Mechanisms of adaptation to a changing world

Adrián Baños Villalba

PhD Thesis

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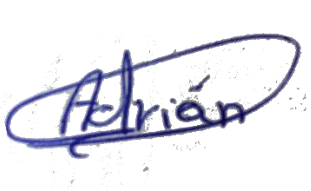
Memoria presentada por el Licenciado en Ciencias Ambientales

Adrián Baños Villalba

para optar al título de Doctor por la Universidad Pablo de Olavide



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CERTIFICAN:

Que los trabajos de investigación desarrollados en la Memoria de Tesis Doctoral "*Mechanisms of adaptation to a changing world*", son aptos para ser presentados por el Ldo. Adrián Baños Villalba ante el Tribunal que en su día se designe, para aspirar al grado de Doctor por la Universidad Pablo de Olavide.

Y para que así conste, y en cumplimento de las disposiciones legales vigentes, firman el presente documento en Sevilla, a 11 de Enero de 2018.

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A mis padres

A Marta

A mi hermano

INDEX

ABSTRACT

The current extinction rate of species on Earth is greater than any of the mass extinctions registered in the fossil record in its entire history. This increased biodiversity loss is caused one way or the other by the human species. Changes in land use, climate or biological invasions are acting worldwide. In this context, understanding the mechanisms by which organisms adapt to the environment and the ecological and evolutionary consequences that these entail is a key factor. In this thesis, this question is approached from two different perspectives.

The first one (**Section 1**) assesses how populations of invasive species adapt to a new environment. Before a population becomes invasive in a non-native area, it must first have passed through the earlier stages of invasion (capture, transport and introduction) before their establishment in this area. These stages could be acting as selective filters of individual variation. In this way, the introduced individuals would not be a random sub-sample of the native population of origin. This could have a great impact on their invasive potential. However, what happens in these earliest invasion stages has hardly ever been studied. To test the hypothesis that selection acts already early during a biological invasion, we followed the individuals of two invasive bird species from their native habitat in Senegal and during these early stages of a potential invasion. We indeed found that selection acts on variation in a gene related to behaviour (**Chapter I**). In addition, we found that selection also acts on many other phenotypic characteristics that could have a great importance for invasive potential, such as sex, age, body size, brain size, beak size and shape, body condition, stress hormone levels and behaviour (**Chapter II**).

The second perspective (**Section 2**) assesses how native populations adapt to environmental changes. For this we studied all the possible mechanisms of adaptation (natural selection, phenotypic plasticity, habitat choice and environment adjustment), but especially focusing on matching habitat choice. This mechanism is based on the non-random dispersal of individuals due to an assessment of variation in their local performance, such that individuals settle

down in those habitats that best match their phenotypes. Despite its eco-evolutionary importance, this mechanism has received almost no research attention. In this thesis, we study how a native population of grasshoppers has adapted in camouflage (a classic form of adaptation to the environment) in the colonization of a new urban environment (one of the most drastic changes in the habitat). We found a population divergence on a micro-geographic scale (differently coloured grasshoppers on distinctly coloured urban substrates) despite the existence of a lot of (presumably homogenising) movement by individuals. In **Chapter III**, we demonstrate that habitat choice, and not other mechanisms such as natural selection or phenotypic plasticity, is the main mechanism that has caused the recent local evolution of camouflage and the micro-geographic population divergence. In addition, we find that habitat choice acts also at a much finer scale, in which individuals improve their camouflage by aligning with certain substrate patterns depending on their degree of colour matching with the substrate, making it a flexible way to increase performance on different spatial scales (**Chapter IV**). However, this matching between phenotype and environment can also be achieved through phenotypic plasticity. In **Chapter V** we show that grasshoppers are able to change their body coloration through successive moults to resemble the substrate on which they live. The degree to which they do so is affected by the risk of predation they are exposed to: experimental increase of risk resulted in an increased phenotypic adjustment.

Taken together, this thesis demonstrates in a convincing and quantitative manner the existence and importance of two neglected mechanisms of adaptation of populations to environmental changes, thereby increasing our understanding of how invasive and native populations adapt to change and ecological opportunities in an increasingly changing world.

RESUMEN

La tasa de extinción actual de las especies en la Tierra es mayor que cualquiera de las extinciones masivas registradas en el registro fósil en toda su historia. Esta mayor pérdida de biodiversidad es causada de una manera u otra forma por la especie humana. Los cambios en el uso de la tierra, el clima o las invasiones biológicas actúan de forma global. En este contexto, entender los mecanismos por los cuales los organismos se adaptan al medio ambiente y las consecuencias ecológicas y evolutivas que implican es un factor clave. En esta tesis, esta cuestión se aborda desde dos perspectivas diferentes.

La primera (**Sección 1**) evalúa cómo las poblaciones de especies invasoras se adaptan a un nuevo entorno. Antes de que una población sea invasora en un área no nativa, primero debe haber pasado por las etapas más tempranas de la invasión (captura, transporte e introducción) antes de su establecimiento en dicha área. Estas etapas podrían actuar como filtros selectivos de variación individual. De esta forma, los individuos introducidos no serían una sub-muestra aleatoria de la población nativa de origen. Esto podría tener un gran impacto en su potencial invasivo. Sin embargo, lo que sucede en estas primeras etapas de invasión casi nunca se ha estudiado. Para testar la hipótesis de que la selección ya actúa en las fases más tempranas durante una invasión biológica, seguimos a los individuos de dos especies de aves invasoras desde su hábitat natural en Senegal y durante estas primeras etapas de una posible invasión. De hecho, encontramos que la selección actúa sobre la variación en un gen relacionado con el comportamiento (**Capítulo I**). Además, encontramos que la selección también actúa sobre muchas otras características fenotípicas que podrían tener una gran importancia para el potencial invasivo, como sexo, edad, tamaño corporal, tamaño del cerebro, tamaño y forma del pico, condición corporal, niveles hormonales de estrés y comportamiento (**Capitulo II**).

La segunda perspectiva (**Sección 2**) evalúa cómo las poblaciones nativas se adaptan a los cambios ambientales. Para esto estudiamos todos los posibles mecanismos de adaptación (selección natural, plasticidad fenotípica, elección del

hábitat y ajuste del ambiente), pero especialmente centrándonos en la elección del hábitat correspondiente. Este mecanismo se basa en la dispersión no aleatoria de individuos debido a una evaluación de la variación en su desempeño local, de modo que los individuos se establecen en los hábitats que mejor se adaptan a sus fenotipos. A pesar de su importancia eco-evolutiva, este mecanismo casi no ha recibido atención de investigación. En esta tesis, estudiamos cómo una población nativa de saltamontes se ha adaptado en camuflaje (una forma clásica de adaptación al ambiente) en la colonización de un nuevo entorno urbano (uno de los cambios más drásticos en el hábitat). Encontramos una divergencia poblacional a escala micro-geográfica (saltamontes de diferentes colores sobre sustratos urbanos de distintos colores) a pesar de la existencia de un gran movimiento (presumiblemente homogeneizador) por parte de los individuos. En el **Capítulo III**, demostramos que la elección del hábitat, y no otros mecanismos como la selección natural o la plasticidad fenotípica, es el principal mecanismo que ha causado la reciente evolución local del camuflaje y la divergencia de la población a escala micro-geográfica. Además, encontramos que la elección del hábitat también actúa a una escala mucho más fina, en la que los individuos mejoran su camuflaje al alinearse con ciertos patrones de sustrato dependiendo de su grado de coincidencia de color con el sustrato, convirtiéndolo en una forma flexible de aumentar el rendimiento en diferentes escalas espaciales (**Capítulo IV**). Sin embargo, esta coincidencia entre el fenotipo y el medio ambiente también se puede lograr a través de la plasticidad fenotípica. En el **Capítulo V** mostramos que los saltamontes son capaces de cambiar la coloración de su cuerpo a través de mudas sucesivas para parecerse al sustrato en el que viven. El grado en que lo hacen se ve afectado por el riesgo de depredación a la que están expuestos: el aumento experimental del riesgo resultó en un aumento del ajuste fenotípico.

En conjunto, esta tesis demuestra de manera convincente y cuantitativa la existencia e importancia de dos mecanismos poco estudiados de adaptación de las poblaciones a los cambios ambientales, aumentando nuestra comprensión de cómo las poblaciones nativas e invasoras se adaptan al cambio y las oportunidades ecológicas en un mundo cada vez más cambiante.

GENERAL INTRODUCTION

Adaptation to the environment is a main characteristic of life on Earth, and the core focus of evolutionary theory. The question of how organisms adapt to their environment is becoming increasingly relevant in the context of recent global change, with components such as changes in land use, climate change and biological invasion. As a result of these changes, global biodiversity is changing at an unprecedented rate, with declines in local species abundance and, critically, species become locally and globally extinct. As a consequence, a biodiversity reduction across the planet is happening. The current biodiversity loss is only comparable, and even higher, in rate and magnitude with the five previous mass extinctions of Earth's history (Barnosky et al. 2011). People and economies are also impacted by these global changes, through costly management of native and invasive species, alteration of natural ecosystems, loss of natural resources and ecosystem services, and threats to public health. Hence, future biodiversity and ecosystem functioning will depend on how organisms (including the human species) respond to these changes.

In such a world of change, populations will be pressured to adapt to altered circumstances or they might go extinct. On the other hand, human-induced environmental change and movement of organisms are also creating many opportunities for populations to enter and colonize new environments. The extent to which they succeed in doing so will have significant impacts on future biodiversity, both by establishing new populations and by driving populations of other species to extinction.

A great deal is already known about how populations adapt to new situations. Adaptation is achieved by obtaining a better match between a population's phenotype distribution and the environment they interact with. An increased phenotype-environment match can be achieved by changing the population's phenotype distribution. This change can occur by natural selection, where some phenotypes are removed because individual survival and reproduction depend on this match (Darwin 1859; Barton et al. 2007; Fig. 1). Also, the change can happen via adaptive phenotypic plasticity, changing the phenotype distribution via a developmental response of individuals to the environment (Schlichting and

Pigliucci 1998; Fig. 1). We can think of no way to change a distribution of elements other than by removing and adding elements (i.e. demographically, as in natural selection) or by changing the existing elements (i.e. developmentally, as in plasticity).

An overlooked but logical alternative to improve phenotype-environment matching is for organisms to actively change their environment. They can either go from one environment to another where their matching is better and increase their fitness, by the process of habitat choice (Edelaar et al. 2008; Fig. 1). Alternatively, they can achieve a better match by making changes to their local environment, adjusting the environment to the requirements of their phenotype (Laland and Sterelny 2006; Fig. 1).

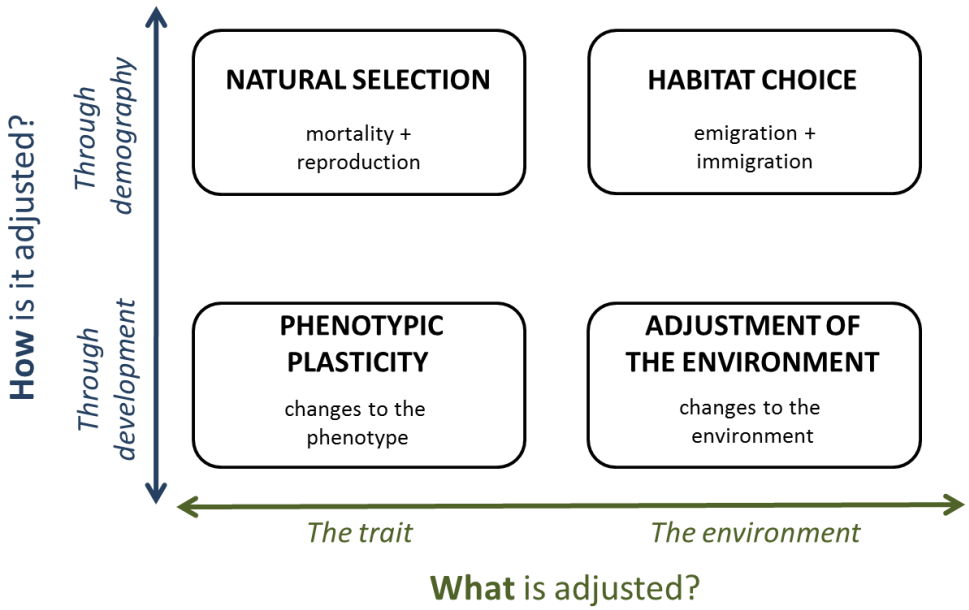

Figure 1. Framework to interrelate the mechanisms of population adaptation to environmental variation, classified by what element changes, and how it changes. Adaptation can be achieved in four ways (panels). (Based on Edelaar & Bolnick, in preparation).

In this conceptual framework (Fig. 1), it is important to note that the evolution of phenotypic plasticity, habitat choice and adjustment of the environment are

mechanisms that have been originally driven by natural selection. However, once these mechanisms exist, in theory they could act independently of each other to achieve locally enhanced fitness. In this way, natural selection would lead to the evolution of processes (habitat choice, plasticity and adjustment of the environment) that would avoid natural selection itself, but changes in environmental conditions prevent this from happening, changing the original selective pressures.

In this PhD thesis, I explore these mechanisms of adaptation from two different perspectives: (I) how invasive populations are adapting to a new environment and (II) how native populations are adapting to environmental change. Specifically, we focus on the importance of two neglected mechanisms of adaptation to environmental change. In the first one, we test for the existence of selection on individuals during the first stages of the biological invasion pathway. In the second one, we test empirically for the existence of habitat choice driving the adaptation of a native population of grasshoppers to an urbanized habitat, and its relative importance compared with natural selection, adaptive phenotypic plasticity and adjustment of the environment. The obtained results increase our understanding of how invasive and native populations adapt to ecological change and opportunity.

Invasive populations adapting to a new environment

Biological invasion is one of the largest consequences *and* causes of global change. Contrary to the deliberate introductions of exotic species of past centuries, much of the current invasions derive from international traffic in exotic species, which has increased in the last decades. Millions of plant and animal specimens, belonging to hundreds of species are extracted annually from nature and transported internationally for trade in pet markets, aquaculture and gardening (Reaser 2008). A small portion of these specimens is finally accidentally released or escapes, forming the seeds of new exotic invasions.

Much research has focused on the factors that contribute to successful invasions. Hypotheses have been proposed focusing on specific factors of the

event (introduction effort or propagule size), on the biotic and abiotic characteristics of the invaded ecosystem, or on characteristics of the invading species (Catford et al. 2009), while other studies have analysed the effects of invasive species on native species, ecosystems and economies. From an applied point of view, since the eradication of invasive populations is costly and often impractical, research efforts have led to the identification of potential invasive species to prevent and avoid future invasions (Kolar and Lodge 2001).

In the process of a biological invasion, different stages are identified (uptake, transport, introduction, establishment and expansion) separated by barriers that act as selective filters that prevent or allow species to move from one stage to another (Fig. 2) (Blackburn et al. 2011).

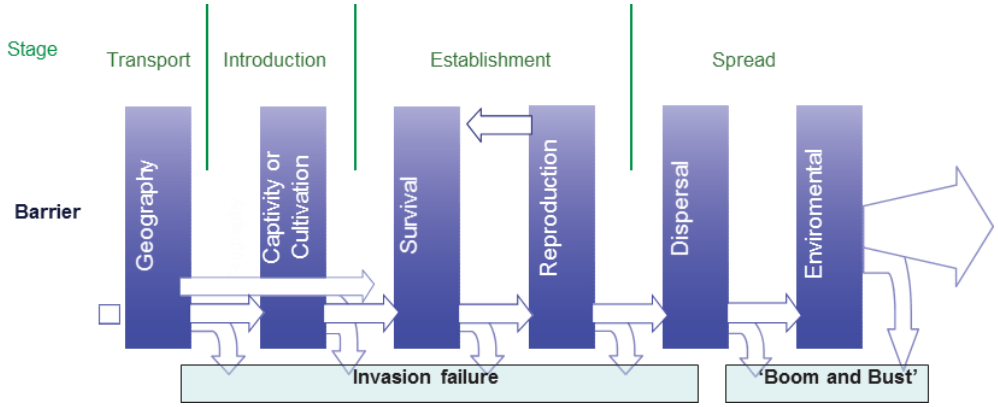

Figure 2. Unified framework for biological invasions. This framework recognises that the invasion process can be divided into a series of stages, and that at each stage there are barriers that need to be overcome for a species or population to pass on to the next stage. Figure from (Blackburn et al. 2011).

Although the early stages of biological invasion (uptake, transport and introduction) have great potential importance (Puth and Post 2005), these have received little research attention, and most studies have focused on the later stages of establishment and expansion. However, the taxonomic biases occurring at these first stages may determine which species will have opportunities to settle and become invasive (Blackburn et al. 2009). Moreover, in the study of this framework, research has focused on the average characteristics of the species, ignoring individual variation within potentially invading species. This despite the

fact that individual variation on invasive potential has been documented, allowing rapid evolutionary changes in morphology, behaviour and life-history traits in established populations (Blackburn et al. 2009), supporting that natural selection (in the novel, non-native environment) acts on this variation.

Likewise, environmental conditions can also induce population changes in the stages *before* the establishment, by causing selection against certain types of individuals. In this way the conditions faced by individuals during the stages of uptake, transport and introduction may impose selective pressures that progressively eliminate individuals with phenotypes and / or genotypes that are less able to cope with new situations, changing the characteristics of the final set of potential invaders, and therefore likely on their probability of establishment and expansion (positively or negatively) (Fig. 3).

Traits that are relevant for invasion success, such as behaviour/personality, immunocompetence, stress-tolerance, morphology, or plasticity, can be under selection during the pre-establishment stages, changing the traits of introduced population Pre-establishment filters can also have additional indirect effects. For example, life history traits and personalities are often associated: more risk-taking individuals reproduce at a younger age and allocate more resources to current reproductive attempts (Wolf et al. 2007). If a selective filter favours more risk-taking individuals, then potentially invasive populations can have a life strategy that will promote fast reproduction. As newly established populations are small and therefore vulnerable to extinction, rapid reproduction increases the likelihood of self-maintenance and expansion. Many of the traits mentioned above are heritable, so that these selection processes may have long-term evolutionary and ecological consequences.

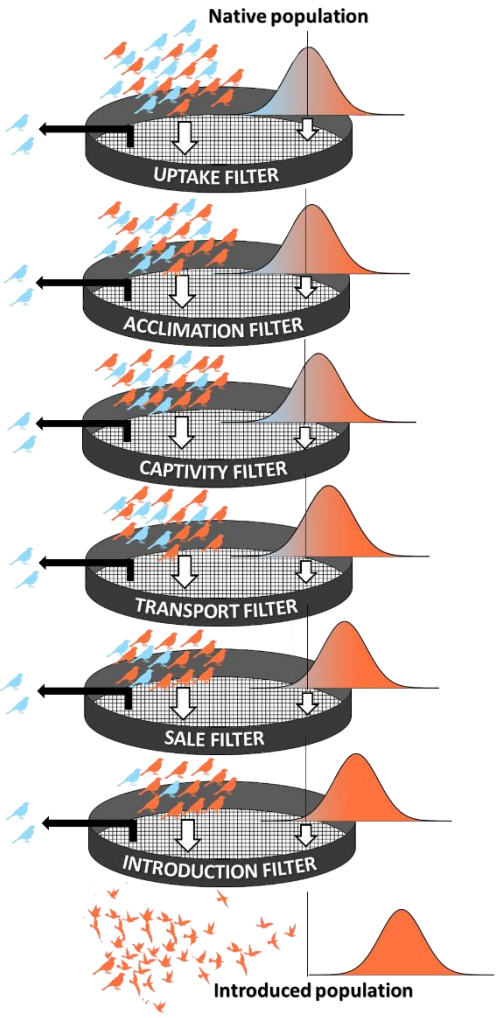

Figure 3. Schematic of certain selective filters acting during the pre-establishment stages of an invasion process. For each filter, one or more selective pressures eliminate certain individuals from the pool of potential invaders. The gradation in colour (for individuals and for the frequency distributions of individual traits) represents differences between the pheno(geno) types.(Figure from Chapter 3).

Furthermore, selection on the non-heritable components that affect individual traits (e.g. condition) prior to establishment could also affect the likelihood of invasion by modulating survival and reproduction of individuals.

Overall, the first stages of biological invasion previous to establishment have been greatly neglected (Puth and Post 2005, Blackburn et al. 2009), although the earliest effects of a process often have the greatest impact upon the final outcome. In our case, those individuals that have not passed a certain invasion stage cannot re-appear during later stages. Hence, if we want to improve our understanding of biological invasion, we need greater attention for what is occurring at the early stages. In addition, there is increasing attention for the importance of individual variation in a number of research fields, and it has been shown that including this extra level of variation has important benefits (Carrete and Tella 2011). However, the field of invasion biology has been lagging behind in this, and historically has mostly focused on species means of traits (Chapple et al. 2012). Individual variation in invasive species has only been

seriously studied in the context of evolutionary change in established populations (e.g. Blackburn et al. 2009), and the results of these studies support that individual variation is present, under natural selection, and relevant for population survival and growth.

In this thesis, I test for the first time whether pre-establishment selection occurs, when it acts, on which traits it acts, and how large its effects are on changing the populations that ultimately would try to establish in novel environments and become invasive species.

Native populations adapting to environmental change

One of the most extreme environmental changes is urbanization, as natural ecosystems are replaced by human-designed landscapes. Many species cannot cope with these changes, leading to local extinctions and biodiversity loss (Sala et al. 2000, McKinney 2002, Ellis et al. 2010). However, this process at the same time constitutes an ideal setting for the study of how populations adapt to environmental changes, as some species have been able to cope with urbanization and the associated changes in abiotic conditions, resources, and natural enemies (McKinney 2002, Shochat et al. 2006, Ellis et al. 2010). Several mechanisms may explain the adaptation of populations to the new conditions arising from the urban environments (Sih et al. 2011, Miranda et al. 2013). One of them is that this new environment can impose natural selection on certain heritable traits. This would lead to a process of local adaptation and a divergence between urban and rural populations (Cheptou et al. 2008, Miranda et al. 2013, Alberti et al. 2017, Brans et al. 2017). Alternatively, the adaptation to the new environments is because the populations that successfully colonize the urban environment have certain adaptive traits. In this way, natural selection in the past in the original environment of the population would have favoured the evolution of some traits that favour survival and reproduction in the new environment, thus creating a population that is pre-adapted to the new conditions. Phenotypic plasticity is one of these pre-adaptations, with individuals adapting their phenotype to have a better match with the environment (Schlichting and Pigliucci 1998). It has been

shown that the degree of plasticity in behaviour or morphology has a great effect on the success of populations in urban environments (Sih et al. 2011, Tuomainen and Candolin 2011, Lowry et al. 2013, Miranda et al. 2013). Habitat choice represents a second kind of pre-adaptation. This mechanism involves individuals changing their habitat (via dispersal) to better match their phenotype. Past natural selection may have favoured genotypes with the morphological and cognitive capacity to choose among available habitats to maximize their expected fitness (Maynard Smith 1966, Ronce 2007, Ravigné et al. 2009, Berdahl et al. 2015, Berner and Thibert-Plante 2015). Once this capacity for habitat choice has evolved, it could subsequently contribute to adaptation in novel contexts (Edelaar and Bolnick 2012, Bolnick and Otto 2013), including urban-rural divergence (Carrete and Tella 2010, Sol et al. 2013). One final kind of pre-adaptation that is possible (Fig. 1) is adjustment of the environment (Laland and Sterelny 2006), in this case, past selection may have favoured organisms with the capacity to modify their environment in order to achieve a better match with their phenotype.

Most research on adaptation, both in the setting of urbanization and in general, has focused on natural selection and adaptive phenotypic plasticity, with well-established empirical examples (Rose and Lauder 1996, Schlichting and Pigliucci 1998), supported by corresponding theory (Schlichting and Pigliucci 1998, Barton et al. 2007). In contrast, habitat choice has received far less research effort, although in theory it may have a great importance for adaptation in general (Edelaar et al. 2008, Bolnick and Otto 2013) and adaptation to urban environments specifically (Carrete and Tella 2010, Sol et al. 2013). This mechanism conflicts with the common assumption that dispersal and gene flow is random (Edelaar and Bolnick 2012), instead stressing that movement and dispersal is not random with respect to genotype and therefore can drive population divergence and adaptation.

In this thesis, I test the presence, functioning, relative importance and consequences of habitat choice driving adaptation to different and novel urban environments. In addition, as far as I know, it is the first empirical study that simultaneously tests the contributions of all four possible mechanisms (natural selection, phenotypic plasticity, habitat choice and adjustment of the environment; Fig. 1) in an integrated approach, and in the same study system.

