p < 0.05; ** p < 0.001; *** p < 0.0001

Table 2 ANOVA for the differences between generations 6 and 11 of Ad and Gro in the five traits analyzed. A1R – age of first reproduction; F1-7 – early fecundity; F8-12 – peak fecundity; RM – male starvation resistance; and RF– female starvation resistance.

Factor	DF	A1R	F1-7	F8-12	RM	RF
Regime	2	0,0051*	0,0000***	0,0004**	0,2968	0,0823^{MS}
Population (Regime)	6	0,5101	0,6849	0,41015	0,1567	0,4805
Generation	1	0,9419	0,9771	0,0011*	0,195474	0,9214
Population (Generation*Regime)	6	0,2941	0,0532^{MS}	0,0414*	0,0474*	0,0285*
Generation*Regime	2	0,3136	0,8514	0,5705	0,1250	0,5539

^{MS} 0.10 > p > 0.05; *p < 0.05; ** p < 0.001; *** p < 0.0001

Juvenile Traits

For development time significant differences were found between each one of the experimental regimes and the control regime (Table 3; Fig. 2a). There were significant differences (p-value < 0.05) between regimes, population and sex for the development time (Table S2). However there were no major differences for viability for any comparison (Table 3; Fig. 2b).

Table 3 Contrasts between the pairs of regimes from the mixed three-way ANOVA done to test the differences between Regime Ad and Gro, at generation 5, and TA for development time and viability. DF = 1 for each comparison.

Comparison	TD	Viability
Ad vs. Gro	0.0005**	0.1091
Ad vs. TA	0.0000***	0.0719[MS]
Gro vs. TA	0.0000***	0.8892

[MS] 0.10 > p > 0.05; *p < 0.05; ** p < 0.001; *** p < 0.0001

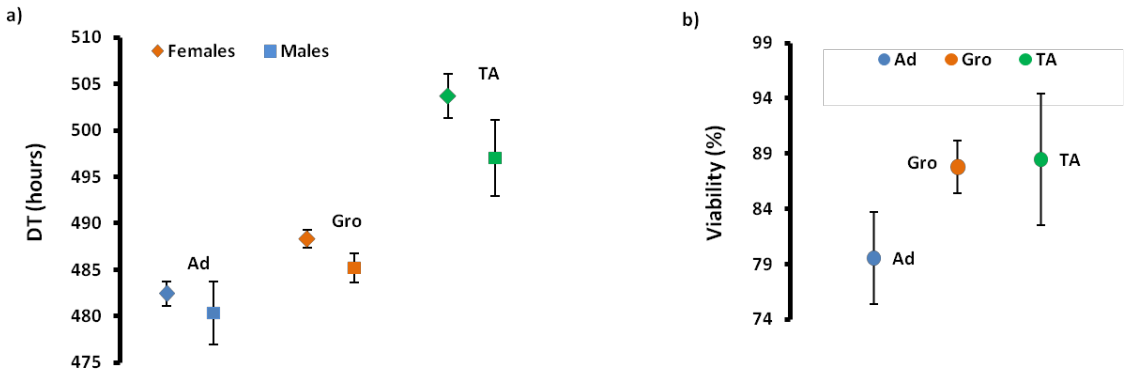

Fig. 2 a) Mean of the development time (hours) and b) mean viability of populations Ad and Gro, at generations 5, and TA. Standard error bars were given by the replicate means.

Evolutionary Trajectories

There were no significant linear temporal changes in either Ad or Gro across the generations assayed, for any of the traits considered (Table S3; Fig. S1; Fig. S2), except for peak fecundity of the Gro regime.

Ad and Gro did not differ significantly either on the average values across generations or on the changes that each presented between generations (Table 4; Fig. 3). The only exception was male starvation resistance, with significant differences between the two regimes across generations as well as a marginally significant difference of slopes between regimes (Table 4; Fig. 3d). In balance Ad and Gro appeared to maintain their differences across generations for early and peak fecundities and female starvation resistance (Fig. 3b, c, e). Also, there was an indication of convergence in male starvation resistance.

Table 4 ANCOVA for the Regime means (difference to the control) of the five traits analysed across generations 4, 6 and 11 of Ad and Gro. A1R – age of first reproduction; F1-7 – early fecundity; F8-12 – peak fecundity; RM – male starvation resistance and RF – female starvation resistance.

Factor	DF	A1R	F1-7	F8-12	RM	RF
Regime	1	0.0949[MS]	0.1921	0.2249	0.0099*	0.3583
Pop (Regime)	4	0.9417	0.2805	0.9888	0.5027	0.3927
Generation	1	0.1522	0.1018	0.1260	0.2777	0.0568[MS]
Regime*Generation	1	0.4904	0.9496	0.9116	0.0647[MS]	0.8454
Pop (Regime*Generation)	4	0.8295	0.1347	0.9635	0.3559	0.1562

[MS] 0.10 > p > 0.05; *p < 0.05; ** p < 0.001; *** p < 0.0001

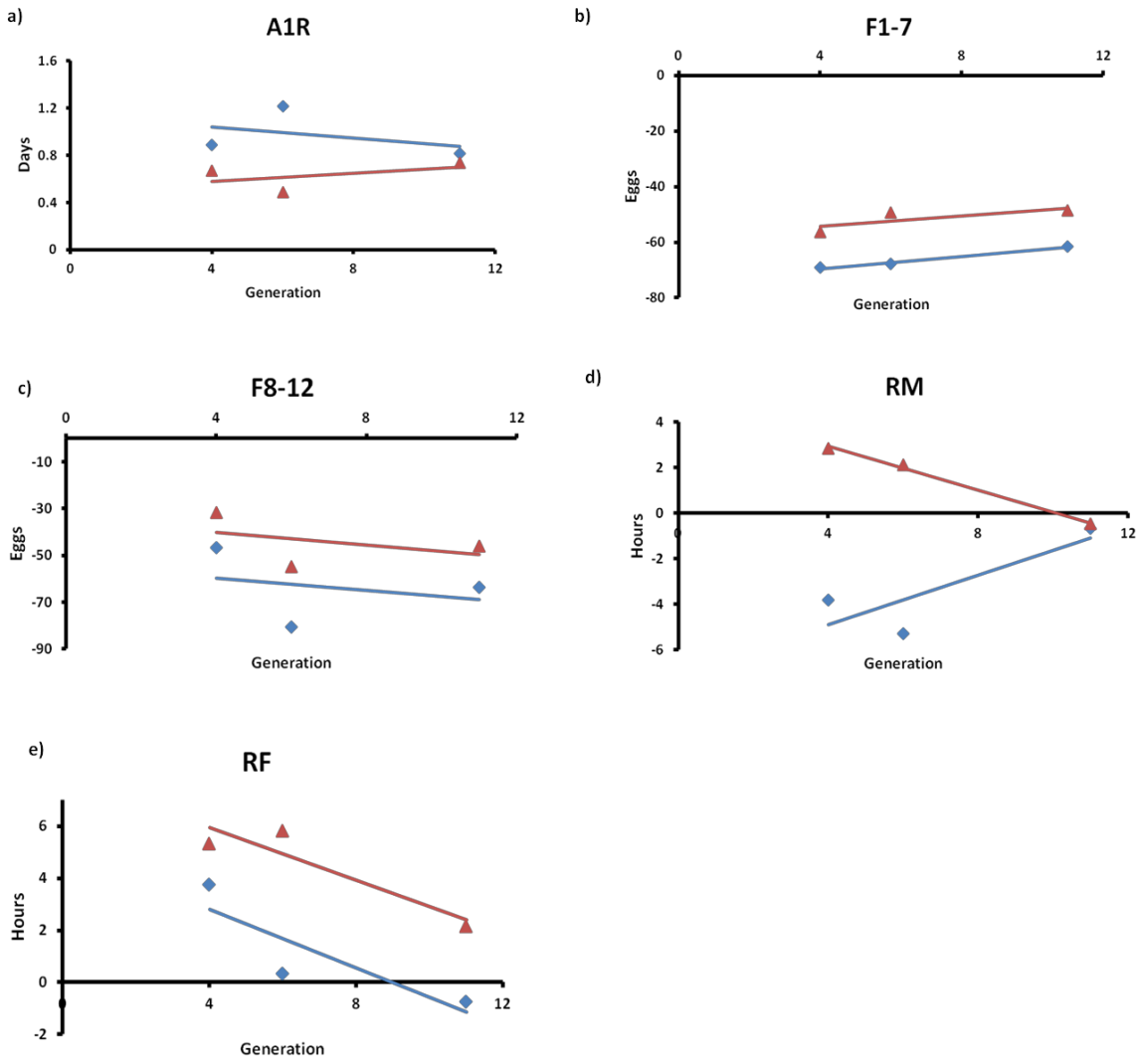

Fig. 3 Evolutionary trajectories of Ad and Gro. The data used correspond to the mean difference between experimental and control regimes, at each generation. a) A1R (Age of first reproduction); b) F1-7 (early fecundity); c) F8-12 (peak fecundity); d) RM (male starvation resistance); e) RF (female starvation resistance). Ad (blue); Gro (red).

The slopes of the differences between Ad and Gro across generations reinforce the aforementioned results, indicating stability of differences between regimes during the period of generations studied (Table 5; Fig. 4). Male starvation resistance did not show significant temporal change of differences between regimes, as might be expected from the analysis presented above.

Table 5 ANCOVA for the difference between Ad and Gro across generations (4, 6, and 11) for all traits analyzed. A1R – age of first reproduction; F1-7 – early fecundity; F8-12 – peak fecundity; RM – male starvation resistance and RF – female starvation resistance.

Factor	DF	A1R	F1-7	F8-12	RM	RF
PD	2	0.6387	0.3605	0.3555	0.4513	0.6607
Generation	1	0.5415	0.9472	0.9130	0.1342	0.7541
PD*Generation	2	0.4626	0.2604	0.2623	0.3315	0.5659

