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Playing evolution in the laboratory: From the first major evolutionary transition to global warming

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Playing evolution in the laboratory: From the first major evolutionary transition to global warming[(a)]

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Abstract – Experimental evolution allows testing hypotheses derived from theory or from observed patterns in nature. We have designed a droplet-based microfluidic "evolution machine" to test how transient compartmentalization ("trait-groups") of independent molecular replicators (likely a critical step in the origin of life) could have prevented the spread of parasitic mutants; that is, inactive RNAs that have been reported to spoil a system of free replicators. In remarkable agreement with the theory, we show that this simple population structure was sufficient to prevent takeover by inactive RNAs. A more complex scenario arises when we use experimental evolution to test field-derived hypotheses; for instance, the idea that temperature is driving genetic spatiotemporal patterns of climate change. In the fly *Drosophila subobscura*, latitudinal clines in gene arrangement frequencies occur worldwide, and more equatorial gene arrangements are becoming more frequent at higher latitudes as a correlated response to climate change. However, the evolution at different constant temperatures in the laboratory was not consistent with patterns in nature, suggesting some limitations of experimental evolution. Finally, also in *D. subobscura*, we show that repeatability in experimental evolution is staggeringly consistent for life history traits, making evolution quite predictable and suggesting that laboratory selection can quickly erase differences between populations. Yet, the genetic paths used to attain the same adaptive phenotypes are complex and unpredictable.

Introduction. – In November 1859, Darwin published the first edition of *The Origin of Species* [1]. One idea of the book, that all complex and sophisticated adaptations of organisms to their surrounding environments are not attributable to a designer but to a "blind" process of natural selection acting on individual differences in a population, defeated previous ideas that seemed basic to man's interpretation of the universe. In a sense, Darwin's hint went along with the conceptual revolution initiated by Adam Smith in his masterpiece *An Inquiry into the Nature and Causes of the Wealth of Nations*, published in 1776 [2]. This book advanced the radical and apparently contradictory idea that a society's wealth is the result of the egoistic individual desire to provide support for him and his family. Darwin's natural selection to promote species' adaptations is somehow reminiscent of Smith's "invisible hand"

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to boost the wealth of nations. Incidentally, these land-mark books can arguably be regarded as the first theories of complexity. Actually, in 1976, two hundred years after the publication of Adam Smith's book, Dawkins published *The Selfish Gene* [3]. This title serves as a metaphor to stress that genes that are evolutionary successful are those that have served their own implicit interest, not necessarily those of the organism.

Although Darwin was a gifted experimentalist and recruited whatever experimental evidence he had to support his reasoning, perhaps the main Darwinian revolution was in comparative biology (besides discovering an algorithm for the generation of organized complexity). This stems from another basic idea of Darwin's book that triggered a new way of thinking about comparisons across species: "descent with modification", which advocated the idea of an evolutionary tree and explicitly emphasised the continuity of the evolutionary process in the biological hierarchy. Modern comparative methods offer invaluable tools in the armamentarium of evolutionary biologists, but they have some shortcomings [4]. The main criticism relates to the difference between patterns and processes in evolution, or correlation and causation, which cannot be easily disentangled by the comparative method.

A complementary approach is experimental evolution, defined as a "research in which populations are studied across multiple generations under defined and reproducible conditions, whether in the laboratory or in nature" ([5], p. 6). It can be used for different purposes such as to learn about the long-term dynamics of adaptation and diversification, to test evolutionary hypotheses derived from theory, or to test hypotheses derived from observed patterns in nature. We are involved in several laboratory and/or nature experiments that aim i) to understand how the problem of parasitic replicators could be solved in the transition from single RNA replicators to networks of replicators; ii) to test whether temperature plays a major role in generating genetic patters in nature; and iii) to unravel the knot that history, chance and selection play in the adaptation to a novel environment.