Study Systems

To have a broad vision about the adaptation of populations to new environmental changes and opportunities, two different study systems were used. To test how invasive populations adapt to a new environment I study two invading bird species, the Blackheaded weaver *Ploceus melanocephalus* and the Yellow-croned bishop *Euplectes afer,* through the first stages of the invasion process. For the study of native populations adapting to environmental changes I studied the colonization by a native grasshopper species, the Azure sand grasshopper *Sphingonotus azurescens,* of an urbanized area.

The Black-headed weaver *Ploceus melanocephalus* and the Yellow-crowned bishop *Euplectes afer* (Fig. 4) are two species of invasive passerine birds occurring as native across wide regions of sub-Saharan Africa.

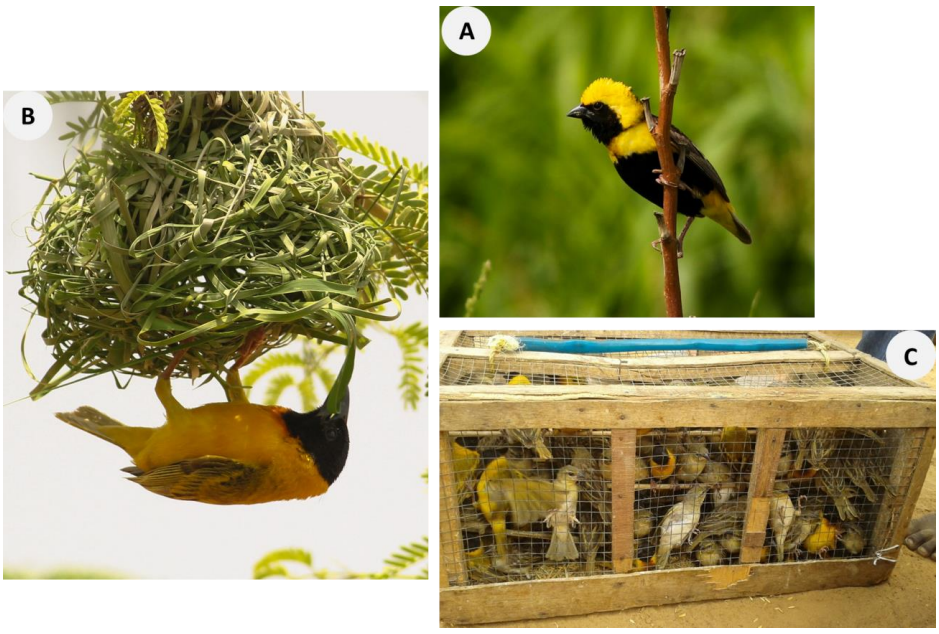

Figure 4. Male individuals of Black-headed weaver *Ploceus melanocephalus* (A) and Yellow-crowned bishop *Euplectes afer* (B) in their native areas at Senegal. (C) Males and females of *Ploceus melanocephalus* captured by Senegalese trappers for the international pet trade.

These species have established large invasive populations in Spain (mostly in the marshes around Seville) and other countries worldwide. They have been introduced by international traffic in pet animals. It is known that Senegal is the source population of these two species according to the records of past imports of wild caught birds to Spain (Abellán et al. 2017). In their native range, both species are common and even considered local agricultural pests, and are currently still captured and exported for the worldwide trade in pet animals.

The Azure sand grasshopper *Sphingonotus azurescens* is a species from the subfamily Oedipodinae (Fig. 5). Its characteristic natural habitat are open soils with scarce vegetation in a Mediterranean climate (Husemann et al. 2013). Contrary to many other grasshopper species, individuals do not perch on plants, but instead are perching and walking on the ground. They have an omnivorous diet feeding on dead invertebrates and live and dead plants. They move mainly by walking slowly, but fly well when disturbed or dispersing.

Figure 5. Variation in body coloration of grasshoppers from the Oedipodinae subfamily, generally with a cryptic colouration with respect of the local substrate.

Their activity is limited to the hotter hours of the day. The life cycle of this species takes one year. The nymphs begin to appear in early spring and become adults after six moults in about six weeks. Reproduction is mainly recorded in September and October, so adults need to survive a long time as non-breeding adults. This would select for a high daily survival rate, which is helped by its cryptic coloration. The variation in body coloration is very large between individuals and between populations, with a continuous variation both in luminosity (from very pale to almost black) and in colour (from bluish-grey to reddish-brown). The coloration of these grasshopper usually resembles that of the local substrate on which they are found, a striking phenomenon that has also been recorded in many other species of the same subfamily (Rowell 1972; Fig. 5).

We have encountered a population of this species of grasshopper adapting to an urbanized environment (Fig. 6) between the towns of Montequinto and Dos Hermanas (province of Seville, Spain: 37,306 º N, 5,932 º E).

Figure 6. Urbanized area in which Azure sand grasshoppers *Sphingonotus azurescens* have adapted to and are common on artificial pavements. The area is composed of distinct types of pavements differing in colour: asphalt roads (A), grey brick paths (B), areas of pale tiles (C), brown brick paths (D), and natural open soils (E).

This humanized habitat is composed of large blocks of areas with natural soils and vegetation, subdivided by paths composed of four different types of pavement: asphalt roads, a brown brick path, a grey brick path and other surfaces and sidewalks made of pale tiles and cement (Figure 6). The area is closed to traffic, but it is common to see people walking. Grasshoppers are relatively common on these pavements and clearly consider them as suitable alternatives to natural soils: adults are commonly displaying, we have seen copulations and egg depositing (into the spaces between bricks and pavements), and nymphs are common in spring. Grasshoppers are also common on the adjacent natural soils, and there is almost certainly frequent exchange between both types of habitat.

OBJECTIVES AND STRUCTURE

The general objective of the present PhD thesis is to increase our understanding of how invasive and native populations adapt to ecological change and opportunity in an increasingly changing world, specifically by testing for the existence and importance of two neglected mechanisms of adaptation to environmental changes and new environments. To address these issues, the thesis is divided in two sections.

In Section 1 I test for the first time whether natural selection occurs during the pre-establishment stages of biological invasion, when it acts, on which traits it acts, and how large its effects are on changing the populations that ultimately would try to establish in novel environments and become invasive species.

Chapter I (published in *Molecular Ecology*) examines selection acting on variation in a gene that are related to invasion-relevant behaviour in the Yellow-crowned bishop *Euplectes afer*, a pet-traded African songbird. Specifically we test for non-random allele frequency changes in a dopamine receptor gene, following the fate of individuals along the early stages of the invasion pathway (trapping, early acclimation and subsequent survival in captivity). We also compared the native Senegalese source population with two independent invasive populations (in Spain and Portugal) to see if pre-establishment selection might explain any genetic difference between source and invasive populations.

Chapter II (submission for publication in preparation) investigates how selection acts on a wide range of phenotypic traits thought to be potentially important for invasion success (sex, age, body/brain/bill size, condition, stress hormone levels, and behaviour) in two avian invaders, *Ploceus melanocephalus* and *Euplectes afer*. For this, we follow the individuals during the pre-establishment stages, as in Chapter I. We also assess the net effect of any selection, comparing the native source population with the surviving individuals at the end of the process.

In Section 2 I assess the presence, functioning and consequences of habitat choice for adaptation to novel and changing environments, and compare their relative importance driving adaptation with respect to alternative mechanisms as natural selection, phenotypic plasticity and adjustment of the environment.

Chapter III ((submitted for publication) explores the importance of habitat choice in the recent evolution of local crypsis and microgeographic population divergence of the Azure sand grasshopper *Sphingonotus azurescens* in the colonization of a novel urban environment. We also test the relative effect of this mechanism compared with present-day natural selection and adaptive phenotypic plasticity, using a combination of descriptive and experimental approaches in the field and the lab, as well as computer simulations.

Chapter IV (published in *Behavioral Ecology*) investigates how the behaviour of habitat choice operates as a function of both individual and environmental variation, at a micro-spatial scale. To address this we focused on the same grasshoppers population adapting to a novel urban environment as studied in Chapter III. We studied in the field how individuals improve their camouflage through habitat choice (by a positioning behaviour) depending on their level of crypsis, with a virtual predation experiment how this behaviour has an adaptive advantage (reducing the predation rate), and how it influences the escape behaviour of individuals in the field.

Chapter V (published in *Behavioral Ecology*) focuses on phenotypic plasticity and how it is modulated depending on the changing environmental conditions. We test if and how grasshoppers change their body colouration to resemble the substrate on they live on during their development from nymph to adult. Moreover, we test how the degree of this plasticity in colour change depends on another feature of the environment, the risk of predation.

SECTION 1

Invasive populations adapting to a new environment

Chapter I

Selection on a behaviour-related gene during the first stages of the biological invasion pathway

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Abstract

Human-induced biological invasions are common worldwide and often have negative impacts on wildlife and human societies. Several studies have shown evidence for selection on invaders after introduction to the new range. However, selective processes already acting prior to introduction have been largely neglected. Here, we tested whether such early selection acts on known behaviour-related gene variants in the yellow-crowned bishop (*Euplectes afer*), a pet-traded African songbird. We tested for nonrandom allele frequency changes after trapping, acclimation and survival in captivity. We also compared the native source population with two independent invasive populations. Allele frequencies of two SNPs in the dopamine receptor D4 (*DRD4*) gene—known to be linked to behavioural activity in response to novelty in this species—significantly changed over all early invasion stages. They also differed between the African native population and the two invading European populations. The two-locus genotype associated with reduced activity declined consistently, but strongest at the trapping stage. Overall genetic diversity did not substantially decrease, and there is little evidence for new alleles in the introduced populations, indicating that selection at the *DRD4* gene predominantly worked on the standing genetic variation already present in the native population. Our study demonstrates selection on a behaviour-related gene during the first stages of a biological invasion. Thus, pre-establishment stages of a biological invasion do not only determine the number of propagules that are introduced (their quantity), but also their phenotypic and genetic characteristics (their quality).

Keywords

Alien species, biological invasion, dopamine receptor D4, *Euplectes afer*, invasion filter, personality, pre-establishment selection, serotonin transporter, wildlife trade, yellow-crowned bishop

Introduction

Biological invasions are characterized by human-induced (unintentional or deliberate) translocations of individuals to non-native ranges where they survive

and reproduce (Blackburn et al. 2011). Due to their negative impacts on biodiversity and human economies, health and well-being (Dyer et al. 2017), biological invasions have been the focus of much study. An extensive literature exists that considers a variety of aspects related to invasions, including factors associated with their success, as well as assessments and intense, controversial discussions of their impacts (Ricciardi et al. 2017). Previous studies have led to a better understanding of the ecology and evolution of invasive species, with knowledge that can be applied to their management. Although these studies have made progress in predicting which factors enhance invasion success and which species may successfully establish and spread in the new area, much of the variability in invasion potential remains unexplained (Hayes and Barry 2008). This may partly be due to the focus on species characteristics, even when substantial variation in invasion potential can be found among populations and can be expected among individuals of the same species (Cardador et al. 2016, Ochocki and Miller 2017).

The invasion process is typically divided into distinct stages, namely uptake (entering transport, including deliberate trapping), transport (including captivity), introduction (including escape), establishment and spread (Blackburn et al. 2011). Recently, it has been hypothesized that phenotypes can be selectively "filtered" while passing through the early stages of the invasion process (Carrete et al. 2012, Chapple et al. 2012). If so, the characteristics of the introduced individuals may be different from those of the native donor population, which could promote or decrease invasion potential and impacts. While some studies have paid attention to selection acting on establishing and spreading populations (i.e. the final invasion stages; Bock et al., 2015), selection during the preestablishment invasion stages has been neglected. This is surprising, because (i) pre-establishment selection might be severe, as suggested for example by the high mortality rates between catching and export for wild-caught birds in the pet trade (7%–62%; Thomsen, Edwards, & Mullikan, 1992), and (ii) pre-establishment selection is important, because any variation that is removed in an earlier stage will no longer be present and exposable to selection in later ones. A good understanding of the selective processes acting during the early stages of the invasion pathway hence may be a key issue to assess invasion potential and impact.

Nonetheless, we are not aware of any empirical study dealing with pre-establishment selection during the invasion process. Such selection seems highly plausible given that individuals with certain behavioural, physiological or morphological traits might be more likely to be caught, to survive transport and captivity or to escape or be released (Carrete et al. 2012, Chapple et al. 2012). For example, it has been shown that variation in risk-taking behaviour causes sampling bias in wild animals (Biro and Dingemanse 2009, Biro 2013, Stuber et al. 2013) and relates to the exploration of novel food sources (Sol et al. 2011). In addition, in many species ‒including invasive ones‒ other behavioural traits affecting invasion potential and impact, such as neophobia, aggression, sociability and dispersal, are often linked to risk-taking behaviour (Duckworth and Badyaev 2007, Réale et al. 2007, Cote et al. 2010).

Here, we investigate pre-establishment selection in an invasive bird, the yellow-crowned bishop (*Euplectes afer*). This songbird naturally occurs across wide regions of sub-Saharan Africa, but has recently and independently established populations in the USA, Venezuela, Jamaica, Puerto Rico, Japan, Italy, Portugal and Spain after escape or release of captive birds (Lever 2005). Nowadays, the wildlife pet trade is a major source of biological invasion among vertebrates, in particular birds (Abellán et al. 2016, Su et al. 2016, Dyer et al. 2017). Specifically, we studied pre-establishment selection on genes that are related to invasion-relevant behaviours such as novelty seeking, activity and harm avoidance. In birds, primary candidates are the dopamine receptor D4 gene (*DRD4*) and the serotonin transporter gene (*SERT*, SLC6A4; Fidler et al., 2007; Korsten et al., 2010; Mueller, Partecke, Hatchwell, Gaston, & Evans, 2013; J. C. Mueller et al., 2013). Indeed, we have previously identified two SNPs in the *DRD4* gene (SNP449 and SNP698, hereafter called candidate SNPs) that had strong and replicated effects on activity after exposure to a novel object in individuals from two invasive populations of the yellow-crowned bishop (Mueller et al. 2014). Hence, we test for frequency changes of these two behaviour- related *DRD4* variants during the invasion process, assuming that these behaviours affect the probability that an individual will be caught and survive in captivity. Heterozygosity at a microsatellite in the second candidate gene *SERT* correlated with flight-initiation distance in

dunnocks (*Prunella modularis*; Holtmann et al., 2016). *SERT* heterozygosity was also higher in blackbirds (*Turdus merula*) from recently colonized urban populations compared to those from the original forest habitat (Mueller et al., 2013).

Any observed allelic shifts can either be signals of selection, or they can be due to neutral random processes, such as genetic drift (Bock et al. 2015). Genetic drift due to small founding population size has the potential to decrease standing genetic diversity in invading populations relative to native populations, but evidence for the importance of this effect in invasions is mixed (Dlugosch et al. 2015) Hence, we first test whether there is an overall loss of genetic diversity between the population of origin (Senegal, SEN) and two introduced populations of *E. afer* from Spain (SPA) and Portugal (POR). Second, to assess the hypothesis that selection already acts during the early invasion stages (Carrete et al. 2012), we test whether the *DRD4* candidate SNPs significantly change their frequency along early stages of the invasion pathway (relative to other markers). To test for selection during uptake, we compare allele frequencies among individuals caught by the traditional trapping methods used by bird exporters (potentially selective given that trapping involves baiting with food and decoy birds, referred to as the TRAP sample) and individuals caught with presumably less-selective mistnets (SEN sample). To test for selection during initial acclimation to captivity, we compare allele frequencies among individuals that successfully acclimated to captivity ($ACCL_{yes}$) and those that died ($ACCL_{no}$). To test for further selection during longterm captivity in storage cages, we compare allele frequencies among individuals that survived captivity ($SURV_{yes}$) and those that did not ($SURV_{no}$). We test for absolute allele frequency changes because we have no clear expectation about the direction of change.

Third, to assess the possibility that early selection (if any) left a genetic signature that is still noticeable after introduction, establishment and spread, we test whether allele frequencies at the *DRD4* candidate SNPs differ between the native (SEN) and the two introduced populations (SPA, POR) in a consistent manner, and if so, whether the change is in the same direction as the allele frequency changes observed during the first stages of the invasion pathway.

Fourth, we test whether heterozygosity at the *SERT* candidate locus changes along the filter steps and whether it is higher in the introduced populations (SPA, POR) than in the native one (SEN). To these ends, we genotyped 335 individuals for nine random microsatellites, the *SERT* candidate microsatellite, and 31 *DRD4* SNPs including the two candidate SNPs previously found to associate with activity in the two invasive populations.

Methods

Sampling of introduced and native populations

Individuals from an invasive Spanish *E. afer* population (SPA, N = 53) were caught with mistnets in January/February 2010 at rice fields close to Seville (Andalusia, Spain) and transferred to communal outdoor aviaries within a few hours. Individuals from an invasive Portuguese population (POR, N = 47, recently mistnetted near Lisbon) were legally purchased in March 2010 on the pet market and transferred to the same aviaries within 3 days. As far as we know, none of the birds died between capture/purchase and blood sampling. These 100 birds are the same individuals scored for behaviour and genotypes as in Mueller et al. (2014).

Individuals from a native Senegalese population (SEN, N = 91) were caught by us with mistnets (Fig. S1b) in September 2014 in the vicinity of Richard Toll, Northern Senegal (16°27'45"N–15°42'03"W). According to the Senegalese bird export company and the CITES trade data (Sanz-Aguilar et al. 2015), this is the same area where this species has been caught for export to Spain and Portugal. All individuals were marked (to avoid resampling of the same individual), blood-sampled (Fig. S1c) from the brachial vein (10–30 µL) and released in situ. Mistnetting is a sampling method that is presumably the least biased with respect to behavioural traits. There are few studies on sampling bias using mistnetting, but Simons, Winney, Nakagawa, Burke, & Schroeder, (2015) did not detect any bias in mistnet-caught birds for their fully monitored island population of house sparrows (*Passer domesticus*). We therefore considered our sample of mistnetted Senegalese birds as the reference for the native population.

Sampling of individuals for the bird trade and follow-up during the first invasion stages

We studied potential selection during three stages of the original invasion pathway via the international exotic bird trade. This involved sampling of birds caught by the Senegalese bird trappers and monitoring the fate of these individuals between trapping and international export, usually 1–3 months later. In stage 1, we accompanied professional local bird trappers working for the Senegalese company that historically exported *E. afer* to Europe and currently to other continents. Between 6 and 13 September 2014, they caught individuals using a traditional clap net baited with seeds and stuffed decoys to attract birds (Fig. S1d-e) in the same area as described for the reference sample (SEN) above. We took blood samples from all these individuals and marked them with uniquely numbered plastic rings. We genotyped a random subset (approximately one-third) of all captured/blood-sampled birds. A first invasion filter of selective uptake can be assessed by comparing these genotyped, traditionally caught birds (TRAP, N = 144) with those caught using mistnets ("trapping" or TRAP-SEN comparison).

In stage 2, we monitored the early survival of these trapped individuals. All individuals were kept at high densities for one week in traditional storage cages (Fig. S1f-g) close to the trapping sites and were then transported 350 km in the same cages (Fig. S1h) to the installations of the bird-trading company in Dakar (about 7-hr driving on the roof of a bus). Therefore, a second invasion filter where selection could take place was a 14- to 18-day period during which individuals either acclimated successfully to entry in captivity and transport (ACCLyes, N = 99) or died (ACCLno, N = 44; one individual was excluded because it lost its ring). Such mortality soon after capture has been documented before (Thomsen et al., 1992) and might select for certain behavioural types. We thus compared the genotypes of the surviving and nonsurviving birds ("acclimation" or ACCLyes _ ACCLno comparison).

In the last stage prior to export, the remaining birds were communally kept in storage cages (Fig. S1f) for 3 months. Thus, a third invasion filter during which

selection was evaluated was this longer-term survival in captivity. Because of its long duration and as most birds had died at the end of this period, we split this period in early mortality/survival (survival in the first 30 days, SURV1) and late mortality/ survival (survival in the next 60 days, SURV2). We assessed selection by comparing the genotypes of individuals that survived with those that died (SURV1yes, N = 54 vs. SURV1no, N = 45; SURV2yes, N = 11 vs. SURV2no, N = 43). Given that the conditions during these two periods were largely the same and to increase statistical power, we then averaged the allele frequency shifts and changes in genetic diversity during these two periods to represent a single invasion filter of long-term survival in captivity.

Genotyping

DNA was extracted from blood samples using the DNeasy blood & tissue kit (Qiagen) for the Spanish and Portuguese samples and a customized magnetic bead technique for the Senegalese birds. We amplified the complete exon 3 of the *DRD4* homologue (621 bp including small pieces of flanking introns) using the primers DRD4_I2F and DRD4_I3R (see Mueller et al., 2014). The PCR products of all birds were directly sequenced using both primers as sequencing primers (sequence see GenBank Accession no. KJ671448). Genotypes of all 31 identified SNP sites were scored. Information about allele names, whether the SNP is synonymous or nonsynonymous, or in an intron or exon (coding status), and major allele frequencies are given in Table S1. Among the 31 SNPs, twelve showed a minor allele frequency > 5% in one of the samples (SEN, TRAP, SPA, POR). Estimated allelic correlations between *DRD4* SNPs are generally weak with most r2 values below 0.5; the average r2 between the candidates SNP449 and SNP698 was 0.14 (Mueller et al., 2014).

We genotyped a microsatellite that is either in exon 1 or in the promoter of the SERT homologue (exact location unknown in this species) using the primers Sert_Ex1_F2 ATCTCCACACATTYCCCAGA and Sert_Ex1_R2 AGGAACCCTAAATCTGCCCTAC (see (Mueller et al., 2013).

To assess population structure, genetic diversity and genetic drift, we increased the number of loci by genotyping an additional nine random autosomal microsatellites: GCSW31, 35, 51, 55 and 57 (Mcrae et al. 2005); WBSW7 (McRae and Amos 1999); and INDIGO 29, 30 and 41 (Sefc, Payne, & Sorenson, 2001; see also Mueller et al., 2014). The sex of all individuals was determined based on plumage characteristics and confirmed by a PCR-based method following Griffiths, Double, Orr, & Dawson, 1998.

Data analyses

First, we evaluated the quality of the genotyping data by chi-square tests with simulated p-values (10,000 permutations on contingency tables with fixed marginals) for Hardy–Weinberg disequilibrium using the R package *genetics* (Warnes 2013, R Core Team 2017). The invasive populations (SPA, POR) and the two samples of the Senegalese population (SEN, TRAP) did not deviate overall from Hardy–Weinberg expectations across all polymorphic loci with a minor allele count (MAC) of more than two (i.e., more than a single minor allele homozygote individual or two heterozygote individuals present in the sample). Eleven of all 89 tests had a $p < .05$ (mostly involving different loci in each one), and none was significant after Bonferroni correction.

To assess population structure, we applied exact tests for allelic differentiation using *Genepop* (Rousset 2008). We visualized population structure with a discriminant analysis of principal components (DAPC) using the R package *adegenet* (Jombart 2008). DAPC first reduces allelic variance of all loci across all individuals (a total of 195 alleles) to the main principal components (we used 50 components explaining 88% of the total variance) and then uses these principal components in discriminant functions to maximize between-group variance while minimizing within-group variance (Jombart et al. 2010). We explored potential genetic substructuring within the populations using the program STRUCTURE with default settings of the underlying model, that is, allowing for admixtured individuals and correlated allele frequencies between genetic clusters (Pritchard et al. 2000). The web tool STRUCTURE HARVESTER was used to combine the

STRUCTURE output of 10 independent runs (Earl and vonHoldt 2012). We also tested for inflated genetic relatedness within the samples SEN, TRAP, SPA and POR by calculating all pairwise maximum-likelihood estimates of relatedness (Milligan 2003) using the R package related (Pew et al. 2015). We compared the mean and distribution of all these values with the correspondent means and distributions of 1,000 random samples of simulated unrelated individuals while maintaining observed allele frequencies and sample sizes.

We also tested whether mean relatedness among the surviving individuals of $ACCL_{yes}$, $SURV1_{yes}$ and $SURV2_{yes}$ is higher in comparison with the traditionally caught birds (TRAP). Here, pairwise relatedness was calculated using the allele frequencies of the TRAP sample as reference. The first test assesses the potential confounding influence of relatedness structure for all samples, whereas the second test evaluates whether surviving individuals tended to be more related. Both effects could lead to nonrandom changes in allele frequencies across all loci.