[MS] 0.10 > p > 0.05; *p < 0.05; ** p < 0.001; *** p < 0.0001

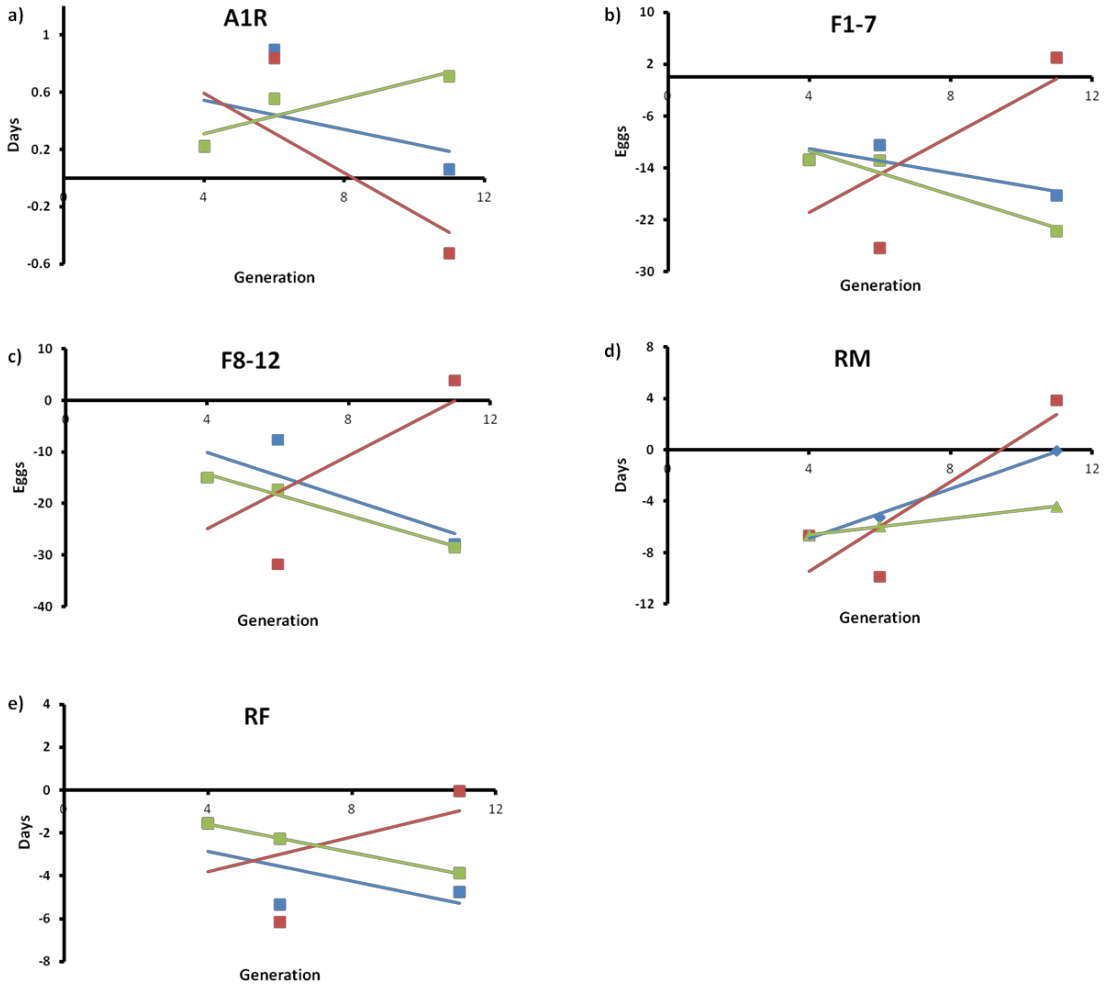

Fig. 4 Evolutionary trajectories of the difference between Ad and Gro. The data used correspond to the difference in mean values between each Ad replicate and its correspondent Gro replicate, at each generation. a) A1R (age of first reproduction); b) F1-7 (early fecundity); c) F8-12 (peak fecundity); d) RM (male starvation resistance); e) RF (female starvation resistance). Ad_1-Gro_1 (blue); Ad_2-Gro_2 (red); Ad_3-Gro_3 (green).

Discussion

Were Ad and Gro initially different?

When populations have different geographic origins they are expected to be different in several traits related to fitness, due to their adaptation to their specific environment (Kawecki & Ebert, 2004). This may also lead to different performances in a novel, common, environment. The populations from Adraga and Groningen exhibit differences in the frequencies of inversion polymorphisms (data not shown) which probably reveal adaptation to different climatic conditions (Rako *et al.*, 2007). However, in the novel, laboratorial environment, at generation 4, Ad and Gro were not very different in the characteristics considered, except in male starvation resistance (Table 1). This apparent absence of differentiation may be due to lack of statistical power since in this assay there were no replicate populations. Obviously this might also be due to the fact that the previous history of the populations does not translate in differences on their performance in the novel environment, due to genotype-by-environment interactions. This illustrates the dangers of extrapolating between studies in the laboratory to those in nature (Matos *et al.*, 2000b).

The populations derived from the Groningen foundation had in general a better performance than the ones derived from the Adraga collection, in all assayed generations. This is an interesting, counterintuitive, observation, as the laboratorial environment, given the mild maintenance temperature, is more similar to the conditions experienced by Ad populations. Indeed, all studies in the Matos laboratory have defined maintenance procedures such that they were adequate for populations that were founded in that same location, such as the control of this study (e.g. see Matos *et al.,* 2002; Simões *et al.,* 2008). However, many factors are involved in differences between populations. Among other factors, size may contribute to the differences observed, both in fecundity and starvation resistance (analyses in progress). Several studies indicate a positive correlation between body size and fecundity in several *Drosophila* species (e.g. Lefranc & Bundgaard, 2000). This may have contributed to the higher fecundity of the Groningen populations. Also, males derived from the Adraga foundation had the lower starvation resistance compared to both females of their own populations and to the populations derived from Groningen (Fig. 1d and 3d). This could be explained again by their smaller body (data not shown), which results in a lower lipid content, leading them to have the worst performance in starvation resistance (Djawdan *et al.*, 1998). Nevertheless both Ad and Gro had lower fecundity than the long established populations across all generations studied. This is expected, considering that the laboratory is just another environment to which populations adapt, and the generations covered by this study were probably not enough for the occurrence of possible convergence between the recently and the long established populations

Both experimental regimes presented a shorter development time compared to the long established populations (Fig. 2a). Considering previous studies it is expected that time of development will increase with lab adaptation (see Simões *et al.*, 2007). Although the development time was not different between Ad and Gro (Table 3), the suggestion of a longer development time of Gro, in both sexes, could be due to the bigger body size of Gro individuals (Robertson, 1963). It is interesting to note that this difference goes in the expected direction of clinal differentiation (Griffiths *et al.,* 2005). But, again, extrapolations between the lab and nature have to be made with caution.

The marginal difference between TA and Ad in viability indicates that Ad has not reached the 'optimum' viability for the laboratory environment (Table 3; Fig. 2b). However Gro shows a similar viability to TA. These results are not consistent with the ancestral environment of the experimental populations, since Ad should

have a better viability at 18˚ than Gro.

The general life-history differences between the Groningen and Adraga recently introduced populations may be due to the fact that Ad and Gro had contrasting adaptive histories, and their specific adaptation to different environments may impose differential performance in a novel environment (Kawecki & Ebert, 2004). An important issue is how much these differences are maintained, increase or disappear as a consequence of adaptive evolution in the novel, common environment.

Are Ad and Gro adapting and converging?

When a population is exposed to a new environment, it is likely that it undergoes evolutionary changes, which translates into adaptation, due to selective pressures (Gilligan & Frankham, 2003). These pressures will be greater the less adapted the population is to this environment and consequently the greater its evolution rate is expected to be, given sufficient standing genetic variance. These changes will be reflected in the characteristics directly or indirectly related to fitness (Travisano *et al.*, 1995). It is also an assumption that when different populations are introduced to a new common environment, they will converge with the long term established populations (e.g. Matos *et al.,* 2002).

Our results demonstrate that there is no clear evolutionary trend for all life-history characteristics during the period covered (Table 4; Fig. 3). This is in contrast with what has been in general observed by Matos and collaborators over the years (Matos *et al.*, 2000a, 2002, 2004; Simões *et al.*, 2007, 2008). Both age of first reproduction, early and peak fecundities have a major influence on fitness, so it was expected they would improve over time. However this improvement was not very accentuated, which could be explained by the lack of additive genetic variance or by the great heterogeneity of the replicas. Female resistance to starvation shows an evolutionary trend of decrease over time. This might seem contradictory to expectations of adaptation. However, Simões *et al.* (2008) showed that that both increase and decrease of starvation resistance may occur through time, suggesting that historical contingencies are stronger for less relevant fitness traits (Travisano *et al.*, 1995). They also showed that for some of the populations studied a slow initial adaptation required more than 15 generations to detect significant evolutionary dynamics. Therefore the lack of the expected evolutionary trend in our study could be due to few generations covered.

In spite of this, there is a suggestion of a temporal trend that seems parallel between the Groningen and Adraga populations for female starvation resistance and early fecundity (Fig. 3). Only more generations will confirm whether parallel evolution is occurring for these characteristics. This could mean that Ad and Gro found different solutions for the same evolutionary problem; approaching to the values of long established laboratory populations Moreover, Ad and Gro evolutionary trends for the male starvation resistance trait indicate convergence from opposite directions and an approximation to the control values for this trait. All these trends are signs of adaptation to the laboratory environment. Age of first reproduction is an exception. Though there is a suggestion of convergence between the new populations, this is due to the improvement of the Adraga populations, getting closer both to the Groningen (that don't change through time) and to the longer established populations.

In summary Ad and Gro regimes are globally different in life-history traits. Despite no clear evolutionary trend for both regimes, our results suggest that different traits evolve differently, either by parallel or convergent evolution. Only more generations in the laboratory will confirm these observations. Also, given

the differences observed in the early performance of these populations, an important issue as a follow up of this study is to analyse at what point these differentiated populations present reproductive barriers, either pre- or post-zygotic, in the novel environment. Incipient barriers may have evolved in the ancestral environment, either by direct or indirect selection processes (Brelsford & Irwin, 2009; Fitzpatrick *et al.*, 2009). These barriers, if present, may be environment dependent. How much can populations initially differentiated in contrasting environments introgress when encountering a novel, common environment, is thus a major question both in evolutionary and conservation terms.

References

Anderson, W. W. 1966. Genetic divergence in M. Vetukhiv's experimental populations of *Drosophila pseudoobscura* 3. Divergence in Body Size. *Genetical Research* **7**: 255-266.

Arthur, A. L., Weeks, A. R., & Sgrò, C. M. 2008. Investigating latitudinal clines for life history and stress resistance traits in *Drosophila simulans* from eastern Australia. *Journal of Evolutionary Biology* **21**: 1470-1479.

Balanyà, J., Serra, L., Gilchrist, G. W., Huey, R. B., Pascual, M., Mestres, F., & Solé, E. 2003. Evolutionary pace of chromosomal polymorphism in colonizing populations of *Drosophila subobscura*: an evolutionary time series. *Evolution* **57**: 1837–1845.

Balanyà, J., Oller, J. M., Huey, R. B., Gilchrist, G. W., & Serra, L. 2006. Global genetic change tracks global climate warming in *Drosophila subobscura*. *Science* **313**: 1773-1775.

Brelsford, A., & Irwin, D. E. 2009. Incipient speciation despite little assortative mating: the yellow-rumped warbler hybrid zone. *Evolution* **63**: 3050-3060.

Budnik, M., Cifuentes, L., & Brncic, D. 1991. Quantitative analysis of genetic differentiation among European and Chilean strains of *Drosophila subobscura*. *Heredity* **67**: 29-33.

Cavicchi, S., Guerra, D., Giorgi, G., & Pezzoli, C. 1985. Temperature-Related Divergence in Experimental Populations of *Drosophila melanogaster*. I. Genetic and Developmental Basis of Wing Size and Shape Variation. *Genetics* **109**: 665-689.

Cohan, F. M., & Hoffmann, A. A. 1989. Uniform Selection as a Diversifying Force in Evolution: Evidence from *Drosophila*. *The American Naturalist* **134**: 613-637.

Djawdan, M., Chippindale, A. K., Rose, M. R., & Bradley, T. J. 1998. Metabolic reserves and evolved stress resistance in *Drosophila melanogaster*. *Physiological Zoology* **71**: 584-594.

Endler, J. A. 1977. *Geographic variation, speciation, and clines*. Princeton University Press.

Fitzpatrick, B. M., Fordyce, J. A., & Gavrilets, S. 2009. Pattern, process and geographic modes of speciation. *Journal of Evolutionary Biology* **22**: 2342-2347.

Fragata, I., Balanyà, J., Rego, C., Matos, M., Rezende, E. L., & Santos, M. 2010. Contrasting patterns of phenotypic variation linked to chromosomal inversions in native and colonizing populations of *Drosophila subobscura*. *Journal of Evolutionary Biology* **23**: 112-123.

Gilchrist, G. W., Huey, R. B., & Serra, L. 2001. Rapid evolution of wing size clines in *Drosophila subobscura*. *Genetica* **112-113**: 273-286.

Gilligan, D. M., & Frankham, R. 2003. Dynamics of genetic adaptation to captivity. *Conservation Genetics* **4**: 189-197.

Griffiths, J. A., Schiffer, M., & Hoffmann, A. A. 2005. Clinal variation and laboratory adaptation in the rainforest species *Drosophila birchii* for stress resistance, wing size, wing shape and development time. *Journal of Evolutionary Bology* **18**: 213-222.

Hoffmann, A. A., Sorensen, J. G., & Loeschcke, V. 2003. Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. *Journal of Thermal Biology* **28**: 175-216.

James, A. C., Azevedo, R. B. R., & Partridge, L. 1997. Genetic and Environmental Responses to Temperature of *Drosophila melanogaster* From a Latitudinal Cline. *Genetics* **146**: 881-890.