The evolutionary transitions in biology have been plagued with potential conflicts between the different units (genes, chromosomes, organelles, cells, etc.), and the difficulty is that these transitions "must be explained in terms of immediate selective advantage to individual replicators" ([6], p. 8). This requires a multi-level selection scenario, an idea that can be traced back to Darwin when he evoked that certain instincts that lead to the death of the individual (*e.g.*, the instinct that drives the bee to sting and thus to die) could have evolved by natural selection because they were "useful to the community" ([1], p. 202). In the next section, we show that compartmentalization of molecular replicators (group selection) is an effective means to overcome the problem of parasitic mutants and prevent population extinction.

We then show how experimental evolution, combined with information about spatiotemporal patterns in nature,

can provide insights on how populations of the fly *D. subobscura* are responding to human-induced climate change. This will also serve to illustrate some problems and potential limitations of experimental evolution when testing field-derived hypotheses. Next, we focus on the long-lasting question about the relative importance of history, chance and selection, which generally are inextricably mixed in most evolving lineages. Using a set of laboratory populations of *D. subobscura*, we show how adaptation to new environmental conditions can be partitioned between these three processes. Because their relative impact defines how predictable evolution is, this is an issue of major relevance in evolutionary [7] and ultimately in conservation terms as well [8]. We also highlight the importance of considering different biological levels, from phenotypes to molecules, to understand adaptive evolution. Finally, we summarize our results.

Experimental multi-level selection on the transition "replicating molecules" → "populations of molecules in compartments". – This is the first major evolutionary transition listed by Maynard Smith and Szathmáry ([6], p. 6). It assumes a preceding history of free independent catalytically active replicators (most probably RNA-like macromolecules) experiencing Darwinian evolution (probably on surfaces; [9]). Earlier systems of free replicators faced three important hitches: accurate transfer of information, ecological coexistence, and evolutionary coexistence.

Being autocatalytic in nature, replicators proliferate and the transfer of sequence information is necessarily error-prone. Both the stability of information and the selection of fitter alternatives require the error rate to be below an error catastrophe threshold. If the product of error rate times the information content is above the threshold, Darwinian selection would be incapable of maintaining useful (functional) information and extinction (information crisis) becomes inevitable [10]. Because in any system of independent replicators the total information content is the product of the number of different templates times the maximum information coded per template, a way to increase complexity and, at the same time, to avoid the information crisis, is to ensure the coexistence of different replicators, each below the error threshold. This is the second hitch since such systems are vulnerable to extinction via stochastic fluctuations, and the information gain might then be non-significant [9]. Furthermore, Maynard Smith [11] raised the problem of fast replicating selfish-mutants (parasites) evolving in the system, which get catalytic support from other molecules but do not contribute to the "common good" and, therefore, can spoil the system.

The emergence through mutation of parasitic replicators is exemplified by the classical studies of *in vitro* evolution of RNA molecules carried out by Spiegelman and his colleagues [12]. They isolated and purified the more than 4000 nucleotides-long single-stranded Qβ RNA that

encodes a number of proteins, including the Qβ replicase. When a Qβ RNA population was maintained in a solution containing Qβ replicase and energy-rich nucleotide triphosphates, new infectious RNA strands were synthesized. However, when the population was kept under perpetual (exponential) growth using serial transfer, resource competition among RNA strains resulted in fast replicating molecules that evolved shorter sequences (83% of the virus genome was lost) and lost their infectiousness; that is, selection acted only on rates of replication and parasitic replicators took over.

To solve the conundrum of coexistence of different replicators, Szathmáry and Demeter [13] proposed a multi-level selection scenario; the stochastic corrector model (SCM) that describes the dynamics of replicators (genes) encapsulated in a reproductive vesicle (compartment, protocell) (fig. S1A given in the Supplementary Material `Supplementarymaterial.pdf` (SM)). As originally devised, the SCM is a traditional group selection model in that there is no mixing of gene content among protocells, and "infected" vesicles simply die out preventing the spreading of the mutant parasites to healthy ones. An intermediate state between a system of independent replicators and the SCM could have been what Wilson [14] called a "trait-group", where, *e.g.*, a population of replicators at generation t is transiently subdivided (encapsulated) into a number of compartments (groups), and compartments' productivity to the next pool of replicators (generation $t+1$) depends on the composition of the compartment (fig. S1B given in the SM). Those compartments containing replicators that contribute to the "well-being" of the group will pass on more replicators to the next pool. Trait-group models help to understand one of the enduring problems in the study of social evolution: how cooperation can be maintained in the presence of free-riders, individuals that take advantage of the more cooperative members of the group but do not reciprocate. Their potential role for the origin of life was suggested over two decades ago [15].