We calculated allele frequencies and genetic diversity (expected heterozygosity) for each population and filter group using the R packages *hierfstat* and *adegenet* (Jombart 2008, Goudet and Jombart 2015). Individuals were randomly permuted between groups to obtain a null distribution for testing differences in heterozygosity. For each invasive-native population comparison and for each filter stage, we calculated changes (delta values) in major allele frequencies such that a positive value indicates an increase and a negative value a decrease along the introduction process: SPA _ SEN (Spain minus Senegal); POR _ SEN (Portugal minus Senegal); TRAP _ SEN (traditionally trapped minus mistnetted); $ACCL_{yes}$ _ $ACCL_{no}$ (surviving acclimation minus nonsurvivors); $SURV_{yes}$ _ $SURV_{no}$ (surviving captivity minus nonsurvivors). Similar delta values were calculated for genetic diversity changes.

For each of the three filter stages (trapping, initial acclimation and longer-term survival) and across all three stages combined, and for each marker, we used a permutation procedure to estimate the likelihood of the observed (or more extreme) absolute allele frequency changes (irrespective of increase or decrease).

The group affiliation of each individual (e.g., TRAP or SEN when assessing the trapping filter) was randomly permuted against the genotypes within each comparison and new delta values were computed; this was repeated 10,000 times. This procedure simulates the random assortment of individuals into the contrasting groups of a specific filter stage (traditionally trapped versus mistnetted or survivors versus nonsurvivors). Similar permutation tests were performed for genetic diversity changes across all filter stages and for comparisons between the introduced and native populations. A table-wide Bonferroni-adjusted significance threshold was calculated by dividing the nominal threshold of .05 by the number of genomic regions (11) or by the effective number of independent polymorphic marker loci (M_{eff}, Li's method) calculated from the distribution of eigenvalues of the matrix of pairwise linkage disequilibrium values between all polymorphic markers in the reference sample SEN (Nyholt 2004, Li and Ji 2005). We first analysed both sexes together, because there was no sex effect on neophobic activity behaviour in the previous association study (Mueller et al., 2014) and females and males did not genetically differ in the Senegalese samples SEN and TRAP and in the invasive samples SPA and POR (allelic differentiation tests across all loci: all four comparisons $p > .29$). *A posteriori* we tested allele frequency changes along the filter and invasive-native comparisons for each sex separately in the same manner as explained above (sample sizes for females and males, respectively, SPA: 20 and 33, POR: 21 and 26, SEN: 48 and 40, TRAP: 49 and 89, $ACCL_{yes}$: 36 and 60, $ACCL_{no}$: 13 and 28, $SURV1_{yes}$: 16 and 37, $SURV1_{no}$: 20 and 23, $SURV2_{yes}$: 1 and 10, $SURV2_{no}$: 15 and 27).

Similar to tests for major allele frequency shifts of the single loci described above, we also used the permutation procedure to test for frequency shifts of functional genotype combinations of the two *DRD4* candidate loci SNP449 and SNP698. We considered frequency changes of the following categories of SNP449-SNP698 genotype combinations with likely different additive activity expressions according to Mueller et al. (2014): high activity (GG-AA), medium-high activity (GG-GA and GA-AA), intermediate activity (GG-GG, AA-AA and GA-GA), medium-low activity (GA-GG and AA-GA) and low activity (AA-GG).

Results

Population structure

We found no overall genetic difference between the two Senegalese samples (the mistnetted sample, SEN and the traditionally trapped birds, TRAP), as indicated by a discriminant analysis (Fig. S2) and by an allelic differentiation test across all loci ($p = .996$). This was expected, given that these two samples came from the same general area. In contrast, the Spanish and the Portuguese populations differed significantly from the two Senegalese populations and from each other (allelic differentiation tests: all five comparisons $p < .05$, Fig. S2).

There was no evidence for a cryptic substructure within the samples of SEN, TRAP, SPA and POR. Posterior probabilities of models assuming more than one genetic subcluster per population were not higher than the model probabilities assuming no substructuring (SEN and TRAP see Fig. S3; SPA and POR see Fig. S1 in Mueller et al., 2014). Mean pairwise relatedness within the samples of SEN, TRAP, SPA and POR ranged from 0.047 to 0.053 and did not differ from expected mean values of simulated random samples (all $p > .84$). Also, the distributions of the observed relatedness values were similar to those of the simulated relatedness values (Fig. S4). Mean relatedness in the surviving filter groups ACCLyes (0.054), SURV1yes (0.056) and SURV2yes (0.076) did not increase more than expected under random subsampling (all $p > .1$). In addition, there were no obvious clusters of genetically related individuals within populations (discriminant analysis, Fig. S2). We thus conclude that it is unlikely that our tests for nonrandom allele frequency shifts among the filter and invasive-native comparisons are confounded by population or relatedness substructuring.

Changes in genetic diversity during different invasion stages

Overall, genetic diversity did not decrease during the first stages of the invasion pathway (from SEN to TRAP, TRAP to ACCLyes, to SURV1yes and to SURV2yes; note that survival was assessed at two stages, whereby statistics were

averaged because the final surviving group was small; see methods). Expected heterozygosity estimates did not differ significantly between the mistnet sample SEN and all other samples (all loci combined; SEN: He = 0.239, TRAP: He = 0.237, ACCLyes: He = 0.238, SURV1yes: He = 0.237, SURV2yes: He = 0.211, permutation test: all $p > .05$).

Across all loci, the expected heterozygosity of the two invasive populations (SPA: He = 0.235, POR: He = 0.238) also did not differ significantly from the mistnetted sample SEN (permutation test: both $p > .05$). There were, however, more losses than gains of SNPs in the invasive populations compared to the native one. Among the total of 24 SNPs found in the equally sized native and invasive samples (SEN and SPA/POR combined), only one SNP was unique to the invasive samples, whereas eight SNPs were present in the Senegal population, but appear to have been lost in the invasive populations (Table S1).

The genetic diversity of the candidate polymorphism in the *SERT* gene did not show a strong change over the different invasion stages (Fig. S5a–c). However, genetic diversity in *SERT* was somewhat larger in the Spanish and Portuguese samples in comparison with the mistnetted SEN sample (combined across both comparisons: $p = .025$; Figs S5d-e and S6). The SEN sample had two individuals with minor alleles (both the same allele), and the similar-sized combined invasive sample (SPA and POR) had four individuals with minor alleles (three different alleles).

Allele frequency shifts during different invasion stages

Figure 1 shows the allele frequency changes of the major alleles of all loci for each invasion stage, that is, during trapping (TRAP vs SEN), acclimation to captivity (ACCLyes vs ACCLno) and survival in captivity (SURVyes vs SURVno). A few loci showed significant changes in single contrasts, but there were only two loci (*DRD4* SNP449 and SNP698) showing repeated allele frequency shifts in the top 10% along two or all three filter stages. The significance of the absolute frequency shifts (irrespective of direction) was evaluated for all major alleles using

a permutation procedure (see Section 2) that controls for sample size and major allele frequency. Each of the two *DRD4* candidate SNPs changed its frequency across the three filter comparisons more than expected by chance (SNP449: p = .0018, SNP698: p = .0018; Figure 2).

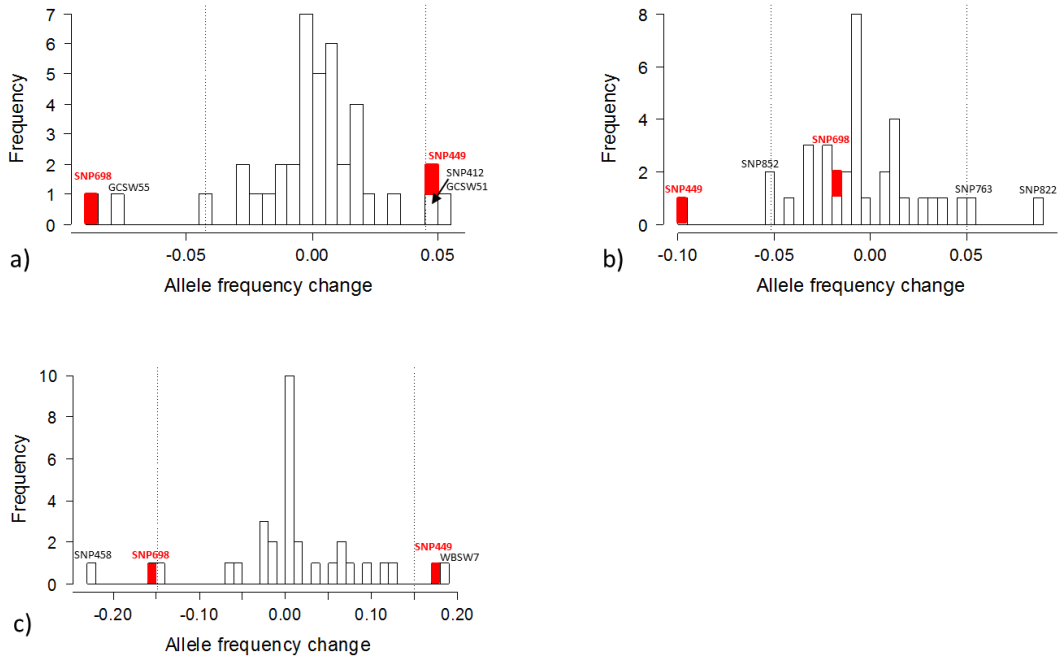

Figure 1. Frequency changes of the major alleles in all polymorphic loci (subset of 31 *DRD4* SNPs, and one *SERT* and nine random microsatellites) for the comparisons of (a) the trapping filter (TRAP _ SEN), (b) the acclimation filter (ACCLyes _ ACCLno) and (c) the survival filter (SURVyes _ SURVno). For the latter, we used averages over two comparisons (SURV1 and SURV2) evaluated at two time points, because the final surviving group was small (see Section 2 for details). The 5% and 95% percentiles are indicated as dotted lines. All markers with changes more extreme than these percentiles are labelled, and the two candidate loci *DRD4* SNP449 and SNP698 are marked in red.

The likelihood that the observed extreme frequency shifts occurred in both candidate SNPs together by chance was very low (p = 1.6 9 10_5). Both candidate SNPs were also among the four table-wide significant markers after adopting a Bonferroni correction for the number of genomic regions tested or for the effective number of independent polymorphic markers tested (Figure 2).

The other table-wide significant loci were another *DRD4* marker from the genic region associated with activity (SNP458; Mueller et al., 2014) and a random microsatellite marker (WBSW7). However, these other two markers showed an extreme allele frequency shift in only one filter comparison. In separate analyses of each filter stage, SNP449 and/or SNP698 were always among the loci with the strongest frequency shifts, although not always significant due to lower power (Fig. S7). Both in females and in males SNP449 or SNP698 was among the loci with strongest frequency shifts, indicating that both sexes contribute to the overall effects (Fig. S8). In single filter comparisons, the frequency changes of each SNP mostly follow the same direction in females and males, but the effect strengths might differ among the sexes. This needs further evaluation given the small sample sizes for each sex.

A comparison of the two invasive populations (SPA and POR) with the native Senegalese population (mistnetted sample SEN) also revealed strong allele frequency shifts for the two *DRD4* candidates, SNP449 and SNP698 (Figure 3). Although allele frequency shifts between the native source population and the invasive populations are expected to be generally stronger across all loci (compared to the first invasion stages) due to the additional scope for genetic drift at intermediate nonmonitored stages, the two candidate *DRD4* SNP449 and SNP698 still belonged to the top 21% of polymorphic markers with the most extreme frequency shifts (Figure 4). The likelihood that the observed allele frequency changes in SNP449 and SNP698 between the native and the invasive samples were due to chance was $p = .021$ and $p = .012$, respectively (permutation test). The likelihood of obtaining the extreme allele shifts in both candidate SNPs together by chance was very low ($p = .0009$). As expected given the smaller sample sizes, the sex-specific analyses mostly show nonsignificant allele frequency changes at the two candidate SNPs (Fig. S9).

The permutation likelihoods in Figures 2 and 4 did not significantly depend on the major allele frequencies across loci when only loci with total minor allele count > 2 (in both subsamples combined for all comparisons) were included. Markers with total minor allele count ≤ 2 were excluded, because a possible single minor allele homozygote produces the same absolute delta value in all simulations

when sample sizes are equal, and thus, this locus has always a likelihood of one. The correlation between the likelihoods and the major allele frequencies of SEN (folded MajAF between 0 and 0.5, i.e., for MajAF > 0.5 we used 1 – MajAF) was not significant: Spearman rho = _.36 (p = .10) for the filter comparisons in Figure 2 and rho = _.18 (p = .37) for the invasive-native comparisons in Figure 4.

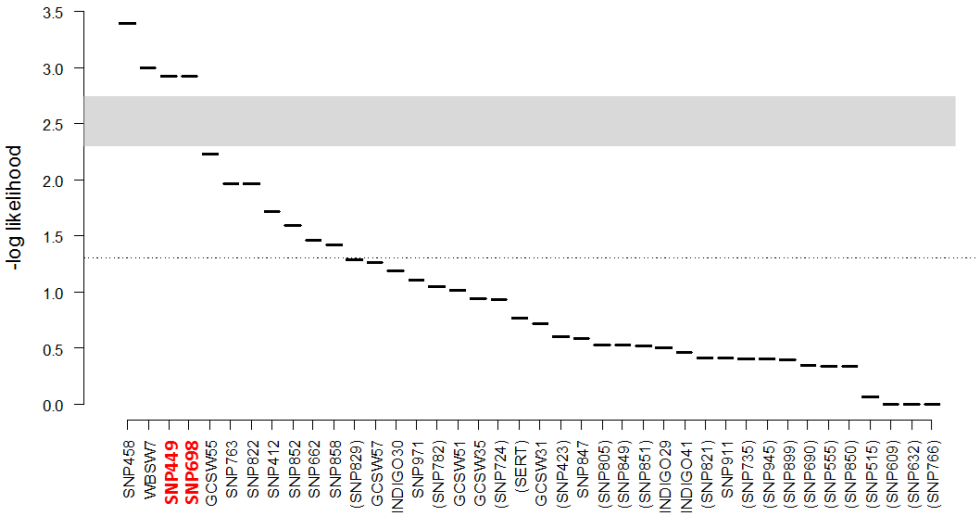

Figure 2. Permutation likelihood (-log) of observed (or more extreme) absolute changes in allele frequency for each locus along the three filter comparisons combined. Candidate *DRD4* SNPs are marked in red. Loci monomorphic in all subsamples of the filter comparisons are excluded. Loci with total minor allele count ≤ 2 in both subsamples combined (see Section 2) of at least one filter contrast are indicated by brackets. Nominal significance level and significance level adjusted for multiple testing (11 genomic regions or 27 independent polymorphic markers) are indicated as a dotted line and a grey bar, respectively.

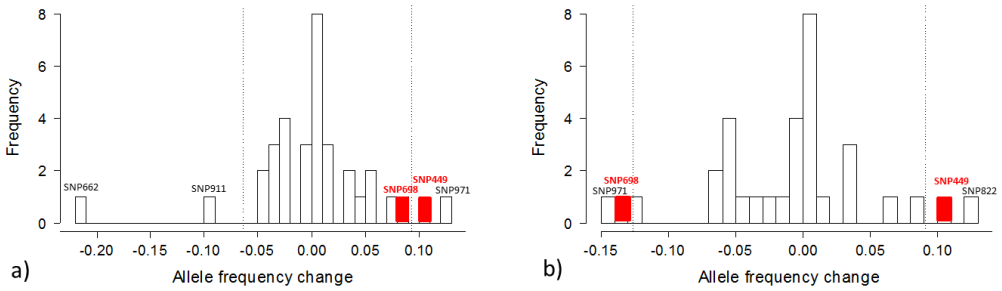

Figure 3. Frequency changes of the major alleles in all polymorphic loci (subset of 31 *DRD4* SNPs, and one *SERT* and nine random microsatellites) for the comparison between the Senegalese native population and (a) the Spanish invasive population (SPA _ SEN) and (b) the Portuguese invasive population (POR _ SEN). The 5% and 95% percentiles are indicated as dotted lines. All markers more extreme than these percentiles are labelled, and the two candidate loci DRD4 SNP449 and SNP698 are marked in red.

Changes in DRD4 SNP genotype combinations during different invasion stages

We now consider five categories of SNP449–SNP698 genotype combinations that likely differ in additive expression of activity (high, medium high, intermediate, medium low, low; see Section 2). The most significant absolute change along the three filter contrasts (trapping, acclimation, long-term survival) was in the low-activity genotype combination (permutation test: $p = .019$). The frequency of the low-activity genotype decreased strongly in the first invasion stage (TRAP-SEN), with smaller changes in the following invasion stages (Figure 5). The Spanish and Portuguese populations also showed a reduced frequency of the low-activity genotype in comparison with the Senegalese sample (Figure 5).

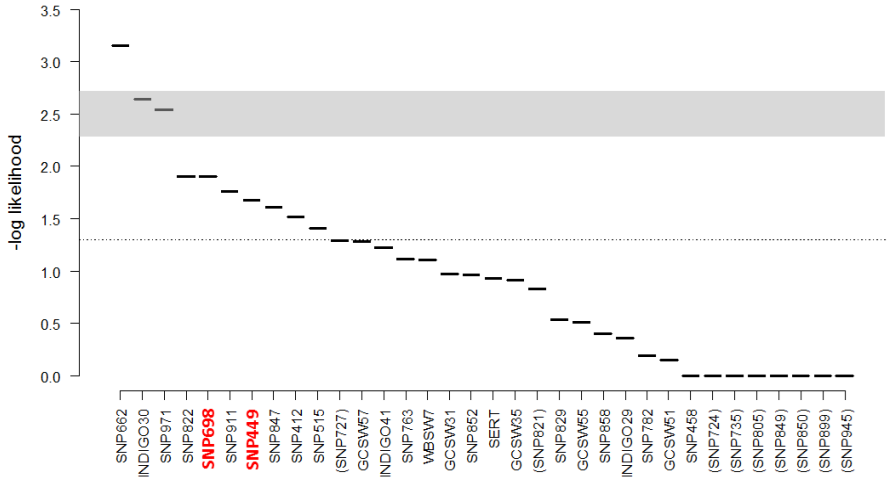

Figure 4. Permutation likelihood (-log) of observed (or more extreme) absolute changes in allele frequency for each locus along the two invasive-native comparisons combined. Candidate *DRD4* SNPs are marked in red. Loci monomorphic in all subsamples are excluded. Loci with total minor allele count ≤ 2 in both subsamples combined (see methods) of at least one invasive-native contrast are indicated by brackets. Nominal significance level and significance level adjusted for multiple testing (11 genomic regions or 27 independent polymorphic markers) are indicated as a dotted line and a grey bar, respectively

Indeed, for the two invasive-native population comparisons combined (SPA-SEN and POR-SEN), the frequency of the genotype combination with low activity showed the largest difference ($p = .041$). The medium-high- and medium-low-activity genotype combinations also significantly changed frequency along the invasion stages ($p = .036$ and $p = .030$, respectively), but their frequency did not differ between the native and invasive populations ($p = .19$ and $p = .29$, respectively). The high and intermediate activity genotype combinations did not show consistent changes, neither for the invasion stages ($p = .15$ and $p = .08$, respectively), nor for the native-invasive population comparisons ($p = .10$ and $p = .23$).

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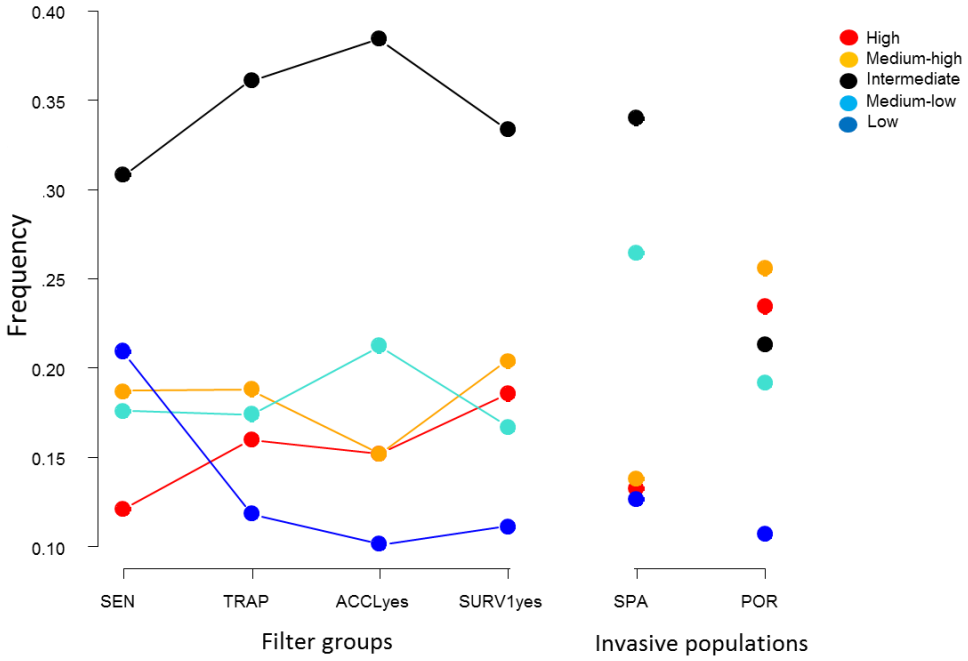

Figure 5. Frequencies of SNP449-SNP698 genotype combinations divided into five categories of predicted activity (according to Mueller et al., 2014) for the Senegal reference population (SEN), the different filtered groups (trapped, acclimation survivors and captivity survivors) and the two invasive populations (Spain and Portugal). The frequency in SURV2yes group is not plotted due to its small sample size.

Discussion

We analysed allelic changes in behaviour-related genes as well as presumably neutral microsatellite loci during the earliest stages of a human-induced biological invasion (i.e., uptake and captivity before introduction) by a well-known biological invader (a pet-traded wild bird, see Abellán, Tella, Carrete, Cardador, & Anadón, 2017 for its invasion process in Spain and Portugal). Among all markers, the two candidate SNPs in the *DRD4* gene were the only variants that showed consistently large, significant changes in allele frequency along two or more

comparisons of selective filters (Figures 1 and 2). Remarkably, these exact same two SNPs explained on average between 11% and 15% of the variation in activity and neophobic behaviour in two replicate invasive populations of this species (Mueller et al., 2014). Specifically, SNP449 which appears to be conserved among bird species, has a high functional potential (Mueller et al., 2014). This suggests that selection on behaviour acts already during the initial invasion stages, as proposed by Carrete et al., 2012; Chapple et al., 2012 As far as we know, this is the first empirical test of pre-establishment selection. Whether pre-establishment selection is common in biological invasions remains to be seen, but this seems likely (Carrete et al. 2012, Chapple et al. 2012). In this system, there is also evidence for sex- or size-biased trapping (A. Baños-Villalba et al., unpublished data). In particular when mortality is high, as in our study (92%), there is potential for strong selection. The observation of significant allele frequency differences at the same two SNPs when comparing two invasive populations with the native population of origin (Figures 3 and 4) suggests that the effects of such pre-establishment selection might be long-lasting. Such selection could therefore potentially affect the probability of successful establishment (e.g., through the degree of behavioural adaptation to novel conditions), the further development of the invasive population (e.g., activity levels may play an important role in range expansion) and its impacts on other species. Hence, our results highlight the importance of studying selective processes during the first stages of a biological invasion, because these stages may not only determine the number of propagules that are introduced (quantity) but also their phenotypic and genetic characteristics (quality).