Kawecki, T. J., & Ebert, D. 2004. Conceptual issues in local adaptation. *Ecology Letters* **7**: 1225-1241.

Kirkpatrick, M., & Barton, N. 2006. Chromosome Inversions, Local Adaptation and Speciation. *Genetics* **173**: 419-434.

Krimbas, C. B., & Powell, J. R. 1992. *Drosophila inversion polymorphism*. CRC Press.

Lankinen, P. 1993. North–south differences in circadian eclosion rhythm in European populations of *Drosophila subobscura*. *Heredity* **71**: 210-218.

Lefranc, A., & Bundgaard, J. 2000. The influence of male and female body size on copulation duration and fecundity in *Drosophila melanogaster*. *Hereditas* **132**: 243-247.

Macarthur, R. H., & Wilson, E. O. 1963. An Equilibrium Theory of Insular Zoogeography. *Evolution* **17**: 373-387.

Matos, M., Rose, M. R., Rocha Pité, M. T., Rego, C., & Avelar, T. 2000a. Adaptation to the laboratory environment in *Drosophila subobscura*. *Journal of Evolutionary Biology* **13**: 9-19.

Matos, M., Rego, C., Levy, A., Teotónio, H., & Rose, M. R. 2000b. An evolutionary no man's land. *Trends in Ecology & Evolution (Personal edition)* **15**: 206.

Matos, M., Avelar, T., & Rose, M. R. 2002. Variation in the rate of convergent evolution: adaptation to a laboratory environment in *Drosophila subobscura*. *Journal of Evolutionary Biology* **15**: 673-682.

Matos, M., Simões, P., Duarte, Ana, Rego, C., Avelar, T., & Rose, M. R. 2004. Convergence to a novel environment: comparative method versus experimental evolution. *Evolution* **58**: 1503-1510.

Misra, R. K., & Reeve, E. C. R. 1964. Clines in body dimensions in populations of *Drosophila subobscura*. *Genetical Research* **5**: 240-256.

Pascual, M., Aquadro, C. F., Soto, V., & Serra, L. 2001. Microsatellite Variation in Colonizing and Palearctic Populations of *Drosophila subobscura*. *Mol. Biol. Evol.* **18**: 731-740.

Pegueroles, G., Papaceit, M., Quintana, A., Guillén, A., Prevosti, A., & Serra, L. 1995. An experimental study of evolution in progress: Clines for quantitative traits in colonizing and Palearctic populations of *Drosophila*. *Evolutionary Ecology* **9**: 453-465.

Prevosti, A. 1974. Chromosomal inversion polymorphism in the southwestern range of *Drosophila subobscura* distribution. *Genetica* **45**: 111-124.

Prevosti, A., L. Serra, M. Aguadé, G. Ribó, F. Mestres & J. Balanyà, 1989. Colonization and establishment of the Palearctic species *Drosophila subobscura* in North and South America, pp. 114–129 in *Evolutionary Biology of Transient and Unstable Populations*, edited by A. Fontdevila. Springer Verlag, Berlin.

Prevosti, A., Serra, L., Segarra, C., Aguade, M., Ribo, G., & Monclus, M. 1990. Clines of Chromosomal Arrangements of *Drosophila subobscura* in South America Evolve Closer to Old World Patterns. *Evolution* **44**: 218-221.

Rako, L., Blacket, M. J., McKechnie, S. W., & Hoffmann, A. A. 2007. Candidate genes and thermal phenotypes: identifying ecologically important genetic variation for thermotolerance in the Australian *Drosophila melanogaster* cline. *Molecular Ecology* **16**: 2948-2957.

Rego, C., Balanyà, J., Fragata, I., Matos, M., Rezende, E. L., & Santos, M. 2010. Clinal patterns of chromosomal inversion polymorphisms in *Drosophila subobscura* are partly associated with thermal preferences and heat stress resistance. *Evolution* **64**: 385-397.

Robertson, F. W. 1963. The ecological genetics of growth in *Drosophila* 6. The genetic correlation between the duration of the larval period and body size in relation to larval diet. *Genetical Research* **4**: 74-92.

Santos, M., Céspedes, W., Balanyà, J., Trotta, V., Calboli, F. C. F., Fontdevila, A., & Serra, L. 2005. Temperature-related genetic changes in laboratory populations of *Drosophila subobscura*: evidence against simple climatic-based explanations for latitudinal clines. *The American Naturalist* **165**: 258-273.

Simões, P., Rose, M. R., Duarte, A, Gonçalves, R., & Matos, M. 2007. Evolutionary domestication in *Drosophila subobscura*. *Journal of Evolutionary Biology* **20**: 758-766.

Simões, P., Santos, J., Fragata, I., Mueller, L. D., Rose, M. R., & Matos, M. 2008. How repeatable is adaptive evolution? The role of geographical origin and founder effects in laboratory adaptation. *Evolution* **62**: 1817-1829.

Simões, P., Santos, J., & Matos, M. 2009. Experimental evolutionary domestication. In T. Garland Jr & M. R. Rose (Eds.), *Experimental Evolution: Concepts, Methods, and Applications of Selection Experiments*. Berkeley: University of California Press (pp. 89-110).

Storz, J. F., Balasingh, J., Bhatz, H., Nathan, P. T., Doss, D. P. S., Prakashzz, A. A., & Kunzx, H. T. 2001. Clinal variation in body size and sexual dimorphism in an Indian fruit bat, *Cynopterus sphinx* (Chiroptera: Pteropodidae). *Biological Journal of the Linnean Society* **72**: 17-31.

Travisano, M., Mongold, J. A., Bennett, A. F., & Lenski, R. E. 1995. Experimental tests of the roles of adaptation, chance, and history in evolution. *Science* **267**: 87-90.

Are reproductive barriers involved in the maintenance of a latitudinal cline?

Abstract

The role of the mechanisms of reproductive barriers in speciation is very important. These can evolve through several ways. One of them is as a by-product of local adaptation, in which individuals discriminate in order to avoid progeny less fit. *Drosophila subobscura* exhibits latitudinal clines for several traits, which can be considered as adaptations to different environments. However, the neutral genetic differentiation among populations along the cline is low. The maintenance of such clines suggests that populations are differently adapted to their latitude, involving some kind of reproductive barrier. When populations from cline extreme invade a new common environment, reproductive barriers may decrease or increase over time. After 5 generations in a new common environment, both populations derived from Adraga (Portugal) and Groningen (Netherlands) demonstrated a preference for homogamic matings. However hybrid breakdown was not detected between populations. From generation 5 to 10 the assortative mating faded away, indicating a relaxation of the selective pressures in the new common environment. Also, that revealed the need to add a temporal component to studies of reproductive isolation

Key-words: reproductive barriers; *Drosophila subobscura*; hybrid breakdown; mating behaviour.

Introduction

Reproductive isolation is a major issue in evolutionary biology, as it can ultimately lead to speciation, which has always been object of intense discussion. There are still questions about the main types and causes of speciation. Specifically, how reproductive barriers evolve and are maintained over time.

The mechanisms that prevent interbreeding between natural populations are called reproductive isolation mechanisms. Despite the fact that these mechanisms can be classified as pre-zygotic or post-zygotic, quoting Mayr (1970), 'an isolating mechanism is rarely an all-or-none affair'. Pre-zygotic mechanisms occur before mating, preventing therefore wastage of gametes and gene flow between populations. Pre-zygotic mechanisms include seasonal and habitat isolation, ethological isolation and mechanical isolation. One of the most important pre-zygotic mechanisms is assortative mating, when individuals choose to mate more often with individuals that are similar to themselves in some specific manner than expected by chance (Burley, 1983). Post-zygotic mechanisms occur after mating, reducing full success of crosses (Mayr, 1970). There are three categories of post-zygotic mechanisms: zygotic mortality, hybrid inviability and hybrid sterility. Due to these aforementioned mechanisms outbreeding depression can arise, which is a phenomenon that occurs when offspring of crossings between genetically differentiated populations, or higher *taxa*, have lower reproductive fitness than progeny from crossings between individuals from the same population (Waser & Price, 1989).

Reproductive barriers can arise through several mechanisms. One of them is when reproductive barriers evolve as a by-product of adaptation (Dodd, 1989; Rice & Hostert, 1993; Rundle *et al.*, 2000) to the environment. In this case, populations in allopatry may develop different mating rituals or mate at different timings. Hence, if individuals from these populations were to meet, the build-up of these differences would

prevent mating. Moreover, in allopatric populations, individuals may also adapt to specific pressures of each environment, which may lead to post-zygotic reproductive barriers. Another mechanism by which reproductive barriers can evolve is by selection acting directly on these traits (Servedio, 2001). Indeed, when populations are locally adapted, they will benefit from not mating with immigrants, hence there may be selection to prevent such mating events. Therefore, pre-zygotic barriers are more likely to evolve in sympatry.

Along a latitudinal cline, the different populations are adapted to their specific environment. Since the differences between populations are gradual along the cline, there is probably gene flow between them (Saccheri *et al.*, 2008). So in order to maintain the local adaptation, reproductive barriers must exist. There are just a few studies that use populations with different genetic backgrounds to test if they are reproductively isolated when inhabiting the same environment (Gefen & Brendzel, 2011). Moreover, the existence of reproductive barriers is frequently studied in just one moment in time, as a fixed trait. It would be interesting to study if there are reproductive barriers between populations with different genetic backgrounds and how they evolve over time when in a new common environment. Since there are still doubts concerning the main types and causes of speciation; specifically how reproductive barriers evolve and are maintained over time. Also how reproductive barriers evolve between populations exposed to the same environment.

Drosophila subobscura is an excellent model to study this thematic, because it has differentiated populations in the extremes of latitudinal clines. This species is originally Paleartic and has colonized South America and later North America about three decades ago (Prevosti *et al.*, 1989). This species exhibits latitudinal clines for chromosomal inversions and many quantitative traits (Prevosti, 1974; Pegueroles *et al.*, 1995; Gilchrist *et al.*, 2001; Santos *et al.*, 2004; Santos *et al.*, 2005). It has been proposed that this cline could be due to historical processes (Krimbas & Powell, 1992). However, the fact that this cline evolved independently in South America (Prevosti *et al.*, 1990) and North America (Gilchrist *et al.*, 2001) suggests that selection may be involved and it has to be strong and consistent along the latitudinal gradient (Balanyà *et al.*, 2003). Nevertheless, in a study using microsatellites no significant genetic differentiation was found between European populations (Pascual *et al.*, 2001). Therefore the maintenance of differences in several traits between populations over time suggests that reproductive barriers may play a role in the maintenance of the cline. Also, there is some indication of post-zygotic isolation between different European populations of *D. subobscura*, namely biased sex-ratio towards females and male sterility in backcrosses due to different chromosomal arrangements (Hauschteck-Jungen, 1990). Moreover, populations from two extremes of the latitudinal cline were confirmed to be differentiated for several characteristics very related to fitness (see previous article of this thesis).

With this study we aimed to determine a) if there are reproductive barriers between two populations from the two extremes of the cline; b) if so to monitor their evolution during adaptation to a new common environment: the laboratory. To these aims, assays of mating behaviour and crosses between the two sets of populations were conducted to determine whether there is a preference in the choice of sexual partners by females and, if so, if this choice entails fitness cost expressed in lower performance of hybrids. Subsequently, a second set of behaviour assays was made after several generations of laboratory adaptation, to determine whether the adaptation to the laboratory changes reproductive barriers, e.g. reduction due to relaxation of selective forces or increase due to evolutionary contingencies.

Material and Methods

Foundation and Maintenance of Populations

The protocols of foundation and maintenance of populations are described in the previous article of this thesis.

Mating behaviour assays

Virgin individuals were sexed within 6-8 hours after emergence and kept in groups of 10 individuals of the same sex, until the time they would be assayed (10 days of age). The first assay of mating behaviour within and across populations was done at the fifth generation after foundation from the wild and the second at generation 10. No-choice and choice mating experiments were done.