Inspired by the natural model of compartmentalisation, together with Andrew Griffiths we developed a droplet-based microfluidic "evolution machine", created by integrating multiple microfluidic modules into microfluidic chips (digital microfluidic systems, consisting of networks of channels of typically 10–$100\,\mu$m diameter). This microfluidic device was in turn applied to show that transient compartmentalisation with selection at the compartment level can keep emerging parasites at bay [16]. In order to partially mimic the supposedly once existing RNA-world, a ribozyme was replicated and tested under three different experimental protocols: i) replication in bulk with no selection for enzymatic activity, ii) replication in compartments without selection for enzymatic activity, and iii) replication in compartments with selection for sufficient enzymatic activity in the individual droplets. The ribozyme applied is the Varkud satellite (VS) transacting ribozyme that can cut an external RNA substrate.

This ribozyme was pasted into the so-called midivariant (MDV-1) that was obtained by selection for replication speed in the Spiegelman experiment (see above). Because a general replicase ribozyme is still lacking, we had to use the Qβ proteinaceous replicase (this trick is, of course, not realistic for the RNA-world scenario). Selection was carried out by fluorescent-activated droplet sorting: the substrate of the VS ribozyme had a fluorescent dye attached to one part and a quencher attached to the other part. Upon successful enzymatic cleavage, the quencher got detached from the dye that was able to glow under laser light. Droplets with a sufficient level of the fluorescent signal were submitted to the harvest of internal content, which then was mixed from the different retained droplets, and they were compartmentalized into the droplets of the next "generation". We emphasize that there was no reproduction at the droplet level, this is why the compartmentalisation was transient.

For the three experimental protocols described above, we found the following respective outcomes: i) fast takeover by non-enzymatic parasitic mutants, ii) slower takeover by "fast" parasites; and iii) protected polymorphism with the coexistence of enzymes and "slow" parasites. In agreement with theory [17], the "multiplicity of infection" (MOI) during droplet loading with templates was critical: with higher MOI, parasites in the mixed phase have a higher chance of getting into the droplets, which almost certainly results in a subcritical overall enzymatic activity, and hence in a subcritical compartment fluorescence for sorting. (We mention in passing that due to the low parasite numbers at the beginning of growth within droplets stochastic effects were found to be important, but these numbers increased to rather high level by replication, which circumstance called for a deterministic approach: our combined model is in surprisingly good agreement with experimental results.)

Thermal evolution in *Drosophila subobscura*: using experimental evolution to test hypotheses derived from observed patterns in nature. – A remarkable episode with *D. subobscura* was that a replicated experiment of invasion happened in nature, when the species was discovered in 1978 in Puerto Montt (Chile) and a few years later, in 1982, near Port Townsend (WA, USA). This invasion helped to decipher the contribution of history, chance and selection in shaping latitudinal clines.

D. subobscura is native to the Palearctic region where it has a wide geographic distribution spanning more than $30°$ of latitude, ranging from Northern Africa to Scandinavia. The frequencies of different gene arrangements (see *Chromosomal inversion polymorphism* and fig. S2 in the SM) in *D. subobscura* show latitudinal clines in Old World populations, but it was not clear whether the patterns were due to selection or historical contingency [18]. Latitudinal gradients in climate (*e.g.*, temperature) might have favoured different gene arrangements at different latitudes, but the problem with this interpretation is that the

southern peninsulas of Europe acted as refugia for many species at the height of the last Weichselian glaciation that ended 11700 years ago; species that rapidly expanded northward as the climate warmed. Genetic differentiation of *D. subobscura* in the refugia and its posterior postglacial expansion might have also contributed to generate the latitudinal clines.