In the first invasion stage (the "uptake" stage), we observed a downward shift in the frequency of the combined *DRD4* genotype associated with low activity in response to novel objects (Figure 5). A reduction in the frequency of the low-activity genotype was also apparent in both invasive populations compared to the original Senegal population. Of note, this suggests that a consistent change in functional genotype combinations of two independent SNPs is possible even though changes at one of the SNPs singly (e.g., at SNP698) can be inconsistent (Figure 3). Our data are thus compatible with a scenario where a single underlying variant of a selected polygenic trait changes frequency, but the direction of the

allele frequency change in each population may depend on changes in all other underlying (mostly unknown) variants of this trait. The concomitant increase in high-activity genotype combinations supports the hypothesis that more active or more response-ready individuals are more likely captured in traps baited with food and with decoy birds than less active ones (Carrete et al., 2012; Mueller et al., 2014). The lower frequency of low-activity genotypes in the invasive populations could therefore represent a long-lasting consequence of this initial trapping effect. However, given the scope for postintroduction adaptation in Spain and Portugal (~25–30 years, which is the equivalent of ~15–30 generations, Sanz-Aguilar, Anadón, Edelaar, Carrete, & Tella, 2014), it is also possible that there was further selection favouring more active types in the new environment and that a new equilibrium of behavioural types has now been established. A similar balancing system of *DRD4* variants (e.g., by negative frequencydependent selection; (van Oers and Mueller 2010) with occasional adaptive shifts has been suggested for great tits *Parus major* (Mueller et al., 2013) and humans (Ding et al. 2002, Wang et al. 2004). Among the 24 SNPs detected in the same-sized native and/or invasive samples (SEN, SPA, POR), only one was unique to the invasive samples, whereas eight appeared only in the native sample and may have been lost in the invasive populations (Table S1). It has been shown that founder events more often lead to loss of rare alleles than to a decrease in heterozygosity (Greenbaum et al. 2014). This indicates that selection on the remaining standing allelic variation seems important here, which can lead to rapid adaptive shifts (Bock et al. 2015). New mutations, however, appear to play a minor role in the genetic changes of the *DRD4* system of *E. afer* during invasion. Mueller et al. (2014) speculated that the observed strong association between the two *DRD4* SNPs and activity-related behaviour in the introduced populations might be partly rooted in the invasion history of these populations. It can be argued that the power to detect genotype–phenotype associations may increase as a result of allele frequency changes (a rare variant with a strong effect might become more common; e.g., Zoledziewska et al., 2015), because of changes in the genomic background (e.g., a general diversity loss may "free" additive genetic variation at epistatically interacting loci, i.e., release cryptic genetic variation; Dlugosch et al., (2015) or because of changes in the ecological environment during invasion (Dlugosch et al. 2015)). We can exclude the first reason, because the two candidate SNPs already had high minor

allele frequencies in the native population. However, our results indicate that a few neighbouring SNPs in the exonic *DRD4* region were lost or changed frequency during the invasion process. This leaves potential for changes in the neighbouring interactive genetic environment (epistasis). Furthermore, genetic variants at other, more distant, loci—in particular rare large-effect alleles could have changed their frequency and thus their interactive influence on the *DRD4* variants (Dlugosch et al. 2015). Only large-scale genomewide genotype–phenotype association studies in the native range of *Euplectes afer* would provide the necessary information. Overall genetic diversity as measured by heterozygosity did not decrease significantly between the native and invasive populations, further supporting that the reported allele frequency changes in the common *DRD4* SNPs are not a mere consequence of genetic drift. Due to the expected disconnect between neutral and adaptive variation among different environments (Leinonen et al. 2008), it might be more informative to investigate specific traitrelated genetic variation along with changes in environmental characteristics (Dlugosch et al. 2015, Estoup et al. 2016). In addition to the *DRD4* gene, we investigated *SERT* as a candidate gene for anxiety, harm avoidance, novelty seeking, and stress sensitivity (Canli and Lesch 2007, Murphy and Moya 2011), aggression (Craig and Halton 2009), distractibility (Maejima et al. 2007), dominance (Miller-Butterworth et al. 2007) and vigilance and cognitive functions (Canli and Lesch 2007, Homberg and Lesch 2011). Genetic diversity at *SERT* was only slightly, but significantly higher in the two invasive than in the native population (Figs S5 and S6). This is similar to findings from blackbird populations which invaded urban areas (Mueller et al., 2013). Although the higher diversity of *SERT* in *E. afer* was not exceptional in comparison with the other tested loci and needs to be verified in future studies, its direction is opposite to that expected by drift. Thus, the invasive populations might have experienced selective bias for rare variants with deviating serotonergic signalling characteristics, similar to urban blackbirds (Mueller et al., 2013). If so, selection would presumably take place during the later stages of the invasion pathway, because we did not obtain statistical support for selection on *SERT* variants during the first stages (Fig. S5a–c). Selection during later stages of the invasion might act via risk-taking behaviour: in dunnocks (*Prunella modularis*), heterozygous females had shorter flight-initiation distances than homozygous females (Holtmann et al. 2016). Interestingly,

heterozygosity of the *SERT* microsatellite homologue was also higher in an invasive dunnock population (in New Zealand) than in the native British one, while all other tested markers showed the opposite pattern (Holtmann et al. 2016). This suggests a similar selection regime to the one in *Euplectes afer*.

In summary, this study provides the first empirical evidence for the operation of selection during the earliest, pre-establishment stages of biological invasions, in this case selection on genetic variation in behaviour. Some of these early selective changes appear maintained in two successful invasive populations, and the reduction in low-activity genotypes could conceivably have influenced invasion success and impact in the habitats where the birds were introduced (Carrete et al. 2012). Selection could also be important in unintentional introductions where nonrandom uptake and survival during transport (e.g., in ships, containers) also represent the first steps of the invasion process (Blackburn et al. 2011, Chapple et al. 2012). Further exploration of this hypothesis is therefore necessary to better understand and effectively manage biological invasions and to gain insight into the evolution of behaviour and other traits in introduced populations.

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Author contributions

All authors contributed to study conception and design. A.B, J.B., M.C., P.E., J.P., J.L.T. carried out field work (Spain and Senegal). J.C.M and B.K. collected the genetic data. J.C.M. and P.E analysed the data. J.C.M., P.E., J.L.T and B.K drafted the manuscript. All additional authors provided comments and approved the final manuscript.

References

Abellán, P., Carrete, M., Anadón, J. D., Cardador, L. and Tella, J. L. 2016. Non-random patterns and temporal trends (1912-2012) in the transport, introduction and establishment of exotic birds in Spain and Portugal. - Divers. Distrib. 22: 263–273.

Abellán, P., Tella, J. L., Carrete, M., Cardador, L. and Anadón, J. D. 2017. Climate matching drives spread rate but not establishment success in recent unintentional bird introductions. - Proc. Natl. Acad. Sci.: 201704815.

Biro, P. A. 2013. Are most samples of animals systematically biased? Consistent individual trait differences bias samples despite random sampling. - Oecologia 171: 339–345.

Biro, P. A. and Dingemanse, N. J. 2009. Sampling bias resulting from animal personality. - Trends Ecol. Evol. in press.

Blackburn, T. M., Pyšek, P., Bacher, S., Carlton, J. T., Duncan, R. P., Jarošík, V.,

Wilson, J. R. U. and Richardson, D. M. 2011. A proposed unified framework for biological invasions. - Trends Ecol. Evol. 26: 333–339.

Bock, D. G., Caseys, C., Cousens, R. D., Hahn, M. A., Heredia, S. M., Hübner, S., Turner, K. G., Whitney, K. D. and Rieseberg, L. H. 2015. What we still don't know about invasion genetics. - Mol. Ecol. 24: 2277–2297.

Canli, T. and Lesch, K. P. 2007. Long story short: The serotonin transporter in emotion regulation and social cognition. - Nat. Neurosci. 10: 1103–1109.

Cardador, L., Carrete, M., Gallardo, B. and Tella, J. L. 2016. Combining trade data and niche modelling improves predictions of the origin and distribution of non-native European populations of a globally invasive species. - J. Biogeogr. 43: 967–978.

Carrete, M., Edelaar, P., Blas, J., Serrano, D., Potti, J., Dingemanse, N. J. and Tella, J. L. 2012. Don't neglect pre-establishment individual selection in deliberate introductions. - Trends Ecol. Evol. 27: 67–68.

Chapple, D. G., Simmonds, S. M. and Wong, B. B. M. 2012. Can behavioral and personality traits influence the success of unintentional species introductions? - Trends Ecol. Evol. 27: 57–62.

Cote, J., Fogarty, S., Weinersmith, K., Brodin, T. and Sih, A. 2010. Personality traits and dispersal tendency in the invasive mosquitofish (Gambusia affinis). - Proc. R. Soc. B Biol. Sci. 277: 1571–1579.

Craig, I. W. and Halton, K. E. 2009. Genetics of human aggressive behaviour. - Hum. Genet. 126: 101–113.

Ding, Y.-C., Chi, H.-C., Grady, D. L., Morishima, A., Kidd, J. R., Kidd, K. K., Flodman, P., Spence, M. A., Schuck, S., Swanson, J. M., Zhang, Y.-P. and Moyzis, R. K. 2002. Evidence of positive selection acting at the human dopamine receptor D4 gene locus. - Proc. Natl. Acad. Sci. U. S. A. 99: 309–14.

Dlugosch, K. M., Anderson, S. R., Braasch, J., Cang, F. A. and Gillette, H. D. 2015. The devil is in the details: Genetic variation in introduced populations and its contributions to invasion. - Mol. Ecol. 24: 2095–2111.

Duckworth, R. A. and Badyaev, A. V. 2007. Coupling of dispersal and aggression facilitates the rapid range expansion of a passerine bird. - Proc. Natl. Acad. Sci. 104: 15017–15022.

Dyer, E. E., Cassey, P., Redding, D. W., Collen, B., Franks, V., Gaston, K. J., Jones, K. E., Kark, S., Orme, C. D. L. and Blackburn, T. M. 2017. The Global Distribution and Drivers of Alien Bird Species Richness. - PLoS Biol. in press.

Earl, D. A. and vonHoldt, B. M. 2012. STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. - Conserv. Genet. Resour. 4: 359–361.

Estoup, A., Ravigné, V., Hufbauer, R., Vitalis, R., Gautier, M. and Facon, B. 2016. Is There a Genetic Paradox of Biological Invasion? - Annu. Rev. Ecol. Evol. Syst. 47: 51–72.

Fidler, A. E., van Oers, K., Drent, P. J., Kuhn, S., Mueller, J. C. and Kempenaers, B. 2007. Drd4 gene polymorphisms are associated with personality variation in a passerine bird. - Proc. R. Soc. B Biol. Sci. 274: 1685–1691.

Goudet, J. and Jombart, T. 2015. hierfstat: Estimation and tests of hierarchical F-statistics. in press.

Greenbaum, G., Templeton, A. R., Zarmi, Y. and Bar-David, S. 2014. Allelic richness following population founding events - A stochastic modeling framework incorporating gene flow and genetic drift. - PLoS One in press.

Griffiths, R., Double, M. C., Orr, K. and Dawson, R. J. G. 1998. A DNA test to sex most birds. - Mol. Ecol. 7: 1071–1075.

Hayes, K. R. and Barry, S. C. 2008. Are there any consistent predictors of invasion success? - Biol. Invasions 10: 483–506.

Holtmann, B., Grosser, S., Lagisz, M., Johnson, S. L., Santos, E. S. A., Lara, C. E., Robertson, B. C. and Nakagawa, S. 2016. Population differentiation and behavioural association of the two "personality" genes DRD4 and SERT in dunnocks (Prunella modularis). - Mol. Ecol. 25: 706–722.

Homberg, J. R. and Lesch, K. P. 2011. Looking on the bright side of serotonin transporter gene variation. - Biol. Psychiatry 69: 513–519.

Jombart, T. 2008. adegenet: a R package for the multivariate analysis of genetic markers. - Bioinformatics 24: 1403–5.

Jombart, T., Devillard, S. and Balloux, F. 2010. Discriminant analysis of principal components: A new method for the analysis of genetically structured populations. - BMC Genet. in press.

Korsten, P., Mueller, J. C., Hermannstädter, C., Bouwman, K. M., Dingemanse, N. J., Drent, P. J., Liedvogel, M., Matthysen, E., Van Oers, K., Van Overveld, T., Patrick, S. C., Quinn, J. L., Sheldon, B. C., Tinbergen, J. M. and Kempenaers, B. 2010. Association between DRD4 gene polymorphism and personality variation in great tits: A test across four wild populations. - Mol. Ecol. 19: 832–843.

Leinonen, T., O'Hara, R. B., Cano, J. M. and Merilä, J. 2008. Comparative studies of quantitative trait and neutral marker divergence: A meta-analysis. - J. Evol. Biol. 21: 1–17.

Lever, C. 2005. Naturalised birds of the world. - T & AD Poyser.

Li, J. and Ji, L. 2005. Adjusting multiple testing in multilocus analyses using the eigenvalues of a correlation matrix. - Heredity (Edinb). 95: 221–227.

Maejima, M., Inoue-Murayama, M., Tonosaki, K., Matsuura, N., Kato, S., Saito, Y., Weiss, A., Murayama, Y. and Ito, S. 2007. Traits and genotypes may predict the successful training of drug detection dogs. - Appl. Anim. Behav. Sci. 107: 287–298.

Mcrae, S. B., Emlen, S. T., Rubenstein, D. R. and Bogdanowicz, S. M. 2005. Polymorphic microsatellite loci in a plural breeder, the grey-capped social weaver (Pseudonigrita arnaudi), isolated with an improved enrichment protocol using fragment size-selection. - Mol. Ecol. Notes 5: 16–20.

McRae, S. B. and Amos, W. 1999. Characterization of hypervariable microsatellites in the cooperatively breeding white-browed sparrow weaver Plocepasser mahali. - Mol. Ecol. 8: 895–906.

Miller-Butterworth, C. M., Kaplan, J. R., Barmada, M. M., Manuck, S. B. and Ferrell, R. E. 2007. The serotonin transporter: Sequence variation in Macaca fascicularis and its relationship to dominance. - Behav. Genet. 37: 678–696.

Milligan, B. G. 2003. Maximum-likelihood estimation of relatedness. - Genetics 163: 1153–1167.

Mueller, J. C., Korsten, P., Hermannstaedter, C., Feulner, T., Dingemanse, N. J., Matthysen, E., Van Oers, K., Van Overveld, T., Patrick, S. C., Quinn, J. L., Riemenschneider, M., Tinbergen, J. M. and Kempenaers, B. 2013a. Haplotype structure, adaptive history and associations with exploratory behaviour of the DRD4 gene region in four great tit (Parus major) populations. - Mol. Ecol. 22: 2797–2809.

Mueller, J. C., Partecke, J., Hatchwell, B. J., Gaston, K. J. and Evans, K. L. 2013b. Candidate gene polymorphisms for behavioural adaptations during urbanization in blackbirds. - Mol. Ecol. 22: 3629–3637.

Mueller, J., Edelaar, P., Carrete, M., Serrano, D., Potti, J., Blas, J., Dingemanse, N. J., Kempenaers, B. and Tella, J. L. 2014. Behaviour-related DRD4 polymorphisms in invasive bird populations. - Mol. Ecol. 23: 2876–2885.

Murphy, D. L. and Moya, P. R. 2011. Human serotonin transporter gene (SLC6A4) variants: their contributions to understanding pharmacogenomic and other functional G×G and G×E differences in health and disease. - Curr. Opin. Pharmacol. 11: 3–10.

Nyholt, D. R. 2004. A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. - Am. J. Hum. Genet. 74: 765–769.

Ochocki, B. M. and Miller, T. E. X. 2017. Rapid evolution of dispersal ability makes biological invasions faster and more variable. - Nat. Commun. in press.

Pew, J., Muir, P. H., Wang, J. and Frasier, T. R. 2015. related: An R package for analysing pairwise relatedness from codominant molecular markers. - Mol. Ecol. Resour. 15: 557–561.

Pritchard, J. K., Stephens, M. and Donnelly, P. 2000. Inference of population structure using multilocus genotype data. - Genetics 155: 945–959.

R Core Team 2017. R: A Language and Environment for Statistical Computing. - R Found. Stat. Comput.: {ISBN} 3-900051-07-0, http://www.R-project.org.

Réale, D., Reader, S. M., Sol, D., McDougall, P. T. and Dingemanse, N. J. 2007. Integrating animal temperament within ecology and evolution. - Biol. Rev. 82: 291–318.

Ricciardi, A., Blackburn, T. M., Carlton, J. T., Dick, J. T. A., Hulme, P. E., Iacarella, J. C., Jeschke, J. M., Liebhold, A. M., Lockwood, J. L., MacIsaac, H. J., Pyšek, P., Richardson, D. M., Ruiz, G. M., Simberloff, D., Sutherland, W. J., Wardle, D. A. and Aldridge, D. C. 2017. Invasion science: a horizon scan of emerging challenges and opportunities. - Trends Ecol. Evol. 32: 464–474.

Rousset, F. 2008. GENEPOP'007: A complete re-implementation of the GENEPOP software for Windows and Linux. - Mol. Ecol. Resour. 8: 103–106.

Sanz-Aguilar, A., Anadón, J., Edelaar, P., Carrete, M. and Tella, J. 2014. Can establishment success be determined through demographic parameters? A case study on five introduced bird species e110019. - PLoS One in press.

Sanz-Aguilar, A., Carrete, M., Edelaar, P., Potti, J. and Tella, J. L. 2015. The empty temporal niche: breeding phenology differs between coexisting native and invasive birds. - Biol. Invasions 17: 3275–3288.

Sefc, K. M., Payne, R. B. and Sorenson, M. D. 2001. Characterization of microsatellite loci in village indigobirds Vidua chalybeata and cross-species amplification in estrildid and ploceid finches. - Mol. Ecol. Notes 1: 252–254.

Simons, M. J. P., Winney, I., Nakagawa, S., Burke, T. and Schroeder, J. 2015. Limited catching bias in a wild population of birds with near-complete census information. - Ecol. Evol. 5: 3500–3506.

Sol, D., Griffin, A. S., Bartomeus, I., Boyce, H., Mayr, E., Lefebvre, L., Duncan, R., Blackburn, T., Cassey, P., Lefebvre, L., Sol, D., Bacher, S., Reader, S., Lefebvre, L., Greenberg, R., Mettke-Hofmann, C., Reale, D., Reader, S., McDougall, P., Dingemanse, N., Shochat, E., Warren, P., Faeth, S., McIntyre, N., Hope, D., Liker, A., Bókony, V., Møller, A., Kark, S., Iwaniuk, A., Schalimtzek, A., Banker, E., Case, T., Lever, C., Tideman, C., Griffin, A., Boyce, H., MacFarlane, G., Biro, P., Dingemanse, N., Bouchard, J., Goodyer, W., Lefebvre, L., Reader, S., Laland, K., Boogert, N., Reader, S., Laland, K., Crawley, M., Arbuckle, J., Laland, K., Reader, S., Reader, S., Laland, K., Boogert, N., Reader, S., Hoppitt, W., Laland, K., II, L. M., Fitzgerald, L., Hone, J., Overington, S., Morand-Ferron, J., Boogert, N., Lefebvre, L., Gosling, S. and Griffin, A. 2011. Exploring or Avoiding Novel Food Resources? The Novelty Conflict in an Invasive Bird (A Iwaniuk, Ed.). - PLoS One 6: e19535.

Stuber, E. F., Araya-Ajoy, Y. G., Mathot, K. J., Mutzel, A., Nicolaus, M., Wijmenga, J. J., Mueller, J. C. and Dingemanse, N. J. 2013. Slow explorers take less risk: A problem of sampling bias in ecological studies. - Behav. Ecol. 24: 1092–1098.

Su, S., Cassey, P. and Blackburn, T. M. 2016. The wildlife pet trade as a driver of introduction and establishment in alien birds in Taiwan. - Biol. Invasions 18:

215–229.

Thomsen, J. B., Edwards, S. R. and Mullikan, T. A. 1992. Perceptions, Conservation and Management of wild birds in trade (T International, Ed.). - Cambridge.

van Oers, K. and Mueller, J. C. 2010. Evolutionary genomics of animal personality. - Philos. Trans. R. Soc. Lond. B. Biol. Sci. 365: 3991–4000.

Wang, E., Ding, Y.-C., Flodman, P., Kidd, J. R., Kidd, K. K., Grady, D. L., Ryder, O. a, Spence, M. a, Swanson, J. M. and Moyzis, R. K. 2004. The genetic architecture of selection at the human dopamine receptor D4 (DRD4) gene locus. - Am. J. Hum. Genet. 74: 931–44.

Warnes, G. 2013. genetics: Population Genetics. R package version 1.3.8.1. in press.

Zoledziewska, M., Sidore, C., Chiang, C. W. K., Sanna, S., Mulas, A., Steri, M., Busonero, F., Marcus, J. H., Marongiu, M., Maschio, A., Del Vecchyo, D. O., Floris, M., Meloni, A., Delitala, A., Concas, M. P., Murgia, F., Biino, G., Vaccargiu, S., Nagaraja, R., Lohmueller, K. E., Timpson, N. J., Soranzo, N., Tachmazidou, I., Dedoussis, G., Zeggini, E., Uzzau, S., Jones, C., Lyons, R., Angius, A., Abecasis, G. R., Novembre, J., Schlessinger, D. and Cucca, F. 2015. Height-reducing variants and selection for short stature in Sardinia. - Nat. Genet. 47: 1352–1356.

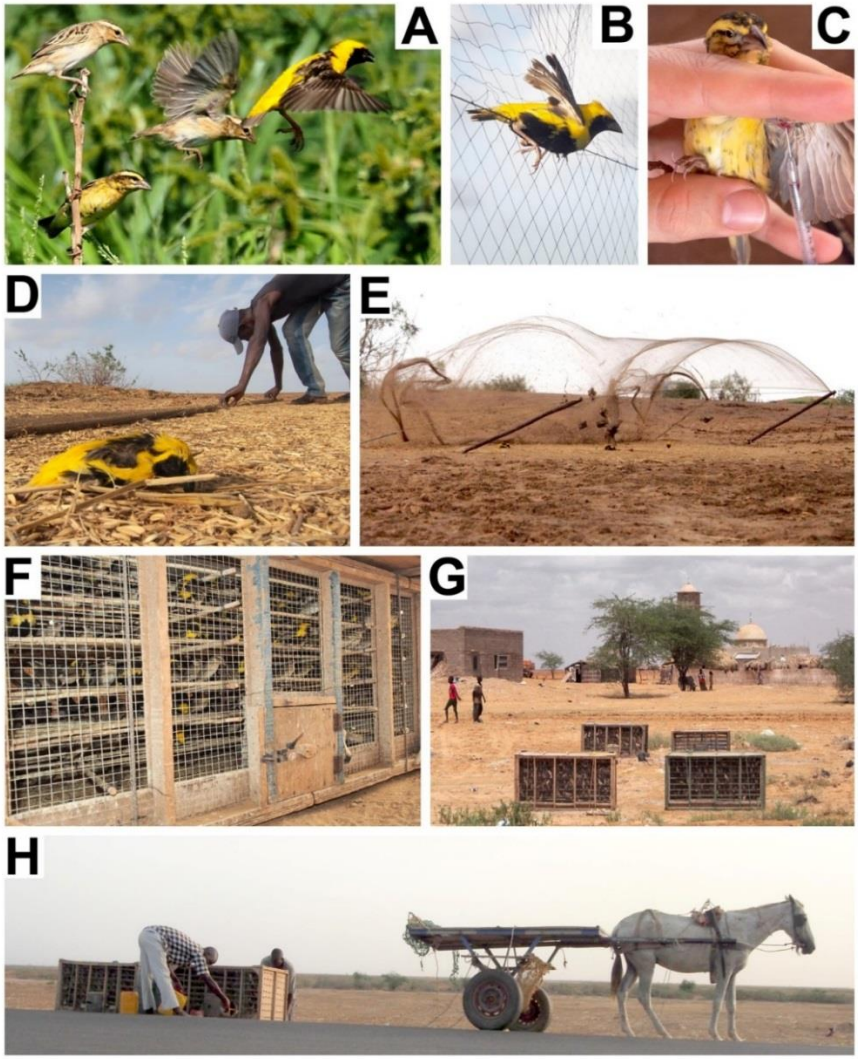
Supporting information

Supplementary table 1. Allele names/numbers, position or coding status and major allele frequencies (MajAF) for each locus (*DRD4* SNP or microsatellite, see Methods) in the reference Senegalese (SEN, N=91), the traditionally trapped Senegalese (TRAP, N=144), the Spanish (SPA, N=53) and the Portuguese (POR, N=47) population.