In the no-choice experiments, one male and one female were placed in each observation vial without CO_2 anaesthesia. The following combinations were assayed: homogamic (Gro x Gro - population from Groningen, Ad x Ad - population from Adraga) and heterogamic (Gro x Ad - populations from Groningen x populations from Adraga, Ad x Gro - populations from Adraga x populations from Groningen – females are always indicated first). The experimental set-up consists of three blocks, grouping the four mating types according to replicate number, so that one replicate of each species was represented in each block (e.g. block 1 consisted of the following mating types: Gro_1 x Gro_1, Ad_1 x Ad_1, Gro_1 x Ad_1, Ad_1 x Gro_1, and similarly for the other two blocks). The several blocks were assayed at different times (including different days). When possible, each block consisted of 25 series of 8 mating pairs, two of each mating types. Due to flies' apathy, the mated pairs were observed during 90 minutes to increase the possibility of mating.

In the female-choice experiments the set-up involved placing one male from each population in the same vial with a female that comes from one of the experimental population. Two days before the assays, males from each population were marked with an innocuous powder, half with green and the other half with red powder. In each vial, one of the males was marked with a green powder and the other with a red powder. Half the vials had the Adraga male marked with green powder and the Groningen male marked with red powder and vice-versa for the other half, to avoid the powder possible influence in performance. The set-up was similar to the no-choice experiments, but consisted of 25 series of 4 mating pairs, two of each possible mating combination (and with the two colour combinations).

Three parameters were measured (in seconds) in these assays: courtship latency (LC) which is the time since the beginning of the assay until the first courtship; courtship duration (CD); and mating duration (MD).

Hybrid Breakdown

This fecundity assay involved a synchronous analysis of both populations, Adraga (Ad) e Groningen (Gro), and their F_1 and F_2 crosses at generation 11 (hereafter called 'generations'). The F_1 individuals were obtained by crossing the parental populations by pairing populations according with replicate number. Individuals from both cross directions were assayed (F_1A – individuals with Ad populations as mother population and F_1B - individuals derived from the reciprocal cross). From the crossing of F_1A individuals and F_1B individuals

resulted F_2A and F_2B individuals respectively. In these assays we analyzed age of first reproduction (A1R), early (F1-6), peak fecundity (F7-9), male and female resistance to starvation (see Matos *et al.*, 2002). Sample was around 24 mated pairs for each population and F_1 and F_2 crosses. Populations and their respective crosses were assayed synchronously.

Statistical analysis

All statistical analyses were performed using Microsoft Excel and Statsoft Statistica.

Mating Behaviour Assays – The effect of the different populations on the parameters LC, CD and ML was tested using a three-way ANOVA with the following model:

$$Y = \mu + B + M + F + B^*F + B^*M + M^*F + B^*M^*F + \epsilon$$

where B is the random factor block with three categories (1, 2, 3) and M and F the fixed factors male and female respectively with two categories (Ad and Gro). This model was used to analyze both no-choice and female-choice data. To test if there were differences in the parameters mentioned above between generation 5 and generation 10, an ANCOVA analysis was used according with the model:

$$Y = \mu + B + M + F + G + B^*F + B^*M + M^*F + M^*G + F^*G + B^*M^*F + M^*F^*G + B^*M^*F^*G + \epsilon$$

where G is the generation fixed factor with two categories (5 and 10).

For the analysis of the female-choice experiments replicated goodness-of-fit tests and G-tests of independence were also done (Sokal & Rohlf, 1995) for each one of the generations. Two different replicated goodness-of-fit tests were done assuming the two competing males are equally likely to mate. First, we compared the number of heterogamic versus homogamic matings; second we compared the number of Ad mated males with the number of Gro mated males. For each test, a heterogeneity G-test was done to test if there was heterogeneity between the different replicates (blocks). Then, a pooled G-test was done to test if the pooled data deviates from the expectation of random mating. G-tests of independence were performed to test differences in each generation and between generations. In each generation two tests were made: the first was to test whether there were differences between Ad and Gro mated males versus the not mated males; and the second to test differences between homogamic and heterogamic matings and the matings that did not occur. Across generations, the G-test of independence was used to test differences between Ad and Gro mated versus the not mated males; between Ad and Gro mated males; and the two types of matings. The G-value was compared to the critical value of a χ^2 distribution for (n - 1) degrees of freedom.

Hybrid Breakdown

To test whether there were differences between the different generations (populations and their crosses) a two-way mixed ANOVA was performed for all traits analysed according with the following model

$$Y = \mu + G + B + G^*B + \epsilon$$

where G is the fixed factor Generation, with three categories (P, F_1 and F_2), and B is the Block, random factor with three categories (1, 2, 3). In order to test for the presence of the composite additive [a], dominance [d], epistasis [e] and maternal [m] effects (Mather & Jinks, 1982), orthogonal contrasts were done between the corresponding means (see Table 1 for contrast coefficients used) (for further details see Rego *et al.*, 2007). The interaction G*B was used as the error term in all analyses.

Table 1 Contrast coefficients for the four composite genetic parameters. [a] – additive effects; [d] – dominance effects; [e] – epistatic effects; [m] – maternal effects.

	Ad	Gro	F_1A	F_1B	F_2A	F_2B
[a]	1	-1	0	0	0	0
[d]	-1	-1	1	1	0	0
[e]	-1	-1	-1	-1	2	2
[m]	0	0	1	-1	0	0

Results

Mating behaviour

From all mating assays performed about 33% pairs copulated.

No-choice experiments

At generation 5 there were significant differences between males in the CL and MD parameters (Table 2). No significant differences were found between females or mating types in neither of the estimated parameters (CL, CD or MD) (Table 2; Fig. 1 left panel). Nevertheless Furthermore, Ad females were courted more often than Gro females (Fig. 1c). Globally, Ad males had a longer mating duration than Gro males.

Table 2 ANOVA for the parameters analysed in the no-choice experiments at generations 5 and 10. CL – courtship latency; CD – courtship duration; MD – mating duration.

	Factor	DF	CL	CD	MD
G5	B	2	-	0,9891	-
	F	1	0,6056	0,5790	0,4001
	M	1	0,0245*	0,9094	0,0024*
	B*F	2	0,6156	0,2121	0,9863
	B*M	2	0,9139	0,2002	0,8722
	F*M	1	0,8988	0,8268	0,1806
	B*F*M	2	0,1706	0,4655	0,6647
G10	B	2	0,7577	0,6444	0,6180
	F	1	0,2957	0,0148*	0,4471
	M	1	0,4678	0,4941	0,3695
	B*F	2	0,7366	0,3106	0,4909
	B*M	2	0,2553	0,0122*	0,5106
	F*M	1	0,9526	0,0002**	0,4036
	B*F*M	2	0,3673	0,9645	0,5349

^{MS} 0.10 > p > 0.05; *p < 0.05; ** p < 0.001

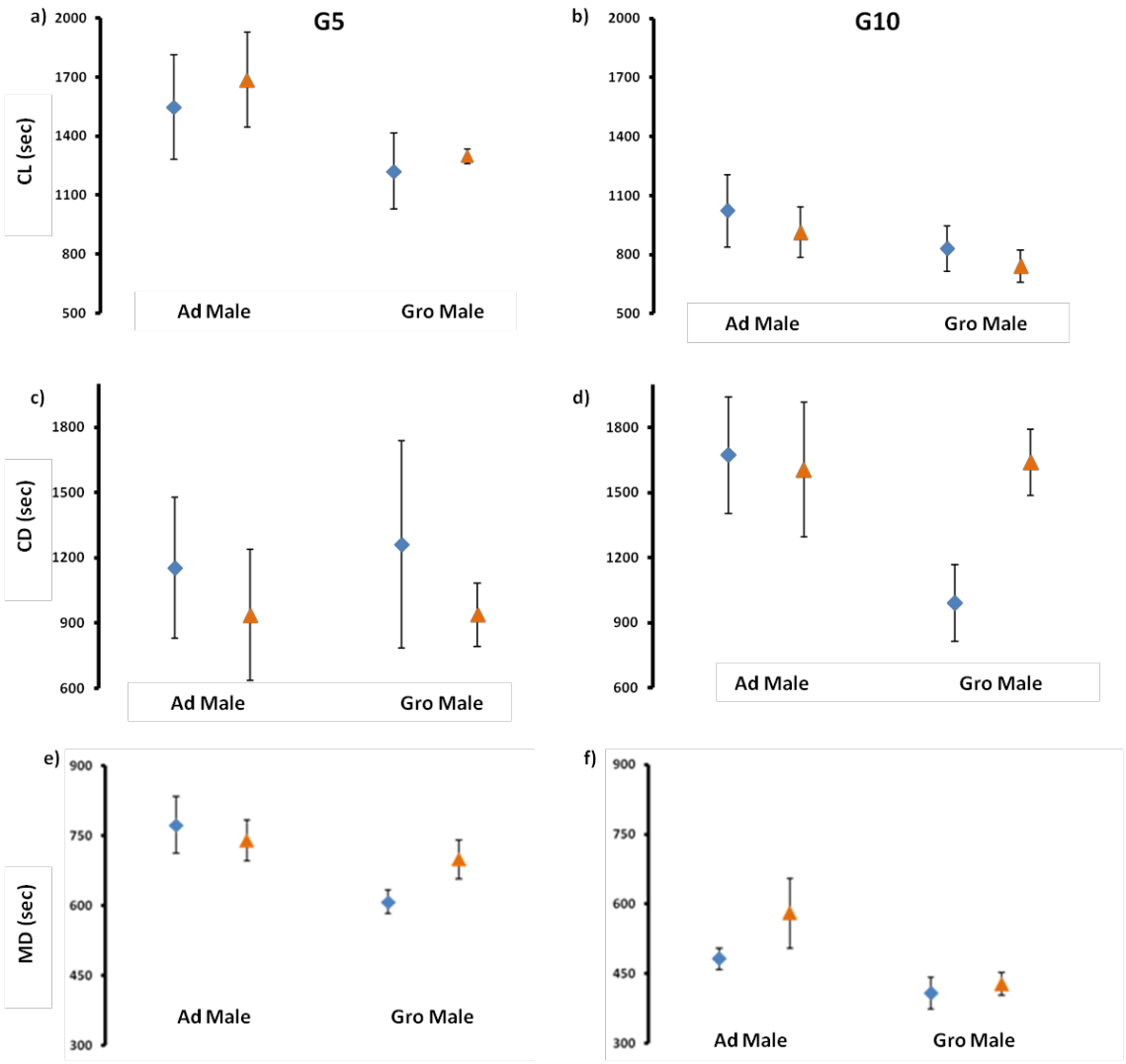

Fig. 1 Means of the no-choice experiments for each combination of male and female at generations 5 (left) and 10 (right). a) and b) courtship latency (CL); c) and d) courtship duration (CD); e) and f) mating duration (MD). Ad female (blue); Gro female (orange).

At generation 10 there were no significant differences between females, males or mating types in CL and MD (Table 2; Fig. 1 b, f). The exception was CD, where there were significant differences between females and mating types. It is also worth noting that Ad females had the shorter CD when courted by Gro males (Fig. 1d).

There were significant differences between generations 5 and 10 in the parameters CL and CD, but not in MD (Table 3). These significant differences reflected a decrease of CL and an increase of CD for both Adraga and Groningen males. Also, there were marginal differences between females across generations.

Table 3 ANOVA for the parameters analysed in the no-choice experiments between generations 5 and 10. CL – courtship latency; CD – courtship duration; MD – mating duration.