The replicated natural experiment that occurred after the invasion and rapid expansion (within approximately three years after its arrival) of *D. subobscura* in the New World helped to test between these alternative interpretations. Repeated collections in Chile and North America, where the species spans about 15° latitude in each location, revealed latitudinal clines that were parallel to those found in the original Palearctic region [19]. Thus, "cold-climate" gene arrangements (*i.e.*, those gene arrangements that show a negative correlation coefficient with maximum temperatures, or a positive correlation coefficient with latitude) were generally the same across continents. This provided compelling evidence that latitudinal gradients in climate are driving the latitudinal clines in gene arrangements.

The most obvious latitudinal gradient in climate is temperature. Additional results on seasonal patterns and long-term trends suggest that temperature is the main selective factor driving the genetic clines, although this interpretation is not without caveats. First, repeated seasonal changes with cold-climate gene arrangements increasing in frequency near winter season and decreasing in summer, are consistent with the latitudinal clines [20]. However, we do not know whether these cyclical patterns are due to hotter summer temperatures *per se*, or to a longer warm and a shorter cool season during which alternative selective factors might play a role.

Second, historical surveys comparing the frequencies of gene arrangements in old and new samples from the same localities indicate that the new collections are more "equator-like", suggesting that the current human-induced global warming is changing the genetic composition of *D. subobscura* populations in the expected direction (*i.e.*, a historical increase in the frequency of warm-climate gene arrangements [20,21]). Again, this interpretation is open to criticism because various complex gene arrangements now appear at relatively high frequencies in some northern locations, where they were undetected in historical surveys [20]. This suggests that the long-term trends in gene arrangements frequencies are caused, at least partially, by migration and not by local adaptation [20].

Third, a strong heat wave in April 2011 affected extensive geographic areas in the world, including Western Europe [22]. It caused a transient shift in the chromosomal gene arrangement polymorphism of *D. subobscura* to the extent that the frequency anomaly of warm-climate inversions (some gene arrangements deviated $+5.4\sigma$ from the average frequency expected in spring) neatly matched the temperature anomaly [22].

Testing spatiotemporal patterns with experimental evolution. If temperature is driving spatiotemporal patterns in inversion frequencies, populations cultured in the laboratory at different temperatures should evolve in the predicted direction: cold-climate gene arrangements increasing in frequency in a cold environment and warm-climate gene arrangements increasing in frequency in a warm environment. This conceptually trivial experiment was performed using a large stock of flies collected in Puerto Montt, the place where *D. subobscura* first appeared in the New World. A set of threefold replicated populations were placed at three constant temperatures (13 °C (cold), 18 °C (optimum) and 22 °C (warm)) and were allowed to evolve for several generations under controlled conditions, and the frequency of chromosomal gene arrangements was estimated at different points in time. The conclusion was clear: although gene arrangement frequencies shifted in the thermal regimes, the results were inconsistent with predictions from nature [23]. For instance, some gene arrangements defined as cold-climate according to spatiotemporal patterns in nature evolved higher frequencies at 22 °C than at 13 °C, just the opposite to what should be expected. Inconsistences also happened for other traits that also display latitudinal clines such as body size, where flies sampled from high latitudes are genetically bigger than their corresponding counterparts at low latitudes. However, flies from the thermal lines did not evolve differences in body size [23]. Why laboratory results did not match natural patterns?