Locus	SNPs: major/minor allele Micros: number alleles	Position/coding status [a]	MajAF SEN	MajAF TRAP	MajAF SPA	MajAF POR
SNP412	G/A	intron	0.867	0.912	0.821	0.947
SNP423	G/A	intron	1	0.993	1	1
SNP449	G/A	S	0.500	0.545	0.604	0.606
SNP458	C/T	S	0.835	0.809	0.830	0.840
SNP515	C/T	S	0.983	0.986	0.934	0.979
SNP555	A/C	NS Asn/His	1	0.996	1	1
SNP609	G/A	NS Val/Met	1	0.996	1	1
SNP632	C/T	S	1	0.996	1	1
SNP662	G/A	S	0.654	0.670	0.443	0.598
SNP690	C/T	NS Arg/Cys	1	0.996	1	1
SNP698	G/A	S	0.582	0.496	0.670	0.447
SNP724	G/A	NS Gly/Asp	0.994	0.983	1	0.989
SNP727	G/T	NS Cys/Phe	1	1	0.972	1
SNP735	G/A	NS Gly/Arg	0.994	1	1	1
SNP763	C/A	NS Pro/Gln	0.972	0.958	0.991	0.936
SNP766	C/A	NS Thr/Asn	1	0.996	1	1
SNP782 [b]	A/G	S	0.978	0.993	0.991	0.979
SNP805	G/A	NS Gly/Glu	0.994	0.996	1	1
SNP821	C/T	S	0.994	1	0.972	0.989
SNP822	G/A	NS Ala/Thr	0.648	0.608	0.726	0.772
SNP829	C/A	NS Ala/Asp	0.983	0.996	1	1
SNP847	G/A	NS Cys/Tyr	0.967	0.979	1	1
SNP849 [c]	G/A	NS Gly/Arg	0.994	0.996	1	1
SNP850 [c]	G/C	NS Gly/Ala	0.994	0.996	1	1
SNP851 [c]	G/A	S	1	0.996	1	1
SNP852	G/A	NS Val/Met	0.879	0.861	0.915	0.819
SNP858	A/G	NS Ser/Gly	0.874	0.844	0.868	0.830
SNP899	G/A	S	0.994	1	1	1
SNP911	C/T	S	0.874	0.882	0.783	0.819
SNP945	C/T	NS Arg/Cys	0.994	1	1	1
SNP971	T/C	S	0.623	0.621	0.745	0.479
SERT	4	promotor or exon	0.989	0.996	0.980	0.978
GCSW35	4	random	0.819	0.837	0.788	0.883
GCSW57	37	random	0.104	0.135	0.163	0.138
GCSW51	5	random	0.698	0.750	0.670	0.691
GCSW55	9	random	0.538	0.461	0.500	0.574
GCSW31	21	random	0.181	0.198	0.226	0.128
INDIGO 30	6	random	0.950	0.951	0.953	0.830
INDIGO 41	30	random	0.104	0.125	0.066	0.053
INDIGO	24	random	0.264	0.240	0.236	0.234

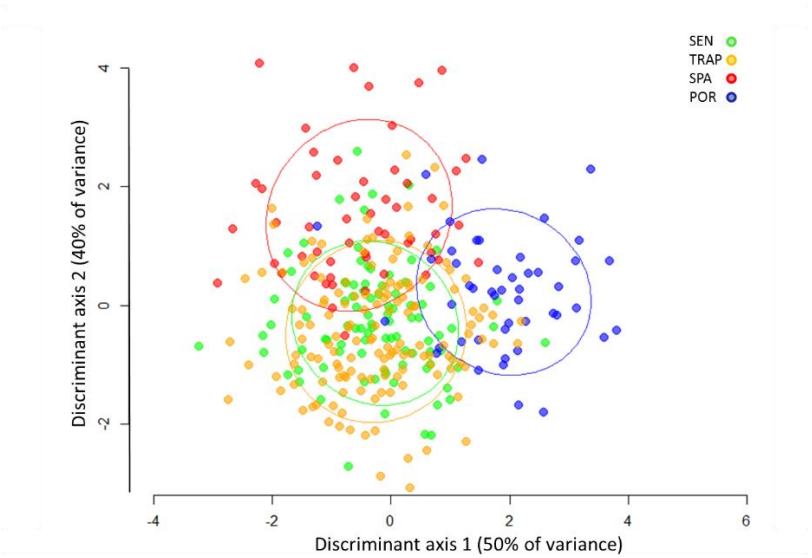
29						
WBSW7	10	random	0.253	0.243	0.311	0.191

[a] S=synonymous coding SNP, NS=non-synonymous coding SNP.
[b] SNP782 is identical with SNP781 in Mueller et al. 2014.
[c] Belong to one codon.

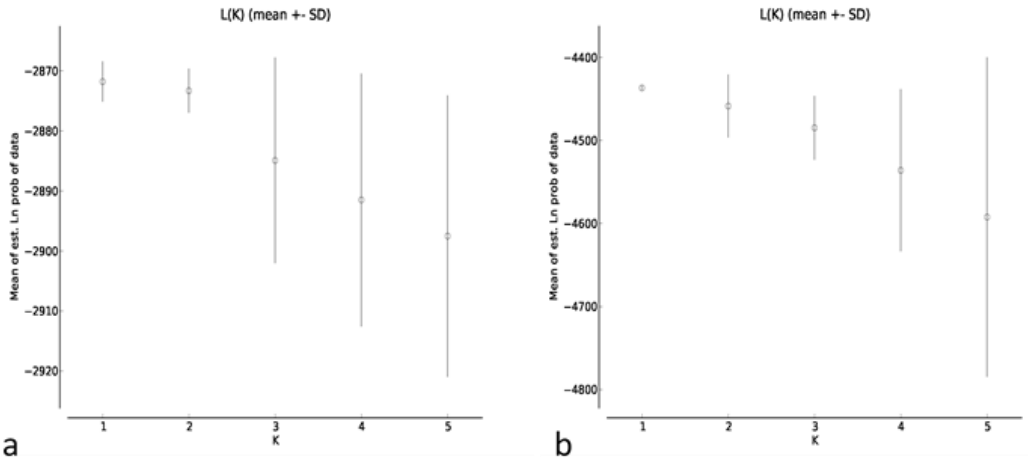


Supplementary figure 1. Photographic summary of field procedures in Senegal. (**A**) A free flock of yellow-crowned bishops near Richard Toll (yellow-coloured birds are males, dull-coloured birds are females or immatures). (**B**) Capture through mistnetting (SEN birds in main text). (**C**) Blood sampling. (**D-E**) Traditional trapping of birds by local Senegalese trappers, using clap nets baited with seeds and stuffed decoys (TRAP birds in main text). (**F-G**) Short-term storage of trapped birds at high densities in traditional cages. (**H**) Transport of birds. The traditional cages are transported 350 km (about 7 hours) to Dakar on the rooftops of public buses (not shown).

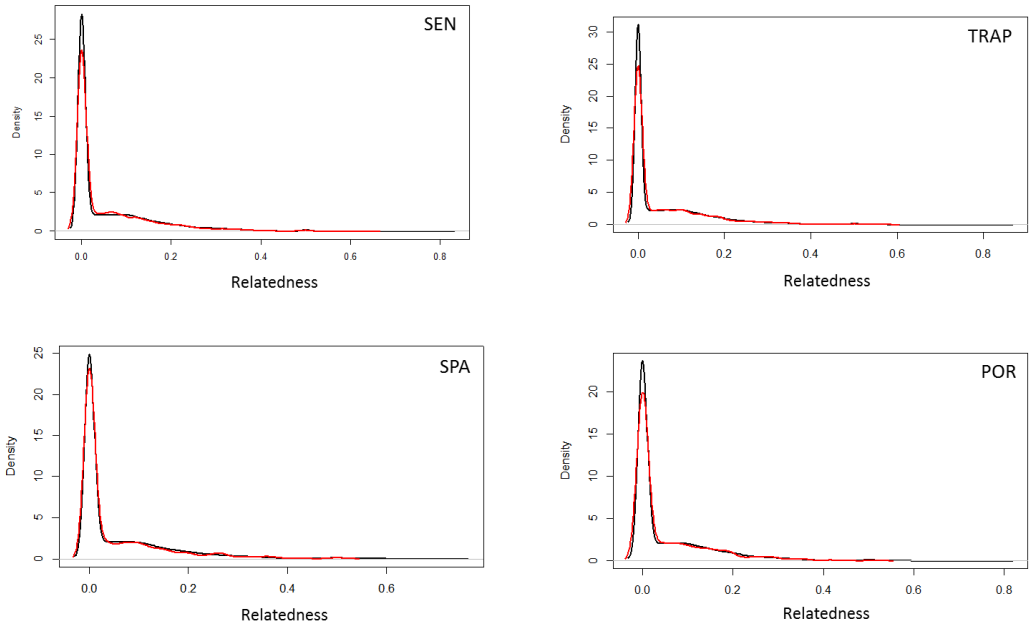
International export usually takes place from Dakar, after 1-3 months of storage in the cages depicted above. Photo credits: Julio Blas.



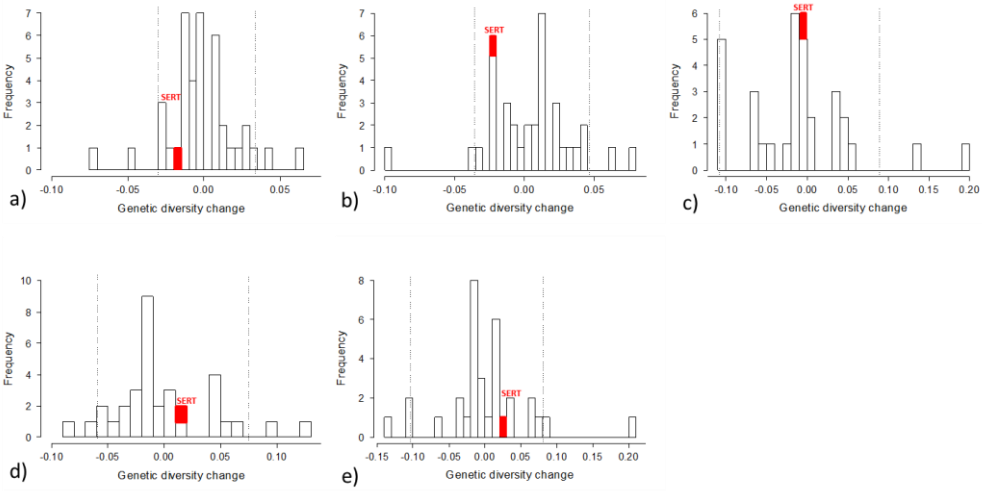
Supplementary figure 2. Discriminant analysis of principal components (DAPC) on individual genotypes of the two Senegalese samples (SEN: sampled by mistnet, TRAP: sampled by clap trap), and the two invasive populations from Spain (SPA) and Portugal (POR).



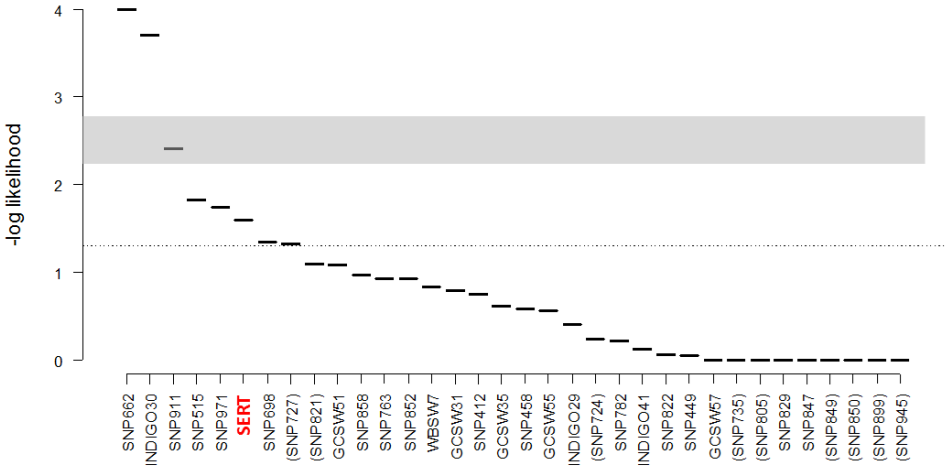
Supplementary figure 3. Mean posterior probabilities (+/- SD) of genotype data of random microsatellites given K subpopulations for the a) mistnet sampled (SEN) and b) traditionally sampled (TRAP) Senegalese birds. The mean probabilities were calculated using S*tructure* with default settings (admixture model with correlated allele frequencies) from 10 replicated runs.



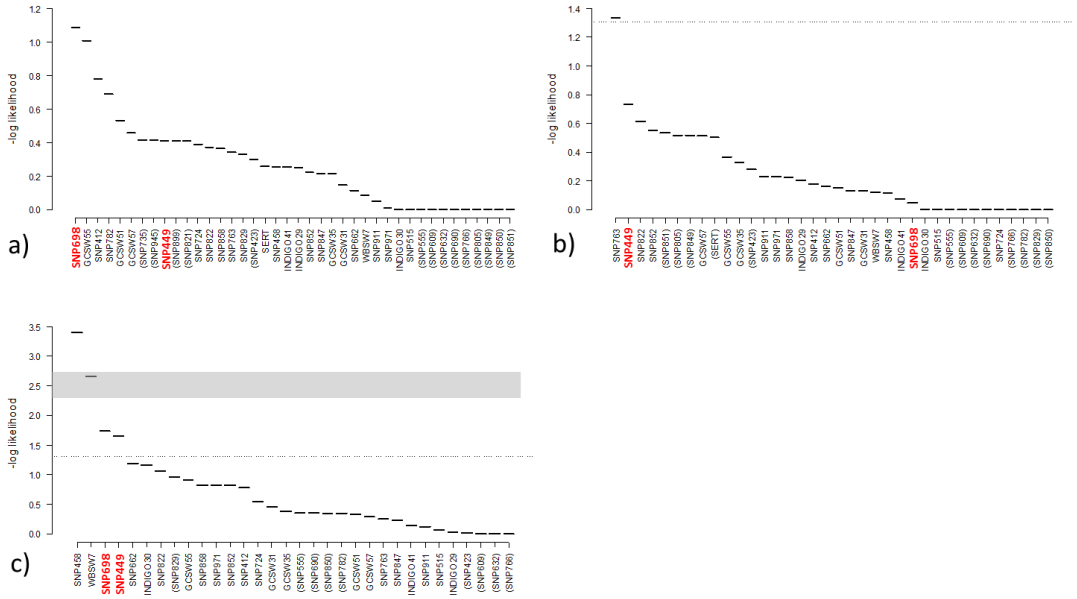
Supplementary figure 4 Density plots of relatedness values in the samples SEN, TRAP, SPA, and POR. Values of the observed data set are indicated in red and values of 1000 simulated random data sets are indicated in black.



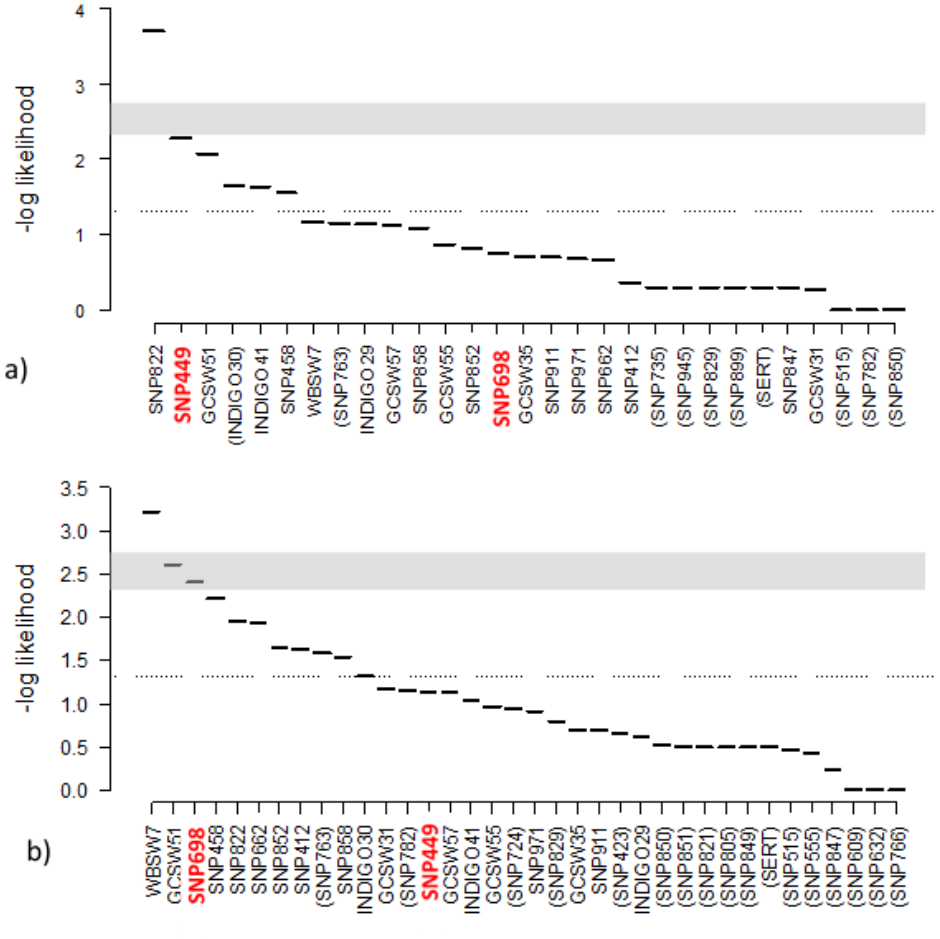
Supplementary figure 5. Genetic diversity changes for each polymorphic locus (subset of 31 *DRD4* SNPs, and one *SERT* and 9 random microstaellites) for the comparison of a) the trapping filter (TRAP – SEN), b) the acclimation filter ($ACCL_{yes}$ – $ACCL_{no}$), c) the survival filter ($SURV_{yes}$ – $SURV_{no}$), d) the invasive-native population comparison (SPA – SEN) and e) the invasive-native population comparison (POR – SEN). The 5% and 95% percentiles are indicated as dotted lines. The candidate locus *SERT* is marked in red.



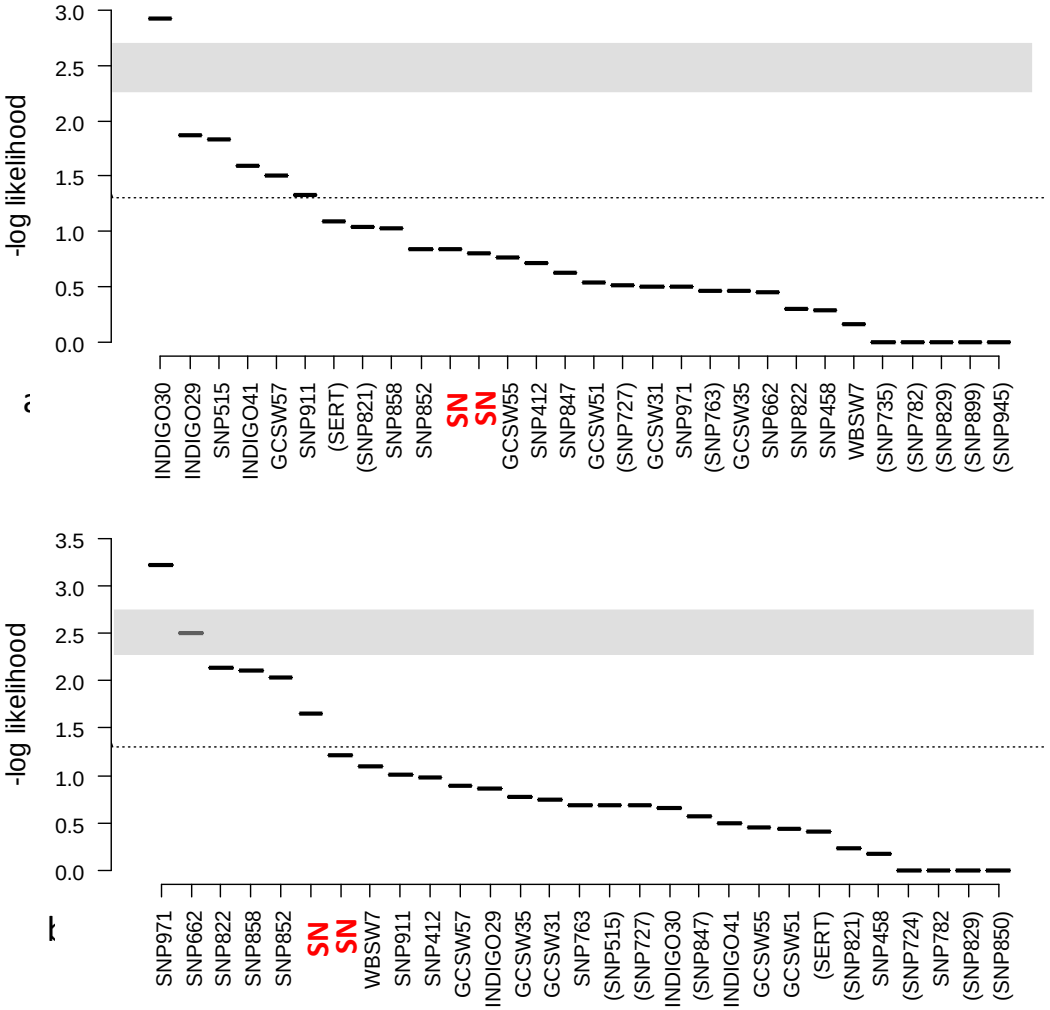
Supplementary figure 6. Permutation likelihood (-log) of observed (or more extreme) increase in genetic diversity at each locus across the two invasive-native population comparisons (SPA – SEN and POR - SEN). The candidate locus *SERT* is marked in red. Loci monomorphic in all subsamples are excluded. Loci with total minor allele count ≤ 2 in both subsamples combined (see Methods) of at least one invasive-native contrast are indicated by brackets. Nominal significance level and significance level adjusted for multiple testing (11 genomic regions or 27 independent polymorphic markers, see Methods) are indicated as a dotted line and a grey bar, respectively.



Supplementary figure 7. Permutation likelihood (-log) of observed (or more extreme) absolute changes in allele frequency for each locus in the comparison of (a) the trapping filter TRAP - SEN, (b) the acclimation filter $ACCL_{yes} - ACCL_{no}$) and (c) the survival filter ($SURV_{yes} - SURV_{no}$). Candidate *DRD4* SNPs are marked in red. Loci monomorphic in both subsamples of each filter comparison are excluded. Loci with total minor allele count ≤ 2 in both subsamples combined (see Methods) are indicated by brackets. Nominal significance level and significance level adjusted for multiple testing (11 genomic regions or 27 independent polymorphic markers) are indicated as a dotted line and a grey bar, respectively.



Supplementary figure 8. Permutation likelihood (- log) of observed (or more extreme) absolute changes in allele frequency for each locus along the three filter comparisons combined in a) females and b) males. Candidate *DRD4* SNPs are marked in red. Loci monomorphic in all subsamples of the filter comparisons are excluded. Loci with total minor allele count ≤ 2 in both subsamples combined (see Methods) of at least one filter contrast are indicated by brackets. Nominal significance level and significance level adjusted for multiple testing (11 genomic regions or 27 independent polymorphic markers) are indicated as a dotted line and a grey bar, respectively.



Supplementary figure 9. Permutation likelihood (-log) of observed (or more extreme) absolute changes in allele frequency for each locus along the two invasive-native comparisons combined in a) females and b) males. Candidate *DRD4* SNPs are marked in red. Loci monomorphic in all subsamples are excluded. Loci with total minor allele count ≤ 2 in both subsamples combined (see Methods) of at least one invasive-native contrast are indicated by brackets. Nominal significance level and significance level adjusted for multiple testing (11 genomic regions or 27 independent polymorphic markers) are indicated as a dotted line and a grey bar, respectively.

Chapter II

Selection on individual variation during the first stages of the biological invasion pathway in two bird species

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Manuscript

Abstract

Biological invasions have become a global problem with large negative impacts on wildlife and human societies. Before a species establishes in a non-native range, all individuals will have to pass through the pre-establishment invasion stages of uptake, transport, and introduction. While virtually neglected, during these early stages there might be selection acting on traits. We test this hypothesis following the fate of a set of individuals of two avian pet-traded invaders, *Ploceus melanocephalus* and *Euplectes afer*, during capture, initial acclimation, and captivity. We find that selection acts on wide range of phenotypic traits thought to be potentially important for invasion success (sex, age, body/brain/bill size, condition, stress hormone levels, and behaviour). Our study demonstrates the existence of early, pre-establishment selection which ultimately could change the composition and thereby the success and/or impact of introduced populations.

Keywords

biological invasion, brain size, feather corticosterone, wildlife trade, pre-establishment selection, invasive potential

Introduction

The worldwide introduction of exotic species is considered a central component of global change that can have severe ecological and socio-economic impacts. Since the eradication of invasive populations is costly and often impractical or impossible, much research has been devoted to the avoiding of future invasions (Kolar and Lodge 2001). This includes the identification of factors that might contribute to successful invasions, like characteristics of the event itself (e.g. introduction effort or propagule number), the biotic and abiotic characteristics of the invaded ecosystem, and the characteristics of the invading species (Catford et al. 2009).