Factor	DF	CL	CD	MD
B	2	0,6730	0,8830	0,5006
F	1	0,4381	0,4431	0,2263
M	1	0,1779	0,5240	0,7333
G	1	0,0008**	0,0173*	0,8799
B*F	2	0,5346	0,2175	0,5063
B*M	2	0,6371	0,4046	0,2634
F*M	1	0,8558	0,4223	0,2085
F*G	1	0,3699	0,0832[MS]	0,3075
M*G	1	0,4423	0,2277	0,9619
B*F*M	2	0,3241	0,5422	0,7624
F*M*G	1	0,8581	0,1914	0,2762
B*F*M*G	8	0,3251	0,7518	0,6965

[MS] 0.10 > p > 0.05; *p < 0.05; ** p < 0.001

Female-choice experiments

At generation 5 there were no significant differences between females, males or mating types in any of the estimated parameters (CL, CD or MD) (Table S1; Fig. S1 left panel). Though not significantly different there is a suggestion that the Gro males mated for less amount of time than the Ad males (Fig. S1e). Gro males had a significantly higher number of matings than Ad males (Table S3; Fig.2). Despite the fact that there were more homogamic than heterogamic matings, the difference between them was only marginally significant (Table S3; Fig. 3).

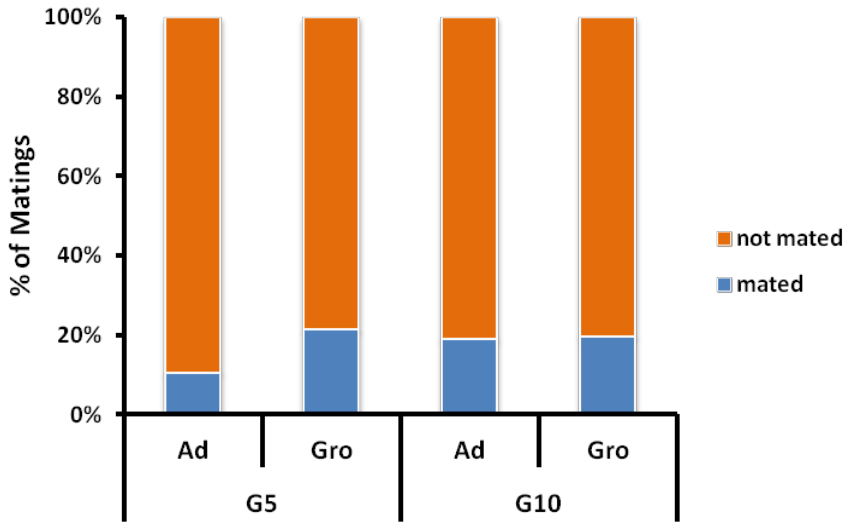

Fig. 2 Percentage of the mated versus the not mated Adraga and Groningen males for the female-choice experiments, at generations 5 and 10.

At generation 10, Gro males presented significantly lower mate duration, and marginally significantly lower court duration than Ad males (Table S1; Fig. S1). Otherwise there were no significant differences in CL, CD and MD between females or mating types. Also, there was no significant difference in the number of homogamic versus heterogamic matings (Fig. 3).

In general there were no significant differences between generations (Table S2), though there were marginally significant differences in LC (Table S2). Ad males had a significant higher number of matings in generations 10 than in generation 5 (Table S5). There were no significant differences between the number of homogamic and heterogamic matings across generations (Table S6).

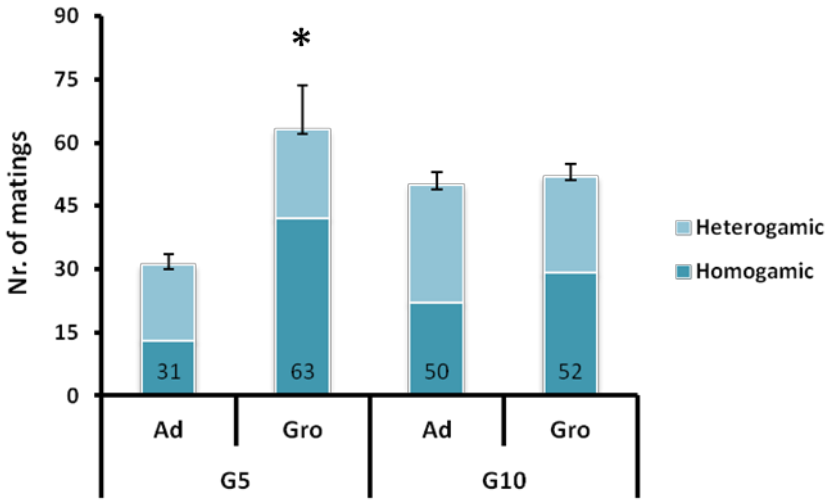

Fig. 3 Number of heterogamic and homogamic matings for Adraga and Groningen males, gat generations 5 and 10.

Hybrid Breakdown

Averages for the fitness traits assayed are plotted in Fig. 4, and statistical analyses are shown in Table 4. The only fitness trait that was significantly different between Ad and Gro was female starvation resistance (Table 4), for which a simple additive model was adequate. However when the analysis is performed just for Ad and Gro parental generations, the differences between Gro and Ad are significant for all traits (analysis is not shown). Also the parental population Gro had a better performance than the Ad in all traits (Fig. 4).

Table 4 ANOVA for the fitness traits assayed (age at first reproduction, fecundity and starvation resistance) measured for six generations (parental populations Ad and Gro, two F_1 hybrids, and two F_2 hybrids) with up to three replicated populations each. Composite genetic parameters were tested from orthogonal linear contrasts (see Table 1). The denominator used to calculate F-values for main effects and contrasts is the corresponding replicate*generation interaction.

	Source of variation	DF	*p-value*
Age of first reproduction	Generation	5	0.6057
	Block	2	0.6205
	Generation*Block	10	0.0023*
	[a]	1	0.1259
	[d]	1	0.5967
	[e]	1	0.5226
	[m]	1	0.8079
Fecundity	Generation	5	0.3697
	Block	2	0.0278*
	Generation*Block	10	0.0052*
	[a]	1	0.0663[MS]
	[d]	1	0.8396
	[e]	1	0.3324
	[m]	1	0.5952
Male starvation resistance	Generation	5	0.1319
	Block	2	0.0981[MS]
	Generation* Block	10	0.1479
	[a]	1	0.0656[MS]
	[d]	1	0.3634
	[e]	1	0.0718[MS]
	[m]	1	0.3110
Female starvation resistance	Generation	5	0.2255
	Block	2	0.1678
	Generation*Block	10	0.7504
	[a]	1	0.0349*
	[d]	1	0.4210
	[e]	1	0.2815
	[m]	1	0.8289

[MS] $0.10 > p > 0.05$; * $p < 0.05$; ** $p < 0.001$

The F_1 and F_2 hybrids from both cross directions did not differ significantly in any of the traits considered. Nevertheless, in general the F_1 hybrids had better performance than the mid-parent value for all traits (Fig. 4). The only exception was the F_1B hybrid population for early fecundity (Fig. 4b). The F_2 hybrids populations had better performance than the mid-parent value for age of the first reproduction and for female starvation resistance (Fig. 4a, d). But they had a worst performance for early fecundity and male starvation resistance (Fig. 4b, c).

There were significant differences in the interaction between blocks and generations, which reduced the statistical power of the analysis.

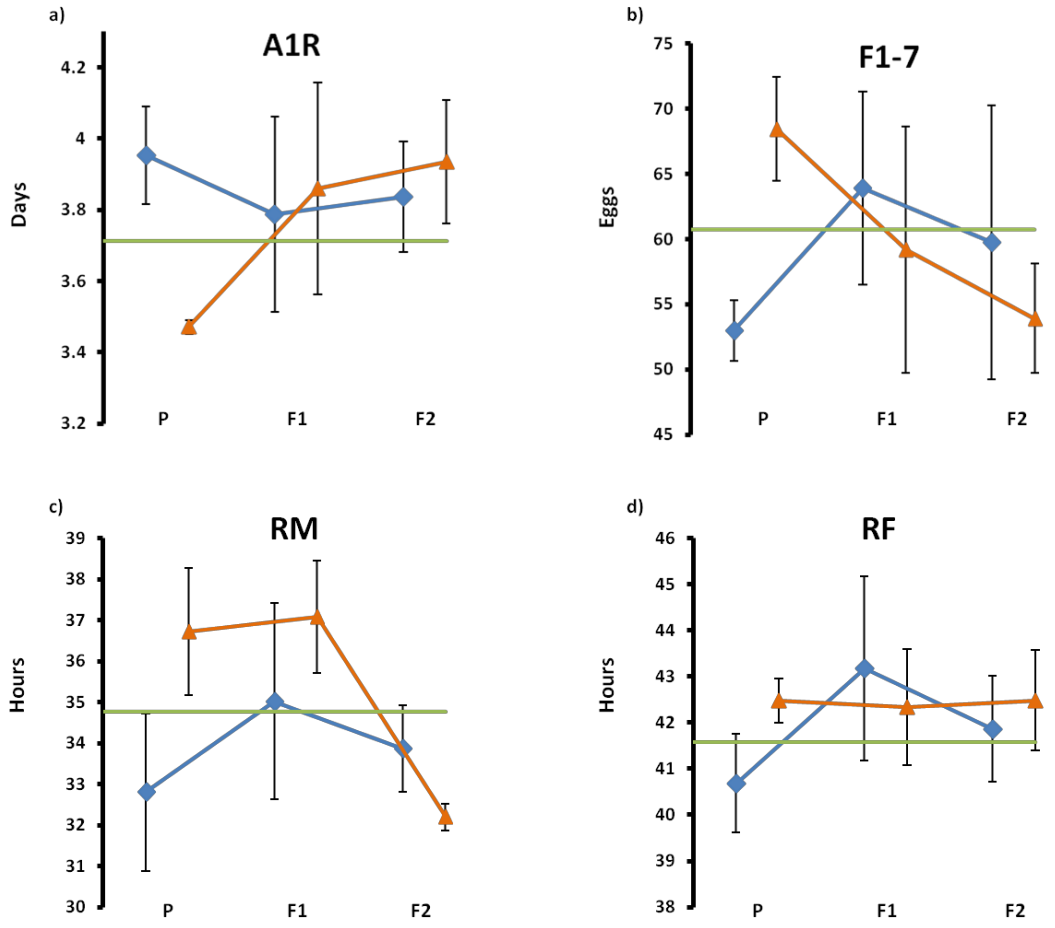

Fig. 4 Generation means for Ad and Gro, and their F_1 and F_2 hybrids from both reciprocal crosses, for all analysed traits: age of first reproduction, early fecundity, peak fecundity and survival. Blue: Ad and F_1 and F_2 hybrids with Ad as maternal population; orange: Gro and $F_1$and F_2 hybrids with Gro as maternal population. Lines connect the dots of the same maternal direction. Standard errors and a line indicating the mid- parent value for each trait are also given.

Discussion

In this study we detected a preference for homogamic matings (assortative mating), but not hybrid breakdown between Adraga and Groningen populations. Moreover, the assortative mating disappear between the two generations assayed, indicating a relaxation of the selective pressures in the new common environment.

Are there reproductive barriers between populations initially differentiated in nature?

There are models that predict the evolution of local adaptation despite the presence of gene flow (Fry, 2003). Also, reproductive barriers may evolve due to different mechanisms, namely as a by-product of adaptation to the local environment (Rundle *et al.*, 2000).

In the no-choice experiments there were differences between males in courtship latency and mating duration at an early generation after foundation. Groningen males started courtship earlier in time than Adraga males, indicating that they are sexually more active. This could be due to Groningen males being under selection to mate faster in nature. However extrapolations between laboratory and nature have to be made with caution (Matos *et al.* 2000). Other alternative is that Groningen populations are simply better in the laboratory environment than Adraga populations (previous article on this thesis). Despite the fact that there were no differences between females, Ad females were courted longer by Groningen males than by Adraga males (Fig. 1c). Also, Adraga females mated for more time with Adraga males than with Groningen males. This may indicate what has been observed before (Knowles & Markow, 2001), that homogamic matings are longer than heterogamic to ensure more sperm transferred, which contributes to a higher fertilization, leading to more reproductive success.