Problems in using experimental evolution to test natural patterns. Besides temperature, other factors (day length, competitors, predators, etc.) also change with latitude and seasonal variation, dispersal, and interactions with other species are important elements in the population dynamics of *D. subobscura*. Keeping flies at constant temperatures could have been problematic because temperature variation over time is just as important as average temperature. Based on empirical and theoretical evidence, fitness increases exponentially with temperature from a critical thermal minimum (CT_{min}) up to a maximum fitness (r_{max}) at the optimum temperature (T_{opt}), and then drops swiftly to the critical thermal maximum (CT_{max}) [24]. The dependence of fitness on temperature can be modelled as a Gaussian function for the increasing portion of the curve up to T_{opt}, and a quadratic decline to zero fitness at the critical thermal maximum (CT_{max}) (see *Thermal performance curves* in the SM).

Two rival hypotheses predict how the maximum fitness of ectotherms evolves in response to thermal adaptation: the compensation hypothesis and the "thermodynamic constraint" or "warmer is better" hypothesis [24]. The basic argument is whether cold-adapted species can overcome the fact that low temperature slows down rates of biochemical reactions. If this were the case, cold-adapted ectotherms with a lower T_{opt} could attain the same maximum fitness than their warm-adapted counterparts do at their optimal temperature, so that r_{max}

would be independent of T_{opt}. We tested these alternative scenarios in the thermal lines and found that flies that evolved at 22 °C had the highest fitness thus supporting the warmer is the better hypothesis [25].

Frazier *et al.* [26] have made some important generalizations. First, cold-adapted insects reach CT_{max} at lower temperatures than do warm-adapted populations. Second, warmer is better for insects and, hence, cold-adapted populations achieve lower r_{max} at their thermal optimum than warm-adapted populations do at their T_{opt}. Third, lower latitude populations experience a narrower range of daily and annual temperatures than high latitude populations and, consequently, cold-adapted populations usually have a wider thermal tolerance. Using Chilean populations of *D. subobscura*, we found latitudinal divergence in thermal preference, with high-latitude flies having a lower mean T_{opt} [27]. However, contrary to expectation we found that high-latitude populations were more tolerant to an acute heat stress (a positive latitudinal cline for CT_{max}) [28].

We also tested whether flies carrying a high number of cold-climate gene arrangements (*i.e.*, we aggregate the different cold-climate arrangements located on the five major chromosomes of *D. subobscura* into the single index "genome-wide cold dose") have a lower CT_{max} and a lower T_{opt} than flies carrying fewer cold-climate gene arrangements. The answer was affirmative in both cases, although the total amount of variance explained by the index was around 1% [29]. What have all these findings to do with the lack of correspondence between results from experimental evolution and patterns in nature? One basic problem is that we do not know the exact dependence of fitness on temperature for the different genotypes, and what we call "cold environment" (13 °C) might not have increased the fitness of cold-adapted gene arrangements above the fitness of warm-adapted gene arrangements (fig. S3 given in the SM).

An additional problem is that the imposed selective thermal regimes might not have been stressful enough. The heat wave that occurred in the spring of 2011 taught us that the genetic constitution of *D. subobscura* flies responds to thermal extremes. Tolerance to thermal extremes might be more important in the evolution and dynamics of the gene arrangement clinal patterns in *D. subobscura* than the average conditions experience in the different environments. By manipulating only one environmental variable in the thermal lines (constant ambient temperature), we obviously missed the effect of other variables such as the daily, seasonal and annual fluctuations in temperature.

History, chance and selection. – The interplay between historical contingencies, chance events and selection ultimately defines to what extent we can predict evolutionary patterns and outcomes. As we are facing an unprecedented impact of human-induced climate change, it becomes urgent to forecast how much the past of natural populations will shape their future. There is an increasing interest to ascertain how foreseeable is evolution, and the emerging picture is that it is surprisingly more predictable than one might expect, particularly at higher levels of organization [7]. However, few studies have quantified the relative roles of history, chance and selection, and their combined effect on predictability. By analysing in real time how replicated populations evolve when facing novel environmental changes, we can i) investigate the similarities and differences in evolutionary patterns across populations from a same source (chance events), ii) estimate the impact of the ancestral state (population history) on evolutionary patterns and processes, and, ultimately, iii) quantify the speed of response to new challenges and evaluate possible limits to this response (selection).