The invasion process is viewed to consist of successive steps (uptake/capture, transport, introduction, establishment and expansion) separated by barriers that act as selective filters that prevent or allow species to move from one stage to

another (Blackburn et al. 2011). However, most studies focus on the later establishment and expansion stages, while the earliest stages (capture, transport and introduction) have received relatively little attention, despite their potential importance (Puth and Post 2005). In fact, taxonomic biases in capture and transport may determine which species have opportunities to settle and become invasive (Blackburn et al. 2009). A stronger focus on these neglected earlier stages is all the more relevant when we realise that any selective events during earlier stages will condition the scope for selection during later stages: variation that is not included or that is removed early on cannot be retrieved later on.

Another dominant feature of the current working hypotheses is to focus on the average characteristics of the species, thereby ignoring individual variation within potential invasive species. This despite the fact that newly established populations have been documented to undergo rapid evolutionary changes in morphological, behavioural, physiological and life-history traits (e.g. Blackburn *et al.* 2009), supporting that individuals do vary in their invasive potential, and that selection acts on this variation. In summary then, studies have neglected (or have been unable) to study the earliest stages of the invasion pathway, and especially so with respect to within-species individual variation. For this reason several calls have been made to investigate this black box of pre-introduction selection on individual variation (Carrete et al. 2012, Chapple et al. 2012).

Such studies would appear to be relevant for several reasons. First, early selection on individual variation could change the composition of the introduced populations that ultimately might establish (or not) in novel environments and thereafter become an invasive species (Fig. 1A). Such selection could enhance or decrease invasive potential, depending on how selection acts and on which traits. Hence, knowledge on early selection could help us to understand why certain species or introductions are successful and others are not. In addition, knowing *how* early selection operates might provide potential for management directed to avoiding further invasions. Second, many comparative studies implicitly assume that early selection can be ignored. When utilising average species traits, the values are collected for native or established populations, while either may not reflect accurately the mean of the population during introduction. Other studies try to infer evolution and adaptation in novel ranges via comparison of invasive and

native populations, thereby ignoring that any encountered differences might have arisen during the early, pre-establishment stages of the invasion, perhaps even with no consequent changes to the population after introduction to the novel range. Hence, as stated before (Carrete et al. 2012, Chapple et al. 2012), there are various reasons why pre-establishment selection on individual variation should no longer be ignored.

Here we test for selective pre-establishment filtering in two avian invaders, the Black-headed weaver (*Ploceus melanocephalus*) and the Yellow-crowned bishop (*Euplectes afer*). These are two African species of passerines that are wild-caught and subsequently traded as pet birds, and that have established many populations worldwide (Sanz-Aguilar et al. 2014, Abellán et al. 2017). Contrary to the deliberate introductions of past centuries, much of the current invasions derive from international traffic in exotic species. Millions of plants and animals, belonging to hundreds of species are extracted annually from nature and transported internationally for trade in pet markets, aquaculture and gardening (Reaser 2008). A small portion of these specimens are accidentally or deliberately released or manage to escape, forming the beginnings of new exotic invasions. We test whether pre-establishment selection occurs, when it acts, and on which traits it acts, by following the fate of a set of individuals that have been characterised for various phenotypic traits during a number of potentially selective events (uptake, initial acclimation and captivity).

In a recent study (Mueller et al. 2017) we investigated and found support for pre-establishment selection on genetic variation in a dopamine receptor gene in the Yellow-crowned bishop, which is related to behavioural variation (Mueller et al. 2014). As far as we know, this is the first published study to investigate pre-establishment selection on individual variation. In this second study we expand to study two species and a wide array of phenotypic traits, thereby considerably generalising the focus of Mueller *et al.* (2017).

We tested for pre-establishment selection on a number of phenotypic traits that we *a priori* thought to be potentially important for invasion success and to be under pre-establishment selection. Individual variation in behavioural responses to stimuli and situations (Réale et al. 2007) could be important for invasion success, especially when associated with dispersion, social organization,

demographic parameters and physiological responses to stress (Réale *et al.* 2007, Dingemanse *et al.* 2010), thereby influencing range expansion into non-native areas (Liebl and Martin 2012) or the exploration of novel food resources (Sol et al. 2011). Although not limited to new situations of risk or stress, behavioural variation may be particularly important in such cases, determining the differential survival of individuals (Réale et al. 2007). Furthermore, how an individual copes with stressful situations is a key determinant of its biological functioning (Blas et al. 2007). Hormone secretion can alleviate the stressful condition, but chronic exposure to these hormones, as may occur after introduction into a new range but also during the early invasion stages, can have negative impacts on performance and survival. Other traits likely to be of relevance to invasion success and to be under selection during the pre-establishment stages are related to morphology. Size and weight could be related to e.g. capture probability, intra- and interspecific competition, resource requirements or resistance to temperature changes. Bill traits (incl. size and shape) may be related to feeding ability (e.g. use of new sources of food) and intra- and inter-specific competition. Brain size in birds appears related to dealing with novel situations, escape strategies (Samia et al. 2015) and the colonization of variable habitats (Fristoe et al. 2017). Finally, the sex, age and reproductive status of individuals are key components of the demographic composition of a newly introduced population, which will influence population survival and growth rate. These traits could also be under pre-establishment selection (differential capture or survival), especially since both study species are sexually dimorphic in breeding plumage and size, and have a delayed sexual maturity.

Methods

Sampling of native populations

Native Senegalese individuals of *P. melanocephalus* and *E. afer* (NATIVE, $N_{P. melanocephalus}$= 394, $N_{E. afer}$= 446) were caught by us with mist nets (Fig. 1.b2) in September 2014 near the vicinity of Richard Toll, northern Senegal (16°27'45" N -15°42'03" W). According to the Senegalese bird export company and the CITES

trade data (Sanz-Aguilar et al. 2014), this is the same area where these species have historically been caught for export. We collected data for the phenotypic characterization of individuals (sex, age, morphometric and behavioral measures, and a feather to measure stress hormone concentration; see section *Characterization of individual phenotypic variation* for details), and after individuals were marked (to avoid resampling of the same individual) all individuals were released in situ. Mist netting is a catching method that is presumably the least biased with respect to behavioral or morphological traits, and therefore the most common method of choice in the scientific monitoring of passerines. For example, Simons *et al.* 2015 did not detect any bias in mist net-caught birds for their fully monitored island population of house sparrows (*Passer domesticus*). We therefore consider our sample of mistnetted Senegalese birds as the best reference for the native population.

Sampling of individuals entering the bird trade and follow-up during the first invasion stages

We studied potential selection during three early stages of the original invasion pathway via the international exotic bird trade by sampling birds caught by Senegalese bird trappers, and monitoring their fate until international export, usually 1-3 months later. In stage 1, we accompanied professional local bird trappers working for the Senegalese company that exports *P. melanocephalus* and *E. afer* to other countries. Between 6-13 September 2014, they caught individuals using a traditional clap net baited with seeds and stuffed decoys to attract birds (Fig. 1.b3-b4) in the same general area as described for the reference sample (NATIVE) above. We characterized the phenotype of all these individuals in the same way as for the native population (see below for details) and marked them with uniquely numbered plastic rings.

A first invasion filter of selective uptake was assessed by comparing the traditionally caught birds (UPTAKE, $N_{P.\ melanocephalus}$= 448, $N_{E.\ afer}$= 529) with those caught by us using mistnets ("trapping" or UPTAKE-NATIVE comparison).

In filter 2, we monitored the early survival of these trapped individuals. All individuals were kept at high densities for about one week in traditional storage

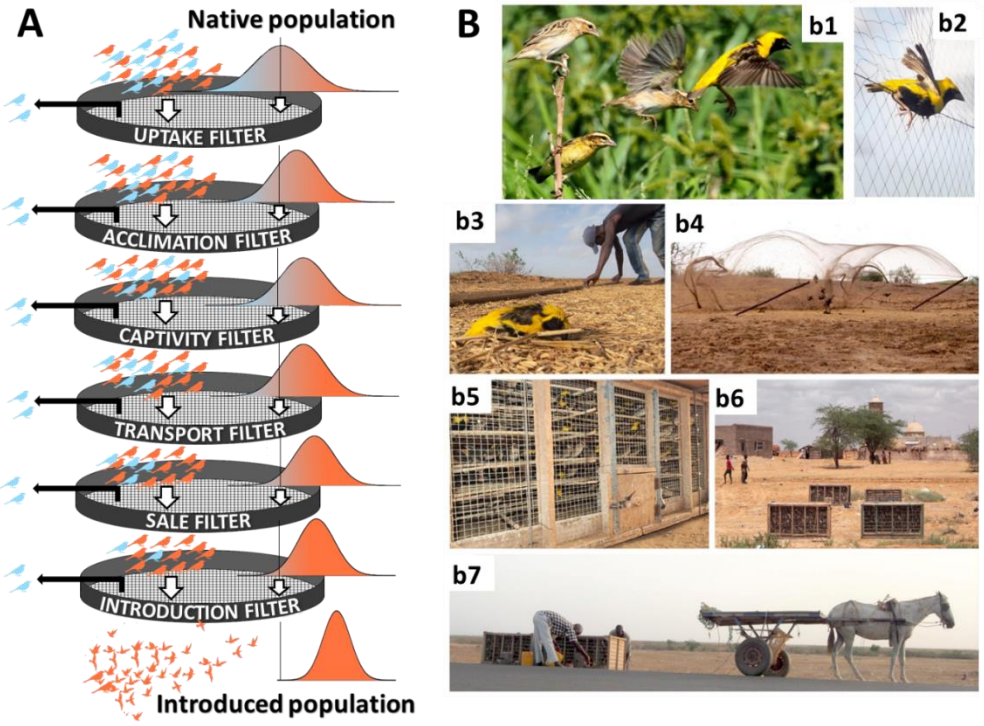

Figure 1. (**A**). Schematic of certain selective filters acting during the pre-establishment stages of an invasion process. For each filter, one or more selective pressures eliminate certain individuals from the pool of potential invaders. The gradation in color (for individuals and for the frequency distributions of individual characters) represents differences between the pheno(geno)types. (**B**). Photographic summary of field procedures in Senegal. (**b1**) A free-flying flock of native yellow-crowned bishops (yellow-colored birds are males; more dull-colored birds are females). (**b2**) Unselective capture through mist-netting (NATIVE birds in main text). (**b3-b4**) Traditional uptake of birds by local Senegalese trappers, using clap nets baited with seeds and stuffed decoys (UPTAKE birds in main text). (**b5-b6**) Short-term storage of trapped birds at high densities in traditional cages (Initial acclimation). (**b7**) Transport of birds. The traditional cages are moved on a horse cart to the nearby road, and then transported 350 Km (ca. 7 hours) to Dakar on the rooftops of public buses (not shown). International export usually takes place from Dakar, after 1-3 months of storage. Photo credits: Julio Blas

cages (Fig. 1.b5-b6) close to the trapping sites and were then transported 350 km in the same cages (Fig. 1.b7) to the installations of the bird-trading company

in Dakar (about seven hours driving on the roof of a bus). Survival was monitored until about one week after arrival in Dakar. Therefore, a second invasion filter where selection could take place was a 14-to-18-day period during which individuals either acclimated successfully to entry in captivity and transport (ACCLyes, $N_{P.\ melanocephalus}$= 235, $N_{E.\ afer}$= 313) or not (ACCLno, $N_{P.\ melanocephalus}$= 80, $N_{E.\ afer}$= 133), and we compared the surviving and non-surviving birds ("initial acclimation" or ACCLyes – ACCLno comparison).

In the last stage we investigated, the remaining birds were communally kept in storage cages (Fig. 1.b5-b6). Typically birds are stored from one to three months before export (according to the Senegalese bird export company). Thus, a third invasion filter during which selection was evaluated was this longer-term survival in captivity. We assessed selection by comparing individuals that survived in the first 30 days with those that did not (SURVyes, $N_{P.\ melanocephalus}$= 143, $N_{E.afer}$= 175 vs. SURVno, $N_{P.\ melanocephalus}$= 92, $N_{E.afer}$= 138).

Finally, to test the cumulative selection (since in each filter selection may act in a different direction) throughout these pre-establishment stages, we compare the native population (those sampled with mist nets) with the population of surviving individuals at the end of the captivity (SURVyes-NATIVE comparison).

Characterization of individual phenotypic variation

In order to test for selection on individual variation during the first stages of the invasion pathway, we measured various traits thought to be potentially important for invasion success. We took morphological measurements of all individuals of the two species just after their capture: wing length, body weight, external skull dimensions (width, height, and length), and beak dimensions (width, height, and length). We use wing length as a proxy of body size, and weight as a proxy of condition (by statistically controlling for body size, see below). We used the skull dimensions to obtain a proxy for brain size, since we could not measure brain size directly in a live individual. We calculated the head volume (cm^3) as the product of head length (minus beak length), head width and head height. This has a high correlation with actual brain case volume of cleaned skulls in the two study species (own data, see Supplementary Information) as well as in another passerine

(Møller 2010). We used the beak measurements (width, height, and length) to obtain proxies for beak size and beak shape. For this, we performed a principal components analysis. For both species, the first axis (with roughly equal loadings and equals signs: $PC1_{P.\ melanocephalus}$= length: 0.77, width: 0.42, height: 0.49 and $PC1_{E.afer}$= length: 0.58, width: 0.56, height: 0.59) is interpreted as beak size (higher PC scores indicate larger beaks:) and the second axis (with opposite signs: $PC2_{P.\ melanocephalus}$= length: 0.63, width: -0.66, height: -0.42 and $PC2_{E.afer}$= length: 0.81, width: -0.47, height: -0.35) as beak shape (larger PC scores indicate more pointed beaks:).The sex of all individuals was determined based on plumage and size characteristics (adults are dimorphic in breeding plumage and, especially *Ploceus*, in size) and confirmed by a PCR-based method following Griffiths *et al.* (1998) for uncertain individuals and all non-breeding individuals. The reproductive stage of individuals (reproductive adult, non- reproductive adult, or juvenile) was also determined by plumage characteristics (presence of adult male breeding plumage, brood patch in females, or juvenile plumage). For the behavioral characterization, we recorded if individuals pecked or tried to escape during manipulation when taking the measurements. In addition, we took the two outermost tail feathers to measure the concentration of the avian stress hormone corticosterone as a measure that reflects the long-term corticosterone serum levels during feather growth (Bortolotti et al. 2009) (following Bortolotti *et al.* (2008) for methods of estimation of feather corticosterone). Corticosterone was measured in a random stratified (by age and sex) subset of individuals (*E. afer* n=142, *P. melanocephalus* n=141),

Data analysis

We used generalized linear models (with a binomial error distribution) using R software (R Core Team 2017) to test the existence of selective filters during the first stages of a biological invasion. Models were fitted for each species and each filter separately. For the first filter, "uptake", we modeled as dependent variable the origin of the individuals (uptake/native) and for the next two filters, "initial acclimation" and "captivity", the dependent variable was the probability of passing the filter, i.e. individual survival during the filter. In addition, to test the cumulative selection throughout this pre-establishment process, we used the

origin of individuals (native/survivor) as dependent variable for the survivors-native comparison. The effects tested were sex (male/female), age (reproductive adult, non-reproductive adult or juvenile), body size (wing length), weight, brain size (skull volume), feather corticosterone concentration, pecks (yes/no), escapes (yes/no), beak size (PC1 scores) and beak shape (PC2 scores). All the continuous variables used in the models were standardized (for each species and comparison separately?) to allow a direct comparison of effect estimates.

Since single, complete models including all variables did not converge we first fitted a basic model including only sex, age, body size and weight as independent variables. To test the other variables, we added each separately to the basic model (see Table 1). In this way, we adjusted separate models for brain size, corticosterone, behavior (including pecks and escapes in the same model) and beak morphometry (including beak size and beak shape in the same model) (Table 1). Hence, these models give the relative effects of the variables tested. However, because the sex or age ratio by itself may be important for the success of an invasive population, we therefore also fitted the models with only one unique independent variable (sex or age).

Results

For the first uptake filter, we observed significant effects in almost all (9) of the phenotypic traits tested in *P. melanocephalus*, and in the majority of traits (6) in *E. afer* (Table 1). For the second and third filter progressively fewer effects reached significance, but in total 21 comparisons reached significance and an additional 6 near-significance (out of 66 comparisons). Cumulatively, selective filters changed the mean of the traits in the surviving individuals for 7 traits in *P. melanocephalus* and 2 traits in *E. afer*. Together these results overwhelmingly show the operation of selection during these first stages of the invasion process, favouring individuals with certain phenotypic traits to enter and continue in the pool of potential invaders.

Table 1. Overview of the effects of several phenotypic traits on the probability of successfully passing a specific selective filter (uptake, initial acclimation and captivity) and of passing all three filters (cumulative selection) as tested in two invasive bird species (*Ploceus melanocephalus* and *Euplectes afer*). The model used and the estimated coefficients are provided (the basic model was composed of sex + age + wing + weight); in bold when significant ($p<0.05$) and underlined when a trend is observed ($0.05<p<0.08$).

Tested effect (+Category of provided coefficient)	**Model**	***Ploceus melanocephalus***				***Euplectes afer***			
		Uptake	**Acclimation**	**Captivity**	**Cumulative selection**	**Uptake**	**Acclimation**	**Captivity**	**Cum sel**
Sex (male)	Pass ~basic model	**-3.329**	-0.533	0.746	**-3.377**	**0.832**	-0.467	0.266	**0**
Age (breeding adult)	Pass~basic model	**-0.865**	0.151	-0.711	**-0.891**	-0.135	0.076	-0.452	-0
Age (chick)		**-0.715**	**1.304**	-0.209	-0.343	**-0.687**	0.551	-0.045	-0
Size	Pass~basic model	**1.387**	-0.248	-0.361	**1.072**	0.145	-0.1	-0.081	0
Weight	Pass~basic model	**1.076**	0.437	0.447	**1.415**	**-0.357**	**1.041**	0.271	0
Brain size	Pass~**brain size** + basic model	**-0.39**	**0.518**	0.185	-0.287	-0.026	**0.294**	-0.064	0
Feather Corticosterone	Pass~**corticosterone** + basic model	**1.238**	-0.48	-0.028	**1.059**	**1.086**	-0.252	-0.484	**0**
Pecks (yes)	Pass~**pecks** + **escapes** + basic model	-1.551	-0.079	1.187	-0.531	-0.061	-1.374	**-1.353**	-0
Escapes (yes)		-0.485	0.937	0.186	-0.257	**-0.583**	**1.295**	0.286	-0
Beak size (PC1)	Pass~**beak size** + **beak shape** + basic model	**0.536**	-0.068	0.192	**0.530**	**0.235**	-0.131	-0.037	0
Beak shape (PC2)		**-0.313**	-0.226	0.04	**-0.329**	-0.05	-0.02	-0.028	-0

Table 1 summarises the estimated coefficients and significances of all tested effects, and below we describe the principal patterns observed. For both species studied, trapped individuals have smaller brain sizes than individuals from the native population, but individuals with bigger brain size are more likely to survive during the next filter of initial acclimation (Table 1, Fig. 2A). A similar pattern arises for both species analyzing feather stress hormone concentration; captured individuals have a higher level of feather corticosterone than the native population, while survival during initial acclimation and captivity is higher for individuals with lower concentrations (Table 1, Fig. 2B), resulting in an overall effect of surviving individuals having higher levels of feather corticosterone than the native population (Table 1).

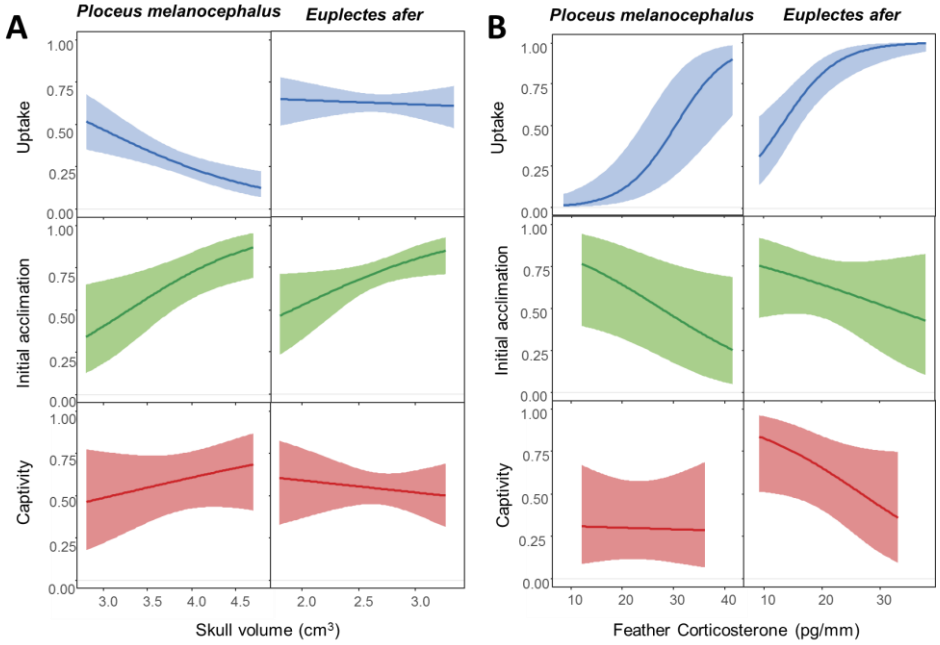

Figure 2. Effects of (**A**) brain size and (**B**) stress hormone levels in feathers across the three selective filters studied (uptake, initial acclimation and captivity) for *Ploceus melanocephalus* and *Euplectes afer*. Lines represent the model predictions (corrected for covariates, see Table 1) and the shadows are the 95% confidence levels.

The effect of sex is different between species: *P. melanocephalus* males have a lower probability to be trapped from the native population, whereas *E. afer* males

have a higher probability and both effects are transferred to the corresponding surviving populations (Table 1). Age effects are similar in both species: breeding adults and chicks have a lower probability to be trapped than non-breeding adults, and chicks are more likely to survive during the initial acclimation (Table 1). In the surviving population of *P. melanocephalus* we observed less breeding adults than in the native population (Table 1). The body size of the individuals has an effect on uptake, with larger individuals more likely to be included in both species, resulting in bigger surviving individuals of *P. melanocephalus* than the native ones (Table 1). *P. melanocephalus* individuals with a larger weight are more likely to be trapped and this effect remains when we look at the cumulative process (survivors-native comparison), while for *E. afer* this effect is the opposite yet individuals with a larger weight do have a higher probability of survival in the initial acclimation filter (Table 1). In both species trapped individuals have a bigger beak than the native populations, and especially in *P. melanocephalus* also a more pointed beak, for which these effects are the same in the survivors-native comparison. Behavioral traits are also selected upon during the filters, especially for *E. afer*, where individuals which attempt to escape during manipulation are less likely to be trapped than native ones and have a higher survival probability during initial acclimation, while individuals that peck have a lower survival probability during the captivity filter.

Models fitted to test only the effect of sex or age (with no other controlling variables) showed that more males are trapped for both species (*P. melanocephalus*: 0.918 (estimate) ± 0.142 (SE), $p<0.001$ and *E. afer*: 0.716 ± 0.142, $p<0.001$), and that *P. melanocephalus* males have a lower survival than females during the acclimation filter (-0.442 ± 0.278, $p<0.001$) but a higher survival during the captivity filter (0.801 ± 0.274, $p=0.003$). With respect to age, chicks of both species are trapped less (*P. melanocephalus*:-1.114 ± 0.177, $p<0.001$ and *E. afer*: -0.672 ± 0.187, $p<0.001$), and for *P. melanocephalus* have a higher survival during acclimation (1.401 ± 0.394, $p<0.001$). *P. melanocephalus* breeding adults have a lower survival in captivity (-0.803 ± 0.342, $p=0.019$) and for *E. afer* they have a higher survival during initial acclimation (0.445 ± 0.224, $p=0.044$).

Discussion

Our results demonstrate the ample existence of selective filters that are already acting during the pre-establishment stages of an invasion process, and affecting a wide range of phenotypic traits. In addition, depending on the trait, these selective forces may be different between the different filters and between the different species, or show a similar pattern. This demonstrates that selective filters could function independently depending on the stage and the species on which they act, selecting the individual characters of the potential invaders.