Against expectations there were no differences between males or females at generation 5 in the female-choice experiment for the parameters analysed. This could be due to the fact that this experimental design is more adequate to estimate the males that mate in competition, rather than the characterization of other parameters. Gro males had more reproductive success than Ad males at generation 5, which could be due to several factors: the inactivity of Adraga males; or the Groningen males being more ready to mate due to the fact that the Groningen populations have a lower age of first reproduction than Adraga populations (previous article on this thesis); or could be due to Groningen males bigger size (data not shown) (Krishna & Hegde, 2003). The higher number of homogamic matings at generation 5 is probably due to the fact that when there is the possibility to choose, the females choose a male of their own population (Nosil *et al.*, 2007). However this tendency was clearer in the Groningen populations than in the Adraga ones, which could indicate that Groningen females are more discriminatory than the Adraga females.

The crosses between Adraga and Groningen populations did not show any indication of hybrid breakdown. This lack of evidence could be due to the fact that these populations are part of a continuous s population than runs through Europe and that in these cases a mating barrier evolves before the hybrid sterility or inviability (Coyne & Orr, 1989). Alternatively, it could be due to the fact that at generation 11 (7^{th} under selection) of common environment is too late to detect hybrid breakdown between these populations. However, the parental populations Ad and Gro seem to be differentiated, which was expected (previous article). This differentiation seems thus to be only additive.

Did reproductive barriers change over time?

The second set of behaviour assays was made five generations after the first one. These assays would determine whether the adaptation to the laboratory changed the assortative mating detected in the previous assay.

In no-choice experiments there were differences for CL and CD parameters across generations. Both Adraga and Groningen males decreased for similar values the courtship latency (CL), indicating convergence for this parameter. This could be due to fact that these populations were being selected for fecundity for six generations (previous article of this thesis); leading them to mate faster at this point since they already had ten days of age. Moreover, the females were courted for a longer period at generation 10 than at generation 5, with the exception of the Adraga female by the Groningen male. The elevated room temperature could have lead to numbness of the flies, explaining the longer courtships (personal observation).

Between generations 5 and 10 there was a decrease of the differences between homogamic and heterogamic matings. This indicates a fade away of the assortative mating occurred at generation 5; which means that there was a relaxation of the selective forces leading to convergence. Also, between generations the number of Adraga mated males increased towards the number of Groningen mated males registered at generation 5, indicating convergence.

In summary, at a very early generation assortative mating was detected, a pre-zygotic barrier, between Adraga and Groningen populations. However hybrid breakdown, a post-zygotic barrier, was not detected. After several generations of adaptation to the common, laboratorial environment, the assortative mating faded away, indicating a relaxation of the selective pressures in the new common environment. Therefore reproductive barriers may play a role in the maintenance of differentiation observed between populations across latitudinal cline over time. Therefore, if there were no essays of two different generations involved in this study, there would be no knowledge of the reproductive barriers' disappearing. That implies that all reproductive isolation's studies need to have a time component, in order to determinate if the reproductive barriers' presence or lack of it maintains itself through time.

References

Balanyà, J., Serra, L., Gilchrist, G. W., Huey, R. B., Pascual, M., Mestres, Francesc, & Solé, E. 2003. Evolutionary Pace of Chromosomal Polymorphism in Colonizing Populations of *Drosophila Subobscura*: an Evolutionary Time Series. *Evolution* **57:** 1837-1845.

Burley, N. 1983. The meaning of assortative mating. *Ethology and Sociobiology* **4:** 191-203.

Coyne, J. A., & Orr, H. A. 1989. Patterns of Speciation in *Drosophila*. *Evolution* **43:** 362-381.

Dodd, D. M. B. 1989. Reproductive Isolation as a Consequence of Adaptive Divergence in *Drosophila pseudoobscura*. *Evolution* **43:** 1308-1311.

Fry, J. D. 2003. Multilocus models of sympatric speciation: Bush versus Rice versus Felsenstein. *Evolution* **57:** 1735-1746.

Gefen, E., & Brendzel, O. 2011. Desiccation resistance and mating behaviour in laboratory populations of *Drosophila simulans* originating from the opposing slopes of Lower Nahal Oren (Israel). *Journal of Evolutionary Biology* **24:** 2110-2117.

Gilchrist, G. W., Huey, R. B., & Serra, L. 2001. Rapid evolution of wing size clines in *Drosophila subobscura*. *Genetica* **112-113:** 273-286.

Hauschteck-Jungen, E. 1990. Postmating reproductive isolation and modification of the "sex ratio" trait in *Drosophila subobscura* induced by the sex chromosome gene arrangement A2+3+5+7. *Genetica* **83:** 31-44.

Knowles, L. L., & Markow, T. A. 2001. Sexually antagonistic coevolution of a postmating-prezygotic reproductive character in desert *Drosophila*. *Proc Natl Acad Sci USA* **98:** 8692-8696.

Krimbas, C. B., & Powell, J. R. 1992. *Drosophila* inversion polymorphism. Boca Raton: CRC Press.

Krishna, M. S., & Hegde, S. N. 2003. Influence of body size in mating success in three sympatric species of *Drosophila*. *Italian Journal of Zoology* **70:** 47-52.

Mather, K., & Jinks, J. L. 1982. *Introduction to biometrical genetics: the study of continuous variation*. London: Chapman and Hall.

Matos, M., Rego, C., Levy, A., Teotónio, H., & Rose, M. R. 2000. An evolutionary no man's land. *Trends in Ecology & Evolution (Personal edition)* **15**: 206.

Matos, M., Avelar, T., & Rose, M. R. 2002. Variation in the rate of convergent evolution: adaptation to a laboratory environment in *Drosophila subobscura*. *Journal of Evolutionary Biology* **15:** 673-682.

Mayr, E. 1970. *Populations, species, and evolution: an abridgment of Animal species and evolution*. Harvard University Press.

Nosil, P., Crespi, B. J., Gries, R., & Gries, G. 2007. Natural selection and divergence in mate preference during speciation. *Genetica* **129:** 309-327.

Pascual, M., Aquadro, C. F., Soto, V., & Serra, L. 2001. Microsatellite variation in colonizing and palearctic populations of *Drosophila subobscura*. *Molecular Biology and Evolution* **18:** 731-740.

Pegueroles, G., Papaceit, M., Quintana, A., Guillén, A., Prevosti, A., & Serra, L. 1995. An experimental study of evolution in progress: Clines for quantitative traits in colonizing and Palearctic populations of *Drosophila*. *Evolutionary Ecology* **9:** 453-465.

Prevosti, A. 1974. Chromosomal inversion polymorphism in the southwestern range of *Drosophila subobscura* distribution. *Genetica* **45**: 111-124.

Prevosti, A., L. Serra, M. Aguadé, G. Ribó, F. Mestres & J. Balanyà, 1989. Colonization and establishment of the Palearctic species *Drosophila subobscura* in North and South America, pp. 114–129 in *Evolutionary Biology of Transient and Unstable Populations*, edited by A. Fontdevila. Springer Verlag, Berlin.

Prevosti, A., Serra, L., Segarra, C., Aguade, Montserrat, Ribo, Griselda, & Monclus, M. 1990. Clines of Chromosomal Arrangements of *Drosophila subobscura* in South America Evolve Closer to Old World Patterns. *Evolution* **44:** 218-221.

Rego, C., Santos, M., & Matos, M. 2007. Quantitative genetics of speciation: additive and non-additive genetic differentiation between *Drosophila madeirensis* and *Drosophila subobscura*. *Genetica* **131:** 167-174.

Rice, W. R., & Hostert, E. E. 1993. Laboratory Experiments on Speciation: What Have We Learnes in 40 Years? *Evolution* **47:** 1637-1653.

Rundle, H. D., Nagel, L., Wenrick Boughman, J., & Schluter, D. 2000. Natural selection and parallel speciation in sympatric sticklebacks. *Science* **287:** 306-308.

Saccheri, I. J., Rousset, F., Watts, P. C., Brakefield, P. M., & Cook, L. M. 2008. Selection and gene flow on a diminishing cline of melanic peppered moths. *Proc Natl Acad Sci USA* **105**: 16212-16217.

Santos, M., Iriarte, P. F., Céspedes, W, Balanyà, J., Fontdevila, A, & Serra, L. 2004. Swift laboratory thermal evolution of wing shape (but not size) in *Drosophila subobscura* and its relationship with chromosomal inversion polymorphism. *Journal of Evolutionary Biology* **17:** 841-855.

Santos, M., Céspedes, Walkiria, Balanyà, J., Trotta, V., Calboli, F. C. F., Fontdevila, A. & Serra, L. 2005. Temperature-related genetic changes in laboratory populations of *Drosophila subobscura*: evidence against simple climatic-based explanations for latitudinal clines. *The American Naturalist* **165:** 258-273.

Servedio, M. R. 2001. Beyond reinforcement: the evolution of premating isolation by direct selection on preferences and postmating, prezygotic incompatibilities. *Evolution* **55:** 1909-1920.

Sokal, R. R., & Rohlf, F. J. 1995. Biometry: The Principles and Practice of Statistics in Biological Research. New York: W. H. Freeman and Company.

Waser, N. M., & Price, M. V. 1989. Optimal Outcrossing in *Ipomopsis aggregata*: Seed Set and Offspring Fitness. *Evolution* **43:** 1097–1109.

Final Remarks

The results of this thesis demonstrated that pre-zygotic reproductive barriers may play a role in the maintenance of the latitudinal cline of *Drosophila subobscura*.

Briefly, it was verified that Adraga and Groningen populations are differentiated populations in the laboratory environment for life-history traits. However, both foundations generally do not exhibit temporal changes over the generations. Moreover, both Adraga and Groningen populations demonstrated having a preference for homogamic matings whether in no-choice whether in female-choice experiments. But there was no indication of a post-zygotic barrier in the form of hybrid breakdown. Also, the pre-zygotic barrier in the form of mating preferences faded away in the second set of mating assays, indicating a relaxation of the selective forces in the new common environment. Therefore, if there were no essays of two different generations involved in this study, there would be no knowledge of the reproductive barriers' disappearing. That implies that all reproductive isolation's studies need to have a time component, in order to determinate if the reproductive barriers' presence or lack of it maintains itself through time.

Furthermore, there were issues poorly understood due to experimental limitations. The preference for homogamic matings at generation 5 could be more significant if we were able to do more crosses. It would have been interesting to have an evolutionary trajectory for mating behaviour, if we have been able to assay more generations. Also, the lack of indications of hybrid breakdown can be misleading, due to the late generation assayed. Maybe if it was done earlier it would be detected, since we observed fewer emergencies for some of the F_1 and F_2 crosses.

The results of the present work raised new questions not only concerning the reproductive barriers along the European cline, but also in the American cline, namely: If there is also a pre-zygotic barrier between populations geographically closer than the populations considered here (considering that this work was done under laboratory adaptation). Also, despite the fact that hybrid breakdown was not detected, if there are differences between hybrids development time and viability and the parental Adraga and Groningen populations. Other interesting questions are: if there are reproductive barriers between sets of European populations and South and North American populations; if there are reproductive barriers between extreme populations in both American continents. And, if there are, if these barriers would fade away as quickly as it happen between Adraga and Groningen populations. Answering these questions may play a role in the follow up process of clarification of the latitudinal cline maintenance and establishment both in Europe and in South and North America.

References

Arthur, A. L., Weeks, A. R., & Sgrò, C. M. 2008. Investigating latitudinal clines for life history and stress resistance traits in *Drosophila simulans* from eastern Australia. *Journal of Evolutionary Biology* **21:** 1470-1479.

Balanyà, J., Serra, L., Gilchrist, G. W., Huey, R. B., Pascual, M., Mestres, Francesc, & Solé, E. 2003. Evolutionary Pace of Chromosomal Polymorphism in Colonizing Populations of *Drosophila Subobscura*: an Evolutionary Time Series. *Evolution* **57:** 1837-1845.