To tackle these issues, we have been analysing for over 20 years how *D. subobscura* populations, recently introduced in a controlled, laboratorial environment, change through time in a panoply of traits such as life-history traits (*e.g.*, fecundity, developmental rate), physiology (stress resistance), behaviour (mating), and morphology (body size). The basic idea is that the laboratory is just another environment, to which populations are subjected and might adapt to. Experimental evolution using recently founded outbred populations from wild collections, such as *D. subobscura*, is particularly informative given the expected high initial standing genetic variation. Our experiments are therefore complementary to classical experiments that use haploid clonal organisms derived from an ancestral clone, where all adaptation happens only through new genetic mutations [30]. Furthermore, adaptive evolution in multicellular organisms might drastically differ from that of unicellular ones [31].

Evolutionary trajectories when experimental populations derive from the same initial source. Originally, our natural source populations were restricted to locations in Portugal, and we have analysed repeated colonisations in the laboratory to characterize their general evolutionary dynamics, as well as their consistency (or absence of) across nearby locations and years. The basic experimental design was quite simple: threefold replication of each founder population by the second generation and periodical assays, particularly for fecundity and starvation resistance (a "surrogate" for survival). We found that evolution is repeatable in the general convergence to longer-term laboratory populations, but also contingent, as differences in evolutionary rates or even patterns were also observed across populations and traits [32]. The analysis of molecular markers showed that major differences quickly arose in the laboratory, as founder populations were quite similar but became genetically differentiated during the first few generations after introducing flies from nature [33]. Bottom line, the rapid evolutionary changes during the earliest generations of the founder event were the major factor behind the differences between our populations later on.

Because all laboratory populations derived from the same "source" natural population, their initial state was

the same. What would be the outcome if the initial populations had contrasting evolutionary histories? How predictable (or not) is evolution as a function of the ancestral state? Despite the relevance of this question, there is a surprising lack of studies addressing it [33].

Evolutionary trajectories from contrasting initial states.

As discussed above, *D. subobscura* shows clear latitudinal clines for body size and inversion polymorphisms. Taking advantage of this natural differentiation, in 2010 we founded *D. subobscura* populations derived from synchronous collections in three European locations of contrasting latitudes: Adraga (Portugal), Montpellier (France) and Groningen (Netherlands), and basically followed the same experimental protocol used in previous studies [34]. The results were astonishing: though our populations started clearly differentiated for life history, morphological and physiological traits, in few generations they became quite similar (fig. 1(A)), swiftly converging among themselves and towards the values of our longer-term populations [34]. In particular, fitness related traits improved at a rate inversely proportional to the initial performance of the populations. On the whole, selection was able to erase the signature of history (fig. S4 given in the SM). Chance events lead to some divergence among replicate populations, but without much impact relative to the main convergent trend across all populations.

Phenotypic solutions to the laboratory environment might be more limited than the number of alternative genetic trajectories to reach the same adaptive phenotype. This seems to be the case because our populations did not converge for gene arrangement frequencies [35] (fig. 1(B)). Molecular genome-wide analysis (only in Adraga and Groningen) also revealed that our populations followed different genomic pathways notwithstanding the fact that they evolved similar adaptive phenotypes [36]. Evolution might thus be highly predictable at one level (phenotype) while widely unpredictable at another (genotypes, at different scales). We might take these results as an empirical proof of what is expected based on theoretical grounds [37]. It also fits the expectations of a rugged fitness landscape, in which populations explore different places and, due to historical contingencies (*e.g.*, epistasis), end up in different, though similar in values, adaptive peaks.