In the uptake filter, we observe that selection favors smaller brains. Although speculative, this might be related with variation in cognitive abilities and escape strategies (Samia et al. 2015). However, in the following stages selection favours larger brains, potentially since this might provide a better coping with novel situations (Sol et al. 2005) that ultimate increases the probability of survival. This might have an impact on the population invasive potential, since bigger brains facilitate the colonization of variable habitats (Fristoe et al. 2017). Selection acts in a similar way for stress resistance. Individuals with higher corticosternone levels are more likely to be captured, which may suggest that these lower-quality individuals are displaced by intraspecific competition towards the areas where they are captured. However, in the following filters, individuals with individuals with lower corticosterone levels have a higher survival probability, possibly due to cumulative acute and chronic stress during the capture-handling transport and captivity strongly that compromise survival (Teixeira et al. 2007). This stress can also modulate the behavioral responses. We found selection on behavioral traits suggesting that a behavior/personality bias can result in how to face challenging unnatural novel situations in theses invasion stages, which may be relevant to success when facing other novel situations in the new area of introduction. One of these new conditions is the food source, in addition to behavior (Sol et al. 2011), and beak morphometry can play an important role in how individuals adapt to a new type of food. In this context, we find that beak morphometry is selected upon in the capture filter, perhaps due to the new source of food used as bait to

attract birds (rice grains). This could also have an impact when these selected individuals arrive in a new non-native area and have to adapt to a new food source. Moreover, we found that individuals are selected by size in the capture, with bigger individuals more likely to be trapped. Individual condition is also under selection in the early stages of the invasion, and it seems logical that individuals with better condition have a greater survival. These biases in size and condition (and likely in overall quality) also can favour the invasion of new areas, e.g. if bigger individuals are more resistance to temperature changes, or if individuals with a better condition have a higher survival. Finally, sex and age biases can have a great effect on the reproduction rate, and thereby on the survival and the expansion of the population that is introduced. While sex has contrary effects in the composite models depending on the species considered, when we just observe the total sex ratio (without controlling for other variables like size which differ between the sexes), more males are trapped for both species. Likewise, more non-breeding adults are trapped. Since both species are polygynous and have a delayed maturation, the reproductive rate is limited by the number of reproductive females and in this case pre-establishment selection could reduce establishment success and the expansion rate of the introduced population.

Hence overall the phenotypic traits on which pre-estabishment selection acts likely have relevance for how individuals cope in newly colonized areas, and therefore this selection likely shapes the future establishment success and invasiveness of any introduced populations. However, studies on how populations undergo micro-evolutionary changes during biological invasions have so far exclusively focused their attention on the later stages of invasion (e.g. Blackburn *et al.* 2009), which has been shown to be very important for the potential impacts of invasions (Faillace and Morin 2016) Nevertheless, as our results show, introduced populations may have already undergone micro-evolutionary changes through selective filters before the establishment stage, and this conditions all subsequent changes related to the adaptation to a new non-native area in the subsequent stages.

Here we demonstrated ample evidence for the existence of pre-establishment selective filters during a biological invasion, in this case one involvingthe international pet traffic as an increasingly important source of biological invasions

(Abellán et al. 2016). However, there is no reason to believe that selection is not equally important in other types of invasions, e.g. unintentional ones, where nonrandom uptake and survival during transport (e.g., in ships, containers) can be easily imagined (Blackburn et al. 2011, Chapple et al. 2012). Therefore, further investigation of what happens during these early stages of invasion, virtually ignored until now, is necessary to better understand and hopefully effectively manage biological invasions.

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Author contributions

All authors contributed to study conception and design. A.B., J.B., M.C., P.E., J.P., J.L.T. carried out field work (Spain and Senegal). A.B and P.E analysed the data. A.B drafted the manuscript. All additional authors provided comments and approved the final manuscript.

References

Abellán, P., Carrete, M., Anadón, J. D., Cardador, L. and Tella, J. L. 2016. Non-random patterns and temporal trends (1912-2012) in the transport, introduction and establishment of exotic birds in Spain and Portugal. - Divers. Distrib. 22: 263–273.

Abellán, P., Tella, J. L., Carrete, M., Cardador, L. and Anadón, J. D. 2017. Climate matching drives spread rate but not establishment success in recent unintentional bird introductions. - Proc. Natl. Acad. Sci.: 201704815.

Blackburn, T. M., Lockwood, J. L. and Cassey, P. 2009. Avian Invasions: The Ecology and Evolution of Exotic Birds.

Blackburn, T. M., Pyšek, P., Bacher, S., Carlton, J. T., Duncan, R. P., Jarošík, V., Wilson, J. R. U. and Richardson, D. M. 2011. A proposed unified framework for biological invasions. - Trends Ecol. Evol. 26: 333–339.

Blas, J., Bortolotti, G. R., Tella, J. L., Baos, R. and Marchant, T. A. 2007. Stress response during development predicts fitness in a wild, long lived vertebrate. - Proc. Natl. Acad. Sci. 104: 8880–8884.

Bortolotti, G. R., Marchant, T. A., Blas, J. and German, T. 2008. Corticosterone in feathers is a long-term, integrated measure of avian stress physiology. - Funct. Ecol. 22: 494–500.

Bortolotti, G. R., Marchant, T., Blas, J. and Cabezas, S. 2009. Tracking stress: localisation, deposition and stability of corticosterone in feathers. - J. Exp. Biol. 212: 1477–1482.

Carrete, M., Edelaar, P., Blas, J., Serrano, D., Potti, J., Dingemanse, N. J. and Tella, J. L. 2012. Don't neglect pre-establishment individual selection in deliberate introductions. - Trends Ecol. Evol. 27: 67–68.

Catford, J. A., Jansson, R. and Nilsson, C. 2009. Reducing redundancy in invasion ecology by integrating hypotheses into a single theoretical framework. - Divers. Distrib. 15: 22–40.

Chapple, D. G., Simmonds, S. M. and Wong, B. B. M. 2012. Can behavioral and personality traits influence the success of unintentional species introductions? - Trends Ecol. Evol. 27: 57–62.

Faillace, C. A. and Morin, P. J. 2016. Evolution alters the consequences of

invasions in experimental communities. - Nat. Ecol. Evol. 1: 13.

Fristoe, T. S., Iwaniuk, A. N. and Botero, C. A. 2017. Big brains stabilize populations and facilitate colonization of variable habitats in birds. - Nat. Ecol. Evol. 1: 1706–1715.

Griffiths, R., Double, M. C., Orr, K. and Dawson, R. J. G. 1998. A DNA test to sex most birds. - Mol. Ecol. 7: 1071–1075.

Kolar, C. S. and Lodge, D. M. 2001. Progress in invasion biology: Predicting invaders. - Trends Ecol. Evol. 16: 199–204.

Liebl, A. L. and Martin, L. B. 2012. Exploratory behaviour and stressor hyper-responsiveness facilitate range expansion of an introduced songbird. - Proc. R. Soc. B Biol. Sci. 279: 4375–4381.

Møller, A. P. 2010. Brain size, head size and behaviour of a passerine bird. - J. Evol. Biol. 23: 625–635.

Mueller, J., Edelaar, P., Carrete, M., Serrano, D., Potti, J., Blas, J., Dingemanse, N. J., Kempenaers, B. and Tella, J. L. 2014. Behaviour-related DRD4 polymorphisms in invasive bird populations. - Mol. Ecol. 23: 2876–2885.

Mueller, J. C., Edelaar, P., Baños-Villalba, A., Carrete, M., Potti, J., Blas, J., Tella, J. L. and Kempenaers, B. 2017. Selection on a behaviour-related gene during the first stages of the biological invasion pathway. - Mol. Ecol. in press.

Puth, L. M. and Post, D. M. 2005. Studying invasion: Have we missed the boat? - Ecol. Lett. 8: 715–721.

R Core Team 2017. R: A Language and Environment for Statistical Computing. - R Found. Stat. Comput.: {ISBN} 3-900051-07-0, http://www.R-project.org.

Réale, D., Reader, S. M., Sol, D., McDougall, P. T. and Dingemanse, N. J. 2007. Integrating animal temperament within ecology and evolution. - Biol. Rev. 82: 291–318.

Reaser, J. K. 2008. Non-native wildlife risk assessment: a call for scientific inquiry. - Front. Ecol. Environ. 6: 466.

Samia, D. S. M., Pape Møller, A. and Blumstein, D. T. 2015. Brain size as a driver of avian escape strategy. - Sci. Rep. 5: 11913.

Sanz-Aguilar, A., Anadón, J., Edelaar, P., Carrete, M. and Tella, J. 2014. Can establishment success be determined through demographic parameters? A case study on five introduced bird species e110019. - PLoS One in press.

Simons, M. J. P., Winney, I., Nakagawa, S., Burke, T. and Schroeder, J. 2015. Limited catching bias in a wild population of birds with near-complete census information. - Ecol. Evol. 5: 3500–3506.

Sol, D., Duncan, R. P., Blackburn, T. M., Cassey, P. and Lefebvre, L. 2005. Big brains, enhanced cognition, and response of birds to novel environments. - Proc. Natl. Acad. Sci. 102: 5460–5465.

Sol, D., Griffin, A. S., Bartomeus, I., Boyce, H., Mayr, E., Lefebvre, L., Duncan, R., Blackburn, T., Cassey, P., Lefebvre, L., Sol, D., Bacher, S., Reader, S., Lefebvre, L., Greenberg, R., Mettke-Hofmann, C., Reale, D., Reader, S., McDougall, P., Dingemanse, N., Shochat, E., Warren, P., Faeth, S., McIntyre, N., Hope, D., Liker, A., Bókony, V., Møller, A., Kark, S., Iwaniuk, A., Schalimtzek, A., Banker, E., Case, T., Lever, C., Tideman, C., Griffin, A., Boyce, H., MacFarlane, G., Biro, P., Dingemanse, N., Bouchard, J., Goodyer, W., Lefebvre, L., Reader, S., Laland, K., Boogert, N., Reader, S., Laland, K., Crawley, M., Arbuckle, J., Laland, K., Reader, S., Reader, S., Laland, K., Boogert, N., Reader, S., Hoppitt, W., Laland, K., II, L. M., Fitzgerald, L., Hone, J., Overington, S., Morand-Ferron, J., Boogert, N., Lefebvre, L., Gosling, S. and Griffin, A. 2011. Exploring or Avoiding Novel Food Resources? The Novelty Conflict in an Invasive Bird (A Iwaniuk, Ed.). - PLoS One 6: e19535.

Teixeira, C. P., de Azevedo, C. S., Mendl, M., Cipreste, C. F. and Young, R. J. 2007. Revisiting translocation and reintroduction programmes: the importance of considering stress. - Anim. Behav. 73: 1–13.

SECTION 2

Native populations adapting to environmental change

Chapter III

Biased dispersal, and not plasticity or natural selection, drives local crypsis following colonization of an urban habitat

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*Shared first authorship as these authors contributed equally to this work

Manuscript

Abstract

An organism's performance depends on how well-suited its phenotype is to its present environment. There are several mechanisms that can improve this phenotype-environment match. However, typically only one or two of these mechanisms are investigated at any time, leaving us with incomplete views of how organisms cope with environmental variation, e.g. caused by urbanization. Here we determine the relative importance of natural selection, phenotypic plasticity and habitat choice in generating crypsis of ground-perching grasshoppers colonizing an urbanized habitat. This is a mosaic of four distinctly colored substrates, and grasshoppers tend to match the color of their local substrate. This crypsis is not a result of present-day natural selection, nor of plasticity. Instead, individuals choose those urban substrates that resemble their own colors. By manipulating grasshopper color, we confirm matching substrate choice. This selective movement actively creates improved crypsis, genetic divergence between substrates, and assortative mating, all over a remarkably small spatial scale of a few meters. Biased dispersal resulting from matching habitat choice can be an important driver of local performance and adaptive population genetic structuring, even when natural selection is presently weak. Evolutionary studies should more fully incorporate that individuals are not only selective targets, but also selective agents.

Keywords

non-random dispersal, matching habitat choice, local adaptation, phenotype-environment correlation, directed gene flow, assortative mating

Introduction

Improving the match between individual traits and environmental characteristics is a central challenge to all life, as it increases ecological performance and thereby fitness (Darwin 1859, Rose and Lauder 1996). Understanding how organisms (including threatened, beneficial and detrimental ones) achieve this is therefore of great fundamental and applied significance in diverse fields of science and policy. The issue is gaining even more importance as natural populations face contemporary human-induced rapid environmental

change (Sala et al. 2000, Sih et al. 2011) and as we increasingly recognize the impacts of adaptive evolution by pathogens, pests, and even tumors (Klein 2013, Faillace and Morin 2016).

Natural selection is a main cause of genetic adaptation at the population level (Darwin 1859, Rose and Lauder 1996, Barton et al. 2007). However, natural selection cannot improve the performance of locally maladapted individuals. Therefore, it has long been recognized that natural selection has favored the evolution of additional demographic and ontogenetic processes that can contribute to an improved fit between individual phenotype and environment (Jones and Probert 1980, Holt 1987, Jaenike and Holt 1991, Schlichting and Pigliucci 1998, Davis and Stamps 2004, Laland and Sterelny 2006, Edelaar et al. 2008, Richardson et al. 2014, Wang and Bradburd 2014). Individuals who alter their environment or their phenotype in beneficial ways can reduce their risk of mortality (or improve their fecundity), pre-emptively evading low fitness. Genotypes that confer this ability may therefore be favored by selection, with the result that in later generations individuals can proactively improve their fitness before selection has a chance to act.

However, past attention for such individual flexible adjustments of phenotype or environment has been rather uneven, and we actually know very little about their relative importance and evolution. For example, even though there are many theoretical studies on the interaction between natural selection and plasticity, or natural selection and habitat choice, only recently have simulation studies started to explore the evolution of plasticity and habitat choice simultaneously (Scheiner 2016, Edelaar et al. 2017a). Similarly, our knowledge on the empirical relative importance of these distinct processes in driving improved local performance is also limited, as most studies typically focus on only one process (e.g. natural selection) or perhaps contrast it with, or exclude, a second (e.g. plasticity). While it could be argued that perhaps authors intuitively know which processes are most important in their study system and therefore just focus on those, nonetheless such partial investigations leave us with an incomplete if not biased view of how organisms actually deal with current and future environmental variation.

A good example of this is the investigation of colonization and adaptation to urban environments. Urbanization is a severe form of habitat change that typically

results in the loss of biodiversity as natural ecosystems are replaced by human-designed landscapes (Sala et al. 2000; McKinney 2002; Ellis et al. 2010). However, some species have been able to cope with urbanization and the associated changes in abiotic conditions, resources, and natural enemies (McKinney 2002; Shochat et al. 2006). Several processes can facilitate the adaptation of urban populations to their novel conditions (Sih et al. 2011, Miranda et al. 2013). First, urban environments can impose strong natural selection on colonizing populations. When this selection acts on heritable traits, urban populations may evolve local adaptations and diverge from their rural progenitors (Cheptou et al. 2008, Miranda et al. 2013, Alberti et al. 2017, Brans et al. 2017). Alternatively, successful urban colonists may have characteristics that pre-adapt them to survive and reproduce in novel urban environments. Phenotypic plasticity is one such pre-adaptation. Past selection in a population's native habitat may have favored the evolution of plasticity, in which individuals adjust their phenotype to better match their habitat (Schlichting and Pigliucci 1998). Plasticity in behavior or morphology can help colonizing populations habituate to and persist in novel urban environments (Tuomainen and Candolin 2011, Lowry et al. 2013, Miranda et al. 2013). Habitat choice represents a second kind of pre-adaptation. The logical mirror image of plasticity (Edelaar et al. 2008), habitat choice involves individuals changing their habitat (via movement) to better match their phenotype. This is the inverse of plasticity, in which individuals change their phenotype (via development) to better match their habitat. Especially when genotypes have the morphological and cognitive capacity to choose among available habitats based on a comparison of local performance (Maynard Smith 1966, Ronce 2007, Ravigné et al. 2009, Edelaar and Bolnick 2012, Berdahl et al. 2015, Berner and Thibert-Plante 2015) this could subsequently contribute to adaptation to novel contexts (Edelaar and Bolnick 2012, Bolnick and Otto 2013). This performance-based habitat choice is sometimes called 'matching habitat choice' (see Edelaar et al. 2008; Akcali and Porter 2017). It has been hypothesized that matching habitat choice could contribute to urban-rural divergence (Carrete and Tella 2010, Sol et al. 2013), for instance if certain genotypes are particularly likely to leave rural habitats to colonize urban sites, or vice versa. However, the data published so far is not definitive because studies have not carefully excluded the effect of alternative mechanisms that can lead to the same observed patterns of adaptation (Wang and

Bradburd 2014). In fact, we are not aware of any studies that have simultaneously tested the contributions of these three main processes (natural selection, plasticity, habitat choice) to phenotype-environment matching, in an integrated approach, in the same study system, in any context (e.g. urbanization, natural variation, novel laboratory environments, etc.). Hence, we still have little data on the relative importance of these processes in driving adaptation in general, and adaptation to urban habitats in particular.

In this article, we quantify the relative importances of natural selection, plasticity, and habitat choice in an urban-colonist population of the Azure Sand Grasshopper (*Sphingonotus azurescens*). We show that matching habitat choice is the cause of adaptation to novel urban environments in this system of ground-perching grasshoppers. These grasshoppers normally live on open natural soils that vary in color. Such habitat heterogeneity drove the evolution of color variability in the grasshoppers, allowing individuals to be locally cryptic on a subset of natural substrates, thereby reducing predation risk (Rowell 1972). At our urban study site, colonizing grasshoppers encountered four different novel urban habitats: sidewalks, foot paths, bike paths, and asphalt roads (Supplementary Fig. 1). These diverse substrates are arranged in a fine-grained mixture of narrow adjoining patches (Fig. 1B,C; Supplementary Fig. 2), which are closed off to traffic and therefore relatively undisturbed, enabling colonization. Our first question is whether the grasshoppers managed to maintain localized crypsis when colonizing these novel urban habitats, as they do on natural soils, even at this very fine spatial scale? If so, did this crypsis arise via present-day natural selection against mismatched individuals, overcoming random movement across the landscape that could prevent or rapidly erode local adaptation? Or is the urban crypsis a result of plasticity, or habitat choice, capabilities that previously evolved in their native environment? To address these questions, we measured the degree of phenotype-environment match in the urban populations, and studied the operation of natural selection, plasticity, and habitat choice. To our knowledge this represents the first study to simultaneously assay the contribution of these long-acknowledged processes. In addition, we do so in the context of colonization of urban environments, for which the available evidence for the operation of different mechanisms is still fragmented.

Methods

A more detailed description of the used methods and materials is found online (Supplementary Appendix I). We first give a brief introduction to this section. We performed regular captures and recaptures of all grasshoppers across our entire study area. New captures were individually marked and photographed for color measurements. These color measures were used to test for crypsis with respect to local substrate. Recaptures allowed us to derive average movement distances, which were compared to simulations of how local crypsis might deteriorate under random movement of various distances. Re. natural selection, recaptures also allowed calculation of field mortality rates, which were compared with the selective mortality rates necessary to maintain crypsis if movement was random. In addition, the potential for selective predation was tested by measuring survival of immobilized grasshoppers in the study area. Re. plasticity, we measured adult color in response to a manipulation of substrate color in adults and lab-bred individuals, to determine the capacity and rate of plasticity, and the heritability of color. Re. habitat choice, we manipulated the color of grasshoppers and determined their response in use of different habitats both in the lab and the field. Finally, we studied whether habitat choice could indirectly lead to assortative mating for color.

Study area

We studied the colonization of urban pavements by grasshoppers in a deserted housing development site in the province of Seville (Spain). Here large blocks of little-vegetated natural soils are subdivided by fenced-off roads composed of four different types of pavement (Fig. 1B,C; Supplementary Fig. 2).

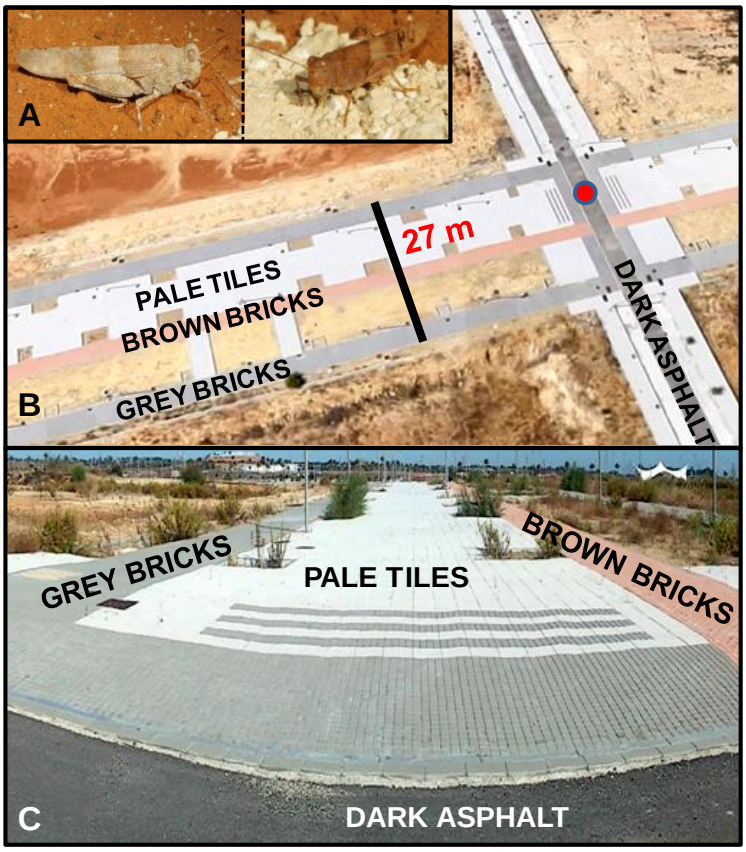

Fig. 1. Study species and study area. (A) Azure Sand Grasshoppers: the left individual was captured on the pale grey soil, the right individual was captured on the brown soil. Dark grey individuals (and all sorts of intermediates) also exist (see Fig. 4A). (B) Aerial view of part of the study area, showing the proximity of the four different linear urban habitats (pavements) in-between large square areas containing natural brown, pale grey and some dark grey soils. (C) A ground view at the position of the red dot in B.

Catch, mark and recapture

We systematically searched for grasshoppers (perched or flushed by us). Captured grasshoppers were individually marked on the posterior part of both fore wings for subsequent visual tracking. Individuals were photographed and released at the location of first encounter. We recorded sex, date of capture, type of substrate on which it was found, and GPS location. The entire study area was

revised for marked and new, unmarked adult grasshoppers ten times from June to October.

Measurement of color and color distances

Grasshoppers and pavements were digitally photographed *in situ* under fixed conditions, and images included an 18% grey standard card. Linearised and normalized mean values of L*a*b in the CIE-Lab color space were extracted from the RAW files using the software Image J (Schneider et al. 2012) and the Mica Toolbox version 1.11, following Troscianko and Stevens (2015). We then calculated chromatic differences between each grasshopper and the average values for each of the four pavements by measuring the Euclidean distances between their L*a*b values (called "delta E"). Since our grasshoppers might be predated on by a wide range of visual predators with very different visual systems, in unknown proportions, we used the same parsimonious route to obtain conservative estimates of delta E values as Lovell et al. (2013). Neither grasshoppers nor natural and urban substrates reflect UV. We also calculated JND (just-noticeable difference) units based on the visual systems of other potential predators (birds, insects and mammals) to show the correlations with the distances for color and luminosity measured on the CIE-Lab color space (Supplementary Appendix I, Figures 8 and 9).

Testing for local adaptation in color

Following Kawecki and Ebert (2004), we tested for local adaptation using two complementary metrics. The home-away contrast tests for local adaptation from the view point of the individuals. We compared for all individuals the delta E value for the habitat in which we first encountered them (observed delta E at home) with the average delta E value each individual would have if it used the other three available habitats in proportion to their availability (predicted delta E away). We used a mixed model to test the effect of the comparison class (home versus away) on delta E values, correcting for the mean differences between

pavement types, and the interaction pavement type * comparison class. We included individual identity as a random effect to account for the two non-independent measurements we have of each individual (its delta E at home and its delta E away). We also tested with a mixed model for the comparison effect for each pavement separately.

The second metric of local adaptation (a resident-immigrant contrast) tests for local adaptation from the view point of the local environment, whether the current resident individuals are better matches than potential immigrants. For this we compared the delta E values of all individuals first encountered on a focal pavement (observed delta E residents) with the delta E values that foreign individuals (first encountered on the other three pavements) would have if they were on the focal pavement (expected delta E immigrants). Tests were done as above.