Dodd, D. M. B. 1989. Reproductive Isolation as a Consequence of Adaptive Divergence in *Drosophila pseudoobscura*. *Evolution* **43:** 1308-1311.

Gefen, E., & Brendzel, O. 2011. Desiccation resistance and mating behaviour in laboratory populations of *Drosophila simulans* originating from the opposing slopes of Lower Nahal Oren (Israel). *Journal of Evolutionary Biology* **24:** 2110-2117.

Gilchrist, G. W., Huey, R. B., & Serra, L. 2001. Rapid evolution of wing size clines in *Drosophila subobscura*. *Genetica* **112-113:** 273-286.

Griffiths, J. A., Schiffer, M., & Hoffmann, A. A. 2005. Clinal variation and laboratory adaptation in the rainforest species *Drosophila birchii* for stress resistance, wing size, wing shape and development time. *Journal of Evolutionary Biology* **18:** 213-222.

Hauschteck-Jungen, E. 1990. Postmating reproductive isolation and modification of the "sex ratio" trait in *Drosophila subobscura* induced by the sex chromosome gene arrangement A2+3+5+7. *Genetica* **83:** 31-44.

James, A. C., Azevedo, R. B. R., & Partridge, L. 1995. Cellular Basis and Developmental Timing in a Size Cline of *Drosophila melanogaster. Genetics* **140:** 659-666.

Karan, D., Munjal, A. K., Gibert, P., Moreteau, B., Parkash, R., & David, J. R. 1998. Latitudinal clines for morphometrical traits in *Drosophila kikkawai*: a study of natural populations from the Indian subcontinent. *Genetical Research* **71:** 31-38.

Krimbas, C. B., & Powell, J. R. 1992. *Drosophila* inversion polymorphism. Boca Raton: CRC Press.

Lankinen, P. 1993. North–south differences in circadian eclosion rhythm in European populations of *Drosophila subobscura*. *Heredity* **71:** 210-218.

Matos, M., Avelar, T., & Rose, M. R. 2002. Variation in the rate of convergent evolution: adaptation to a laboratory environment in *Drosophila subobscura*. *Journal of Evolutionary Biology* **15:** 673-682.

Misra, R. K., & Reeve, E. C. R. 1964. Clines in body dimensions in populations of *Drosophilia subobscura*. *Genetical Research* **5:** 240-256.

Pascual, M., Aquadro, C. F., Soto, V., & Serra, L. 2001. Microsatellite variation in colonizing and palearctic populations of *Drosophila subobscura*. *Molecular Biology and Evolution* **18:** 731-740.

Pegueroles, G., Papaceit, M., Quintana, A., Guillén, A., Prevosti, A., & Serra, L. 1995. An experimental study of evolution in progress: Clines for quantitative traits in colonizing and Palearctic populations of *Drosophila*. *Evolutionary Ecology* **9:** 453-465.

Prevosti, A. 1966. Chromosomal polymorphism in western Mediterranean populations of *Drosophila subobscura*. *GenetRes* **7:** 149-158

Prevosti, A. 1974. Chromosomal Inversion Polymorphism in the southwestern range of *Drosophila subobscura* distribution. *Genetica* **45:** 111-124.

Prevosti, A., Serra, L., Segarra, C., Aguade, Montserrat, Ribo, Griselda, & Monclus, M. 1990. Clines of Chromosomal Arrangements of *Drosophila subobscura* in South America Evolve Closer to Old World Patterns. *Evolution* **44:** 218-221.

Rako, L., Blacket, M. J., McKechnie, S. W., & Hoffmann, A. A. 2007. Candidate genes and thermal phenotypes: identifying ecologically important genetic variation for thermotolerance in the Australian *Drosophila melanogaster* cline. *Molecular Ecology* **16:** 2948-2957.

Rice, W. R., & Hostert, E. E. 1993. Laboratory Experiments on Speciation: What Have We Learnes in 40 Years? *Evolution* **47:** 1637-1653.

Rundle, H. D., Nagel, L., Wenrick Boughman, J., & Schluter, D. 2000. Natural selection and parallel speciation in sympatric sticklebacks. *Science* **287:** 306-308.

Servedio, M. R. 2001. Beyond reinforcement: the evolution of premating isolation by direct selection on preferences and postmating, prezygotic incompatibilities. *Evolution* **55:** 1909-1920.

Simões, P., Rose, M. R., Duarte, A., Gonçalves, R., & Matos, M. 2007. Evolutionary domestication in *Drosophila subobscura. Journal of Evolutionary Biology* **20:** 758-766.

Simões, P., Santos, J., Fragata, I., Mueller, L. D., Rose, M. R., & Matos, M. 2008. How repeatable is adaptive evolution? The role of geographical origin and founder effects in laboratory adaptation. *Evolution* **62:** 1817-1829.

Simões, P., Santos, J., & Matos, M. 2009. Experimental evolutionary domestication. In T. Garland Jr & M. R. Rose (Eds.), Experimental Evolution: Concepts, Methods, and Applications of Selection Experiments. Berkeley: University of California Press (pp. 89-110).

Appendix I - Supplementary material of the 1st Research Article

Table S2 ANOVA for the differences between Regimes in the five traits analyzed at generations 4, 6 and 11. A1R – age of first reproduction; F1-7 – early fecundity; F8-12 – peak fecundity; RM and RF – male and female starvation resistance.

	Factor	DF	A1R	F1-7	F8-12	RM	RF
G4	Regime	2	0.0906[MS]	0.0000***	0.0004**	0.0254*	0.0436*
G6	Regime	2	0,0094**	0,0001**	0,0008**	0,1376	0,0570[MS]
	Population (Regime)	6	0,4672	0,1975	0,0098*	0,0000***	0,0074*
G11	Regime	2	0,0518[MS]	0,0008**	0,0043*	0,9535	0,5539
	Population (Regime)	6	0,0335*	0,0464*	0,0610[MS]	0,1396	0,0872[MS]

[MS] 0.10 > p > 0.05; *p < 0.05; ** p < 0.001; *** p < 0.0001

Table S2 ANOVAs for the differences between Regimes for development time and for viability of populations Ad and Gro, at generation 5, and TA.

	Factor	DF	*p-value*
Development Time	Regime	2	0,0030*
	Population (Regime)	6	0,0365*
	Sex	1	0,0181*
	Population (Regime*Sex)	6	0,3902
	Regime*Sex	2	0,3429
Viability	Regime	2	0,3517
	Population (Regime)	6	0,1562

[MS] 0.10 > p > 0.05; *p < 0.05; ** p < 0.001; *** p < 0.0001

Table S3 ANCOVA for the Population means across generations (4, 6 and 11) of each Regime. Five traits were analysed. A1R – age of first reproduction; F1-7 – early fecundity; F8-12 – peak fecundity; RM and RF – male and female starvation resistance.

		DF	A1R	F1-7	F8-12	RM	RF
Ad	Pop	2	0.8828	0.3522	0.8871	0.4883	0.5599
	Generation	1	0.2314	0.3486	0.4576	0.4041	0.0678[MS]
	Pop*Generation	2	0.8092	0.2567	0.7985	0.4643	0.4503
Gro	Pop	2	0.6477	0.0618[MS]	0.9962	0.4785	0.3517
	Generation	1	0.5305	0.1443	0.0158*	0.1402	0.3152
	Pop*Generation	2	0.4010	0.0126*	0.9950	0.3257	0.1780

[MS] 0.10 > p > 0.05; *p < 0.05; ** p < 0.001; *** p < 0.0001

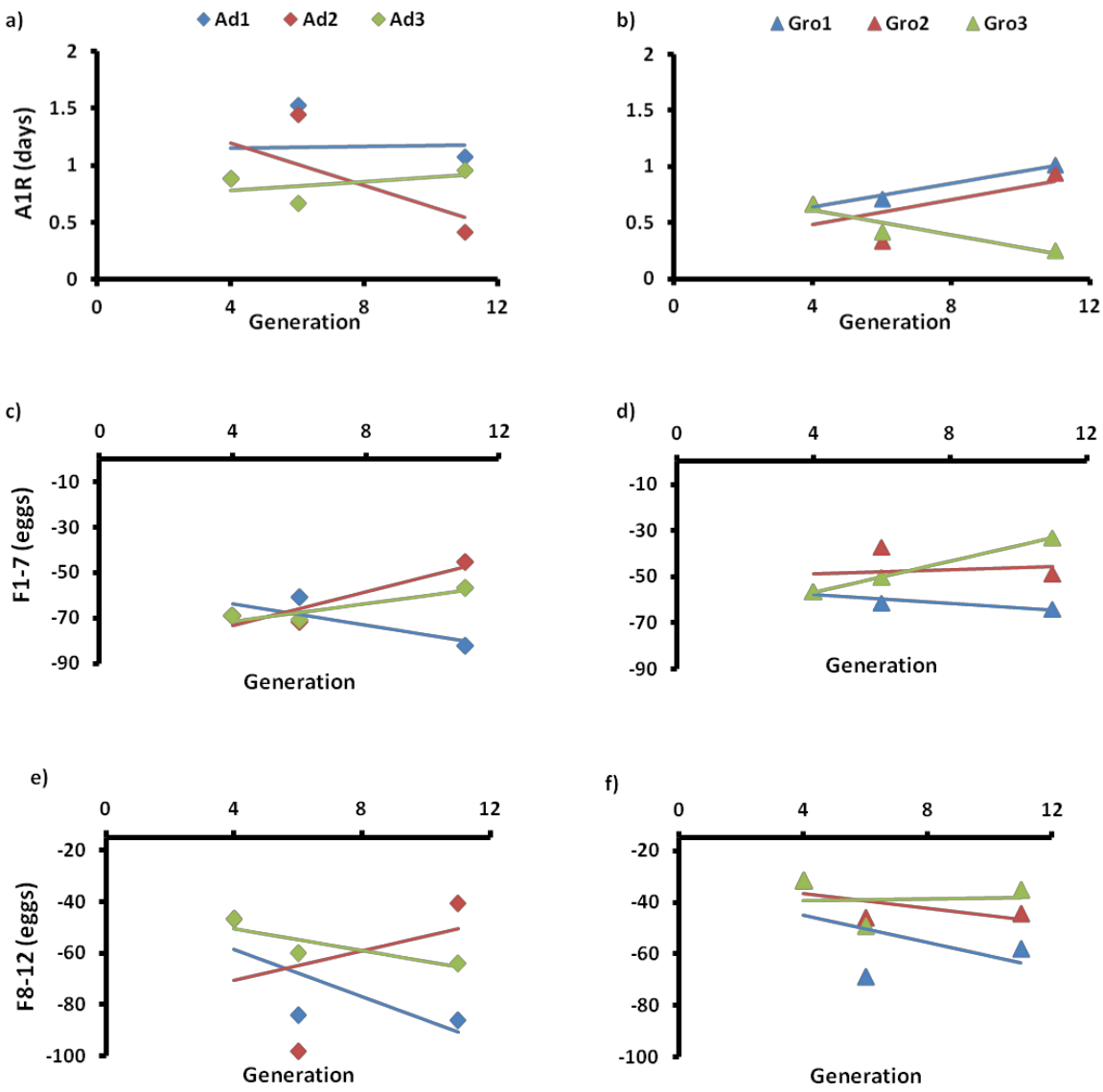

Fig. S1 Evolutionary trajectories of Ad and Gro, respectively. The data used correspond to the difference in mean values between each replica and its replica-control, in each generation. a) and b) A1R (age of first reproduction) of Ad and Gro respectively; c) and d) F1-7 (early fecundity) of Ad and Gro respectively; e) and f) F8-12 (peak fecundity) of Ad and Gro respectively. Replicate 1 (blue); Replicate 2 (red); Replicate 3 (green).