Besides exploring the roles of history, chance and selection using a spatial dimension, experimental evolution also allows identifying repetitive patterns in time. This is what we are studying now. In 2013, three years after the previous experiments, we sampled flies from Adraga and Groningen and founded new laboratory populations. We found quite predictable phenotypic evolutionary patterns [38], suggesting that spatiotemporal convergence at the phenotypic level is robust. However, these new populations showed a tendency to converge for inversion frequencies, in contrast to what we found before. We are now studying the populations at the molecular level to see whether there is a similar trend.
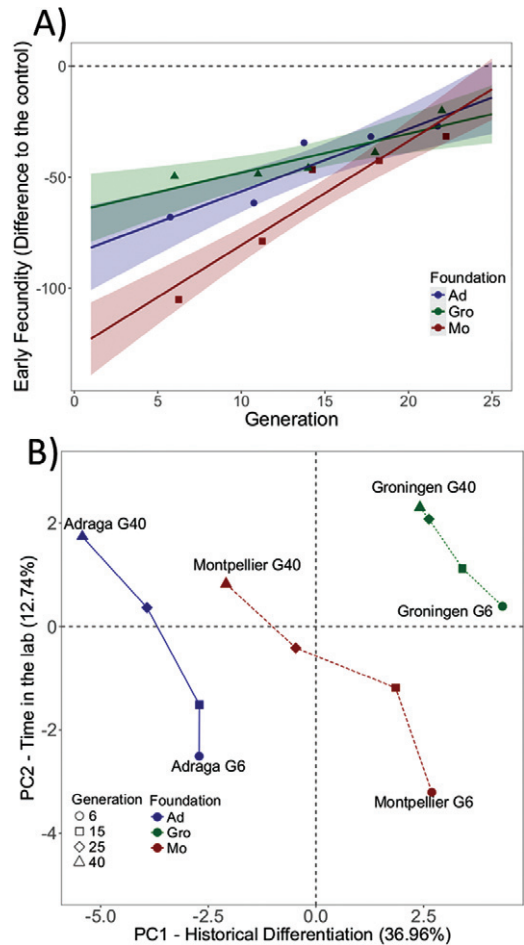

Fig. 1: (Colour online) Evolutionary trajectories of Adraga, Groningen and Montpellier populations founded in 2010. (A) Trajectories for early fecundity. (B) Multivariate evolutionary trajectories for 29 gene arrangements distributed on the five major chromosomes of *D. subobscura* using Principal Component Analysis.

The clear repeatability of phenotypic evolution in the laboratory environment suggests that we can predict evolutionary outcomes under common selective pressures. A number of questions remain. Based on earlier phases of evolution, how will populations change later on? More challenging, how much can the knowledge of a population's initial evolutionary pattern help to predict both short-term and long-term evolution of another population? Combining real-time evolution experiments with a theoretical framework, we can test expectations on the predictability of such evolutionary patterns. Assuming there is a single phenotypic optimum, models that include deceleration of the evolutionary rate with time are expected to be the best predictors of evolution in our case [39]. Moreover, this would suggest that it is possible to use short-term evolutionary patterns to estimate longer-term evolution within and across populations founded in different years and from different sources.

Concluding remarks. – We have shown how experimental approaches allow the re-instantiation of at

least some critical, hypothetical intermediate stages of the earliest evolution of molecular replicators. The effects of transient compartmentalization have been clearly demonstrated by experiments, in remarkable agreement with theory. A next step would be to experimentally realize the phase of reproducing compartments to show the working of the SCM [13] *in vitro*. Further questions can be experimentally addressed, including the origin of chromosomes and early evolution of genetic coding [6].

Using the laboratory as a simple environment, we have illustrated that populations are able to walk to the same phenotypic adaptive peak, but the genetic paths they used to reach it are complex and unpredictable [38]. Even in populations with high standing genetic variation, the predictability of evolution is dependent on the biological level analysed.

Experimental evolution does have, however, its own limitations and has to be combined with other approaches. The lack of correspondence between spatiotemporal patterns in nature and results in the laboratory [23] could have been due to our (in retrospect) naive assumption that average environmental temperature is all that matters. This "failure" fostered additional experiments that, together with observed evolutionary responses to a heat wave, suggest that more complex evolutionary scenarios (*e.g.*, subject the flies to constant and fluctuating thermal environments) need to be explored.

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