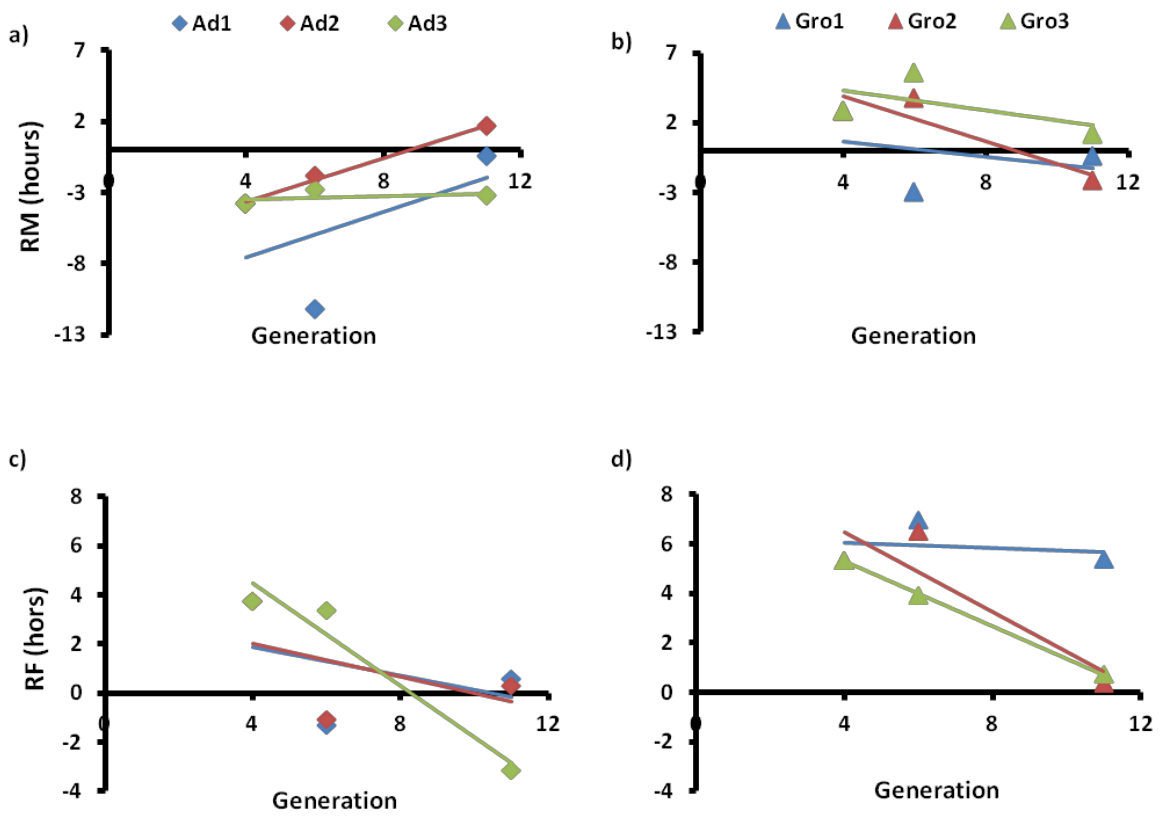

Fig. S2 Evolutionary trajectories of Ad and Gro, respectively. The data used correspond to the difference in mean values between each replica and its replica-control, in each generation. a) and b) MR (male starvation resistance) of Ad and Gro respectively; c) and d) FR (female starvation resistance) of Ad and Gro respectively.

Appendix II - Supplementary material of the 2st Research Article

Table S1 ANOVA for the parameters analysed in the female-choice experiments at generations 5 and 10. CL – courtship latency; CD – courtship duration; MD – mating duration.

	Factors	DF	CL	CD	MD
G5	B	2	-	-	0,6586
	F	1	0,4262	0,5762	0,3055
	M	1	0,5327	0,5221	0,9900
	B*F	2	0,7021	0,8030	0,5197
	B*M	2	0,9696	0,6117	0,4828
	F*M	1	0,3248	0,8697	0,4726
	B*F*M	2	0,3891	0,3894	0,0067*
G10	B	2	0,2041	-	0,5942
	F	1	0,9676	0,2491	0,6732
	M	1	0,1930	0,0892 MS	0,0481*
	B*F	2	0,2180	0,9491	0,5206
	B*M	2	0,1581	0,7138	0,6079
	F*M	1	0,0987MS	0,7334	0,4605
	B*F*M	2	0,8876	0,0866MS	0,6309

MS 0.10 > p > 0.05; *p < 0.05; ** p < 0.001

Table S2 ANOVA for the parameters analysed in the female-choice experiments between generations 5 and 10. CL – courtship latency; CD – courtship duration; MD – mating duration.

Factors	DF	CL	CD	MD
B	2	-	0,6898	0,7205
F	1	0,3344	0,4604	0,3128
M	1	0,1913	0,5735	0,8157
G	1	0,0542 MS	0,3700	0,1065
B*F	2	0,7880	0,4985	0,5478
B*M	2	0,8566	0,5428	0,4975
F*M	1	0,4806	0,4261	0,4607
F*G	1	0,5461	0,3679	0,1604
M*G	1	0,2044	0,7004	0,5170
B*F*M	2	0,4080	0,3195	0,0010**
F*M*G	1	0,6931	0,3287	0,2378
B*F*M*G	8	0,7650	0,6342	0,0023*

MS 0.10 > p > 0.05; *p < 0.05; ** p < 0.001

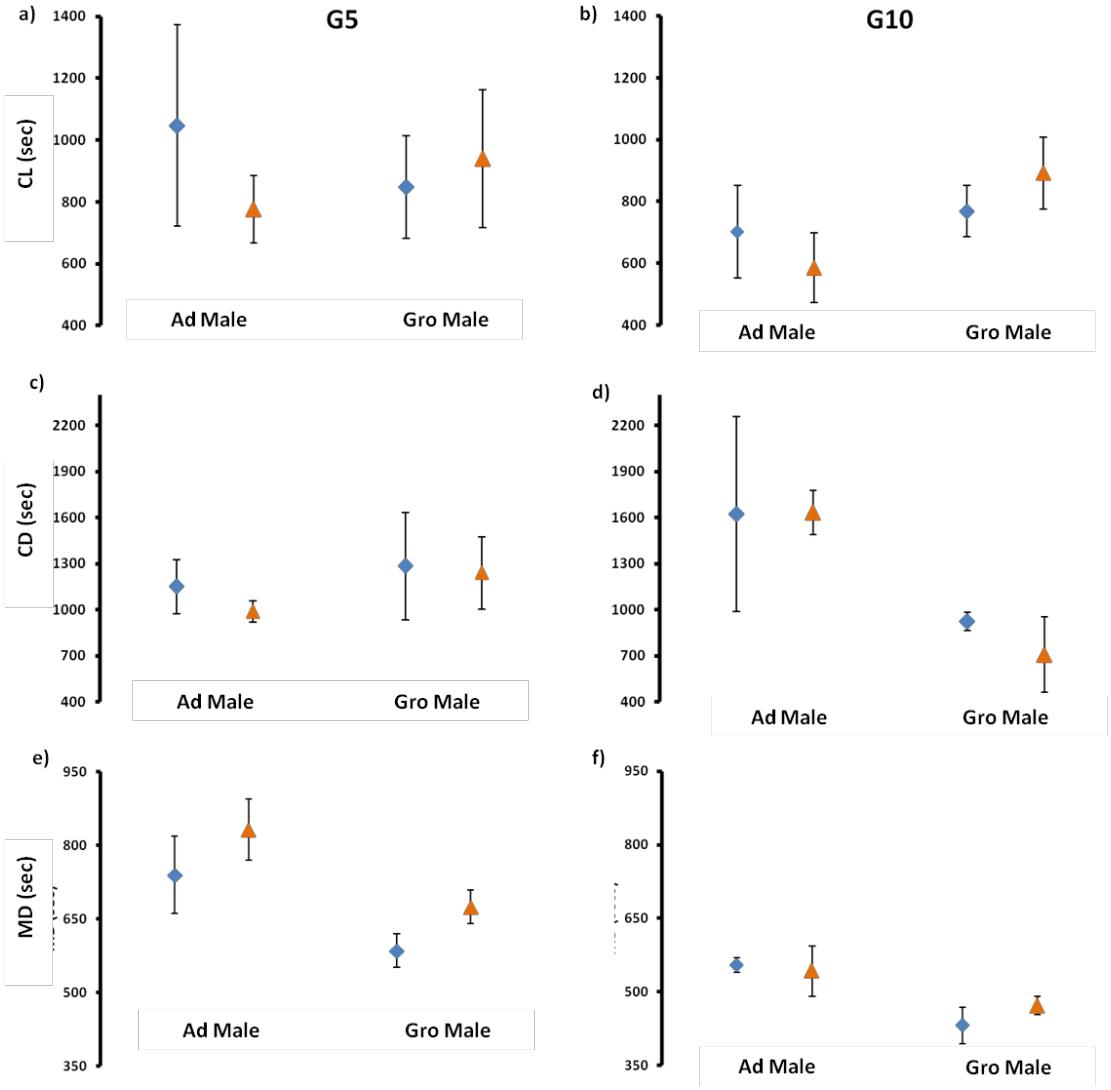

Fig. S1 Means of the female-choice experiments for each combination of male and female at generations 5 (left) and 10 (right). a) and b) courtship latency (CL); c) and d) courtship duration (CD); e) and f) mating duration (MD).

Table S3 Replicate Goodness-of-fit tests of female-choice experiments at generations 5 and 10. HO vs. HE – number of homogamic vs. number of heterogamic matings; ♂ Gro vs. ♂Ad – number of Gro males matings vs. number of Ad matings.

	Comparison	GH	DF	*p*	GP	DF	*p*
G5	HO vs. HE	0,7222	2	0,6969	2,7367	1	0,0981[MS]
	♂ Gro vs. ♂ Ad	2.2549	2	0.3239	11.1144	1	0.0009**
G10	HO vs. HE	0,7829	2	0,6761	0	1	1
	♂ Gro vs. ♂ Ad	0.0249	2	0.9876	0.0392	1	0.8430

[MS] 0.10 > p > 0.05; *p < 0.05; ** p < 0.001

Table S4 G-test of independence of female-choice experiments at generations 5 and 10. ♂ Gro vs. ♂Ad - number of Gro and Ad mated males vs. not mated number of Gro and Ad not mated males; HO vs. HE – number of mated homogamic and heterogamic males vs. number of not mated homogamic and heterogamic males.

	Comparison	Gi	DF	*p*
G5	♂ Gro vs. ♂ Ad	13,1721	1	0,0003**
	HO vs. HE	3,2509	1	0,0714[MS]
G10	♂ Gro vs. ♂ Ad	0,0486	1	0,8255
	HO vs. HE	0,4697	1	0,4933

[MS] 0.10 > p > 0.05; *p < 0.05; ** p < 0.001

Table S5 G-test of independence of female-choice experiments between generations 5 and 10. ♂Ad – number of mated and not mated Ad mated males at generation 5 vs. number of mated and not mated Ad mated males at generation 10; ♂ Gro – number of mated and not mated Gro mated males at generation 5 vs. number of mated and not mated Gro mated males at generation 10.

	Comparison	Gi	DF	*p*
♂ Ad	M vs. NM	8.1094	1	0.0044*
♂ Gro	M vs. NM	0.2156	1	0.6424

[MS] 0.10 > p > 0.05; *p < 0.05; ** p < 0.001

Table S6 G-test of independence of female-choice experiments between generations 5 and 10. ♂ Gro vs. ♂Ad – number of mated Ad and Gro males at generation 5 vs. number of mated Ad and Gro males at generation 10; HO vs. HE – number of homogamic and heterogamic matings at generation 5 vs. number of homogamic and heterogamic matings at generation 10.

Comparison	Gi	DF	*p*
♂ Gro vs. ♂ Ad	5.2258	1	0.0223*
HO vs. HE	1.4291	1	0.2319

[MS] 0.10 > p > 0.05; *p < 0.05; ** p < 0.001