Calculation of daily grasshopper movement

We followed Börger and Fryxell (2012) in using Net Squared Displacement as a synthetic measure of animal movement rate. The Mean Net Squared Displacement (MSD) is an exponential function of time multiplied by a diffusion constant: $MSD = D*t^{a}$. We fitted our data to the linear double-logarithmic form of this function by a mixed model, including individual identity to deal with the repeated measures of some individuals. The likely detection bias against individuals that have moved greater distances back to natural soils or out of the study area means that our estimate of daily movement is conservative with respect to our inferences.

Simulation of population homogenization with increasing movement

We simulated the spatially explicit effects of movement if it were random (i.e. no habitat choice) with custom code written in the R environment (R Core Team

2017). Each individual for which we had recapture data (N=72) was simulated as starting at its original observation site (coordinates and type of pavement as recorded in the field), then moving in a random direction and distance. The random distance is drawn from a flat distribution ranging from zero to a certain maximum distance. This random movement determines the color of the destination pavement, as determined by a map of pavements at the field site. If the simulated individual does not land on pavement within the study area, the initial movement is repeated until accepted. We then calculate the individual´s visual distance between its color and that of its new substrate (see below). This is repeated for all individuals, and the average visual distance after a single bout of movement of the population is calculated for 1,000 of such independent, uncorrelated repeats. This was done for a range of maximum distances of movement up to 200 meters.

Simulation of necessary mortality rates to obtain observed population divergence

To simulate divergence by natural selection alone, initially all marked and phenotyped grasshoppers from across the four pavement types are introduced onto a single focal pavement. Next, stabilizing selection is exerted on these individuals, with fitness distributed normally around the optimum (delta E = 0), according to the standard function (see e.g. Estes and Arnold 2007): fitness = $\exp(-(\text{delta E})^2/(2\omega^2))$, where ω^2 is the variance of the fitness function and delta E is an individual´s measure of maladaptation in coloration on the focal pavement. Actual death or survival of each individual was subsequently stochastically determined by a draw from the binomial distribution, with a probability of survival equal to its relative fitness as calculated with the fitness function. We visually determined the range of probable values for selection strength ω that could have resulted in the observed mean delta E value for the focal habitat, and then derived which mortality rates this strength of selection would imply.

Measuring predation rate with decoy grasshoppers

We placed dried, dead grasshoppers (N=45) on the street pavements with the aid of some Blue Tag poster fixing material, and counted how many individuals were removed the next day, presumably by predators. The experiment was repeated in a natural area (with a high density of lizards observed) (N=45).

Measuring survival with multistate capture-recapture modelling

We fitted multi-state capture-recapture models to our data on the live grasshoppers (N=272) using the program Mark (White and Burnham 1999, Lebreton and Pradel 2002). We used as state variable whether a grasshopper used the pavement on which it was most cryptic (lowest delta E value) or not. For each of the three parameters (recapture probability, probability to switch states, survival probability) we fitted a model where the parameter was either state-dependent or not, yielding 8 possible models. We did not fit time-dependence to avoid overparameterization, but did correct for the unequal number of days between each capture occasion and included sex-dependence for recapture probability, giving 16 possible models. We calculated the model-weighted average and lower and upper 95% confidence limits for each parameter.

Measurement of rate of phenotypic plasticity in adult coloration

Young adults were randomly assigned to boxes painted black on the inside (N=20), or boxes painted white on the inside (N=20). In nymphs this results in the development of matching colors (Rowell 1972, Edelaar et al. 2017b) (see also Supplementary Fig. 6). We took pictures of each adult at regular intervals. To test if visual distance to its box (delta E) diminished over time we fitted the interaction between time and color treatment, while allowing for random intercepts and random slopes for each individual. Sex ($p=0.98$) was excluded in the final model.

Heritability of adult coloration

Groups of pale reddish-brown grasshoppers and dark blue-grey grasshoppers were allowed to mate freely within each group. Halfway development, nymphs were placed in reddish-brown or bluish-grey rearing boxes. Pictures were taken once the new adults (N=85) were a few days old and color was fully developed. We tested if adult coloration is influenced by color of the rearing environment and coloration of the parent population, allowing for effects of sex and batch (fixed effects), and clutch and rearing box identity (random effects).

Manipulation of grasshopper color

We experimentally altered grasshopper color to conduct habitat choice experiments that decouple individual genotype from individual color. We conducted these experiments in both the laboratory and the urban field site (see below for details). Color manipulation was done in two different ways. (i) We applied pigments externally by using pale or dark aquarelle paint. These dry quickly, give a very natural final look, are also not UV reflecting, and do not contain solvents which might harm the condition and behavior of the grasshopper. We painted the areas likely visible to the grasshoppers (N=40). We have noted no additional mortality or unusual behavior in painted individuals. (ii) Following Yerushalmi and Pener (2001), we injected individuals with the hormone corazonin to induce the deposition of dark pigments into the cuticle by the individuals themselves. Control individuals were not injected.

Laboratory habitat use experiment

Habitat use as a function of individual coloration was measured in a small rectangular transparent plastic box where each long side was filled with a layer of a pale or a dark substrate. To prevent visual disturbance, the sides of the box were covered with a strip of paper of matching colors. Grasshoppers were placed

individually in the centre of a box, and left for about 30 minutes to habituate. We next recorded the position of the grasshopper every 15 minutes, 20 times. Individuals were made to jump after each moment of data collection in order to obtain more independent measures of habitat use. No food or water was provided during choice trials to prevent this from influencing habitat use. For the painted grasshoppers, we modeled the use of the dark habitat as a binomial response variable with a generalized linear mixed model, with color manipulation and rearing substrate (the substrate of collection in the field) as fixed effects, and the identity of the individual (replicated data from different days) and the rearing box in the lab as random effects. Habitat use of grasshoppers injected with corazonin was modeled and tested the same way, except that we modeled corazonin injection (yes/no) and date as fixed effects (all were reared on the same substrate), and the rearing box and experimental box as random effects.

Habitat selection in the field after manipulating grasshopper color

We tested habitat selection of color-manipulated grasshoppers in the field by releasing them in a 115-metre long street composed of a 7 meter wide central area of dark asphalt (similar to the color of grasshoppers made darker by injection with corazonin, mean delta $E < 10$), with strips of pale pavement of 5.5 meters on either side (similar to the color of control, pale grasshoppers, mean delta $E < 10$). We released individuals on the border of the two pavements in the morning. The next morning we recorded the type of pavement of recaptured individuals. This experiment was repeated in two different years (in total $N=112$, with 41 recaptured). We modeled habitat use (binomial response variable) with a generalized linear mixed model, with color manipulation, sex and year as fixed effects, and identity of rearing box in the lab as random effect.

Male-female mating interactions

A successful mating typically starts with the detection of a female by a male. In order to determine over which distances males can detect females, we made a female to jump and fly a few meters. As soon as she landed, we looked for any males that responded to her, and classified its initial distance to the female. This was repeated until we had data from a few different males, after which a new female was used, etcetera. There was no effect of female identity on the male response distances (as tested by a random effect in a mixed model), so we treated all observations (N=83) as equal. We constructed a generalized response curve using as an index the number of responses observed per number of males assumed available.

Results

Population divergence despite large scope for dispersal-mediated homogenization

We observed significant spatial structure in grasshopper coloration across the four different urban pavements, leading to local crypsis (Fig. 2A). Grasshoppers overall were more cryptic on their home pavement than they would be on other pavements (Fig. 2B; $N_{individuals} = 272$, $F_{1,536} = 282.4$, $p < 10^{-15}$). The same is true for asphalt, brown bricks, and grey bricks when tested separately (all $p < 0.0001$), and is replicated across different sections of pavement (Supplementary Fig. 3). The one exception was that grasshoppers observed on pale tiles would typically be more cryptic elsewhere (which may explain their lower density on pale tiles). Their persistence on this substrate despite poorer matching might be due to the relatively greater available surface area of this pale tiles substrate (Fig. 1B) and/or alternative benefits of pale substrate that offset lower crypsis (e.g., lower midday surface temperatures). Also comparing residents versus potential immigrants

(following Kawecki and Ebert 2004), resident grasshoppers overall were more cryptic than potential immigrant grasshoppers from other substrates would be (Fig. 2C, $N_{individuals} = 272$, $F_{1,850.5} = 66.8$, $p < 10^{-14}$). This resident advantage is most pronounced for grasshoppers from asphalt ($N_{individuals} = 41$, $F_{1,270} = 83.4$, $p < 10^{-15}$) but this time also true for grasshoppers from pale tiles ($N_{individuals} = 152$, $F_{1,270} = 65.4$, $p < 10^{-13}$).

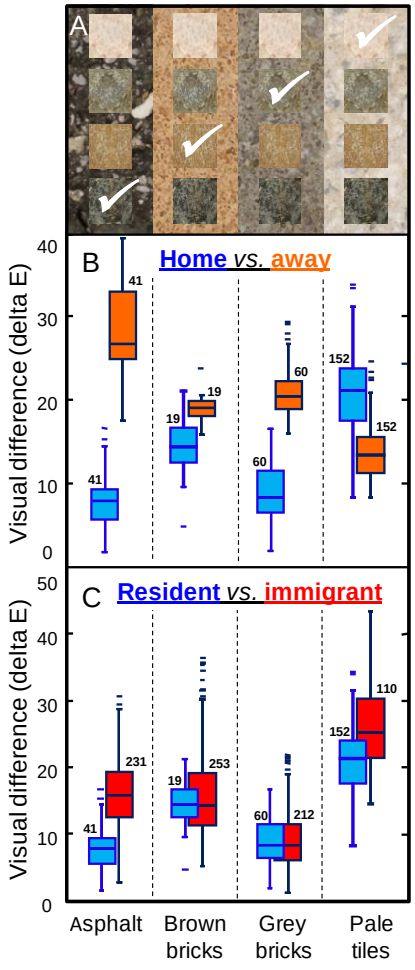

Fig. 2. Grasshoppers are more cryptic than expected by chance as they colonize novel, urban habitats. (A) Background images are representative of each of the four street habitats, with representative parts of four grasshopper individuals positioned on top (small square images of the thorax). For each individual (same individual per row) the white tick-mark indicates in which habitat it is most cryptic (i.e. lowest visual distance). (B) Comparing crypsis in own versus other habitats. Blue box plots: observed visual differences between color of the grasshoppers and color of their local habitats (home). Orange box plots: predicted visual differences if individuals were using the other three habitats in proportion to their availability (away). Visual differences are expressed as delta E (a quantification of color differences, see Methods) (C) Comparing crypsis between resident versus potential immigrant grasshoppers. Blue box plots: observed visual differences, identical to B (local residents). Red box plots: predicted visual differences if all the grasshoppers from the other three habitats would use the focal habitat (potential immigrants). Sample sizes are given for each box plot; $N_{total} = 272$. All comparisons are significant at $p < 0.0001$, except resident vs. immigrant for brown bricks ($p=0.35$) and grey bricks ($p=0.74$). Tukey-type box plots: middle line = median; box = central 50% of values = interquartile range; whiskers = highest and lowest value within 1.5*interquartile range; dashes = values within 3*interquartile range.

This microgeographic variation in grasshopper color is surprising, because the grasshoppers are highly mobile. Analysis of mark-resighting data showed that the

grasshoppers move on average 12.3 meter/day (95% CI 6.1-24.9; Supplementary Fig. 4), which exceeds the spatial grain of substrate heterogeneity. Spatially-explicit simulations predict that at this rate, random movement across the heterogeneous urban landscape should prevent or rapidly erode local cryptic coloration (Fig. 3). Yet, local crypsis persists despite the observed movements, suggesting that spatial structure must be maintained by one or more powerful processes.

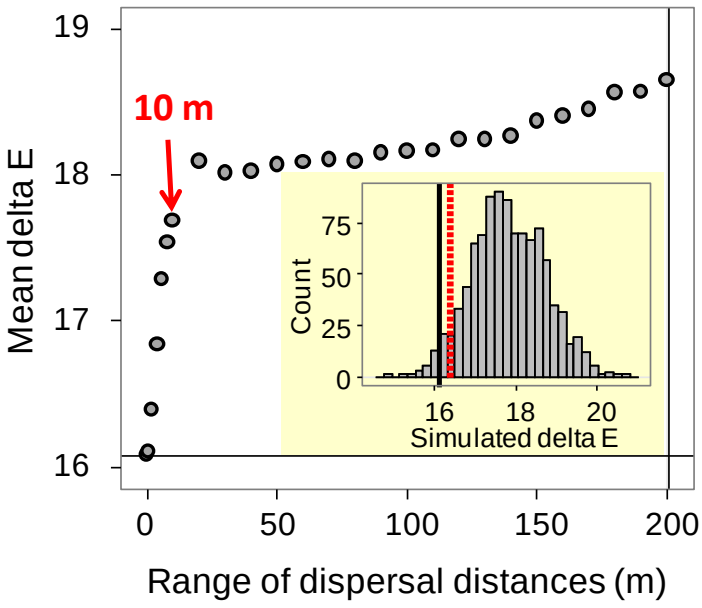

Fig. 3. Local similarity in color rapidly decreases with increasing movement distances, if these movements are random. Main panel: as grasshoppers (N=72) are simulated to move greater distances in random directions, average local crypsis (grey dots, expressed in delta E) rapidly decreases and starts to asymptote when the range of allowed dispersal distances (a flat distribution) has a maximum of 10 meters (i.e. a mean of 5 meters). Observed movement in the field (Supplementary Fig. 4) was on average 12.3 meters per day. The horizontal line indicates the average delta E as observed in the field. The vertical line indicates the greatest dispersal distance recorded in the field (biased downward, because of relatively small size of study area). The plotted results (mean over N=1,000 simulation runs) are highly accurate: an independent replicate (N=1,000 runs) yielded nearly indistinguishable values (not shown), so the irregularity in the relationship is due to the use of the exact spatial configuration of the study site for the simulations. Inset: histogram of resulting mean delta E values of 1,000 simulation runs, using the observed average movement per day (12.3 meters) as the upper limit of the flat distribution from which to sample random dispersal distances. Even with this conservative setting, the observed delta E from the field (vertical black line) is smaller ($p=0.022$) than the lower 5% limit of simulated values (red dotted line): local similarity is significantly reduced already after one day of random movement.

Little to no support for natural selection on color and plasticity of color as drivers of trait-environment matching

Ongoing divergent natural selection on grasshopper color (e.g. by selective predation) would classically be the default hypothesis to explain apparent local adaptation (crypsis) despite high potential for gene flow (Wang and Bradburd 2014). However, we obtained several lines of evidence which indicate that natural selection on crypsis is currently weak or even absent in the urban setting. Simulations showed that natural selection on grasshopper color would have to be unrealistically strong to maintain the observed microgeographic divergence in the face of random movement (e.g., mortality on asphalt would have to be at least 77-87% per day to explain observed spatial structure: Supplementary Fig. 5). Such very strong selection is inconsistent with the low observed 3.8% daily mortality rate in the field (state-dependent capture-recapture model, 95% CI = 2.9-5.2%, N=272). These estimated mortality rates did not differ between substrates conferring higher versus lower crypsis (confidence intervals were nearly identical). Lastly, we found negligible daily predation rates on dead grasshoppers that we placed on the urban substrates, in contrast to high predation on those at a natural site (4.4% vs. 40.0% respectively; N=90, binomial model: p=0.00007). With such low predation in the urban habitats (where grasshopper predators appear sparse), the opportunity for selective mortality to drive crypsis is very low, in stark contrast to rural habitats.

Another classical explanation, rapid phenotypic plasticity (color change to match the utilized pavement), is also insufficient to explain observed color divergence. First, color differences among grasshoppers from the field are heritable in a common laboratory environment (brightness: $\chi^2_1=10.5$, p=0.0012; red versus blue reflectance: $\chi^2_1=15.4$, $p < 0.0001$; Supplementary Fig. 6). Second, plasticity of color in adults is two orders of magnitude too slow/weak to maintain color matching if their movements were random with respect to habitat (Supplementary Fig. 7). Additionally, plasticity is unidirectional: adults only

darkened and did not lighten, so plasticity could not generate the observed crypsis on pale tiles (Fig. 2C).

Support for matching habitat choice

With contemporary natural selection and phenotypic plasticity eliminated as plausible drivers of the observed local crypsis, a remaining explanation is habitat choice. We experimentally confirmed this inference using laboratory and field experiments. In a laboratory substrate choice experiment, adult grasshoppers that were painted darker made a greater use of dark habitat than grasshoppers painted paler (Fig. 4A; N = 30, $\chi = 2.58$, p = 0.0098). The same effect was observed in grasshoppers darkened via corazonin hormone injection (54% darker, $p<0.00001$; Fig. 4B), which also used the dark habitat more (Fig. 4B; N=52, $p < 10^{-6}$). Importantly, this effect was also seen in our field site (Fig. 4C): after release, corazonin-darkened grasshoppers predominantly used the dark asphalt habitat (70.4%, N=17), whereas pale control grasshoppers predominantly used the adjacent pale substrates (71.4%, N=14; p = 0.0017). This strong habitat divergence occurred in a single day, too fast for selective predation to have much impact (especially given the observed negligible mortality rates).

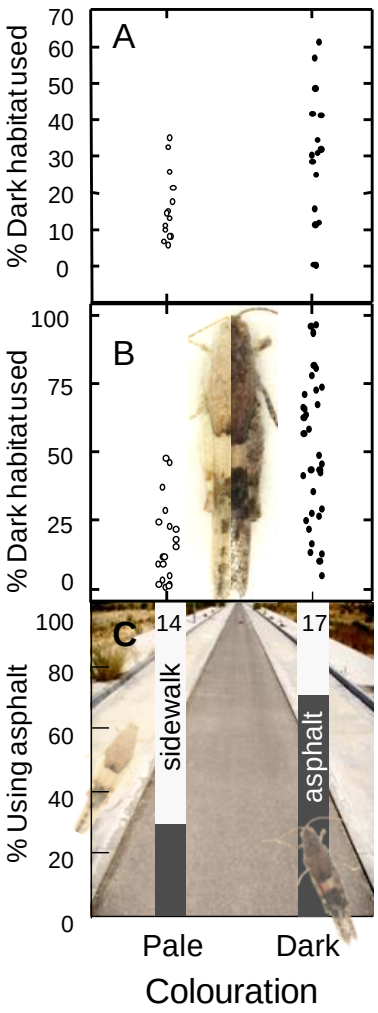

Fig. 4. Manipulating the phenotype of grasshoppers causes them to change habitat use in the expected direction. (A) Grasshoppers (N=30) painted dark (dark dots, right) are found more often on the dark laboratory habitat than grasshoppers painted pale (pale dots, left). (B) Grasshoppers (N=52) injected with the hormone corazonin (right half of image and dark dots) become darker and are found on the dark laboratory habitat more often than untreated, pale grasshoppers (left half of image and pale dots). (C) Grasshoppers darkened by corazonin (right individual, N=17) are mostly recaptured on dark asphalt, whereas pale control individuals (left individual, N=14) are mostly recaptured on pale parking spaces and sidewalks. Background image: the dark asphalt road bordered by pale parking spaces and sidewalks.

Consequence of habitat choice for population structuring

The experimentally demonstrated habitat choice can be very effective at generating fine-scale divergence among different pavements in the genes that underlie heritable color variation, despite very high opportunity for gene flow. This divergence can be strengthened by spatially structured assortative mating. Male grasshoppers readily approach any moving female for mating, but their response rapidly declined with increasing distance from the female, and females more than four meters away were no longer detected (Fig. 5). This spatial restriction on mate finding, set within the fine-scale urban landscape (Fig. 1 and 4C), should indirectly induce positive assortative mating between individuals with similar phenotypes (and genotypes), because they choose to utilize similar pavements and are therefore more likely to encounter one another.

Discussion

We found microgeographic spatial structure in heritable grasshopper color in recently colonized adjacent but distinctly-colored urban habitats (Fig. 2B,C). The spatial variation in color increases grasshopper's similarity to their background (Fig. 2A), and hence appears to improve crypsis (see Edelaar et al. 2017a; Baños-Villalba et al. 2018 for supporting evidence). Typically, such crypsis would be attributed to divergent natural selection due to predators removing individuals with mismatched colors from the different substrates (Rowell 1972, Cox and Cox 1974). Yet, we found no evidence this crypsis was driven by present-day selection, as observed mortality was far too low to achieve the degree of observed crypsis in the face of such high potential gene flow. Also, mortality was not higher for mismatched individuals. Nor is crypsis caused by color plasticity, which was slow and unidirectional (Fig. S7; see also Peralta-Rincon et al. 2017). Instead, grasshopper color differences between urban substrates are maintained by habitat choice. This choice is self-referential: when we experimentally alter grasshopper color (Fig. 4) they change their substrate use accordingly. Thus, these grasshoppers can evaluate their own crypsis against a substrate, and act accordingly.

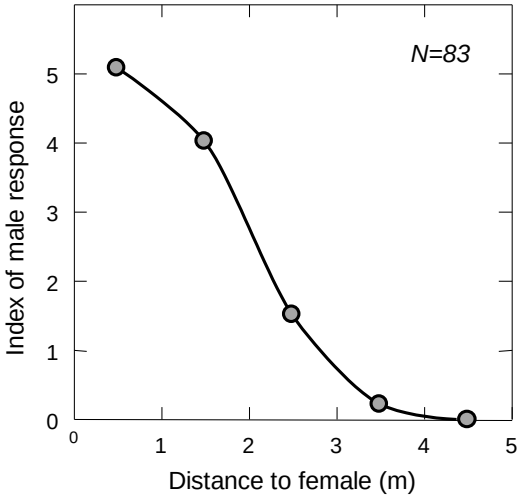

Fig. 5. Greater distance between potential partners reduces likelihood of mating. Male response (N=83 observations) to moving females rapidly declines with increasing distances, and is absent at distances greater than 4 meters. This should favor mating between individuals sharing the same pavement type. Since pavement is selected as a function of an individual´s phenotype (see main text), this should then indirectly favor positive assortative mating between individuals with similar phenotypes. This small spatial scale of mate finding and assortative mating supports an interpretation of the distinct pavements of being somewhat independent populations with a reduced level of gene flow among them.

Such self-referential habitat choice may be facilitated by the protruding round eyes and mobile heads of grasshoppers (Fig. 1A), allowing them to view, compare and evaluate the color of their body relative to that of the substrate. As the average use of dark substrate by darkened individuals was sometimes below parity (Fig. 4A) or above parity (Fig. 4C), it does not seem that darkening just resulted in random substrate use. Color-dependent substrate choice was previously demonstrated for some grasshoppers, but only in lab settings (Gillis 1982, Karpestam et al. 2012). The continuous variation in color present in our colonizing grasshoppers can be explained by the diverse soil colors of the nearest natural habitat (Fig. 1B). By coincidence, the colors of the urban substrates newly made available coincided with the range of colors of the grasshoppers (Fig. 2A): if the urban substrates were very differently colored, we predict that the grasshoppers would have avoided the novel environments in order to maintain crypsis.

Our results provide a strong combination of observational and experimental evidence for the hypothesis that the colonization of and adaptation to urban habitats is enhanced by specific individuals actively preferring certain urban habitats. Theory has long suggested that biased dispersal can drive population genetic structure and adaptation to different environments in general (Maynard Smith 1966, Ronce 2007, Edelaar et al. 2008, Armsworth 2009, Kerr and Godfrey-Smith 2009, Ravigné et al. 2009, Edelaar and Bolnick 2012, Bolnick and Otto 2013, Berdahl et al. 2015, Berner and Thibert-Plante 2015). While several recent studies have drawn attention to the possible role of biased dispersal in the colonization of urban habitats (Carrete and Tella 2010, Sol et al. 2013), we know of no previous studies where all alternative hypotheses were tested, as we did here. In doing so, we found negligible support for effects of present-day natural selection and plasticity. This is not to say that in general these alternative drivers play a minor role during the colonization of urban habitats, and several studies have found evidence that does support their operation (Cheptou et al. 2008, Sih et al. 2011, Lowry et al. 2013, Miranda et al. 2013, Alberti et al. 2017). Nonetheless, our study not only provides some of the first evidence that biased dispersal contributes to colonization and evolution in urban environments, it also shows that its contribution can be large, and even dominant. Even in natural settings few if any studies have simultaneously measured the effects of selection, plasticity, and

habitat choice. Our empirical results suggest that pre-existing habitat choice and dispersal behavior (put in place by previous selection) may generally play a key role in adaptation to new environments, as long anticipated by theory.

Habitat choice has its limitations (e.g. access to distinct habitats, and costs and limits to movement and choice: Bonte et al. 2012; Edelaar et al. 2017b), but is favored when individuals have easy access to more than one type of environment. It is therefore expected to act especially at smaller spatial scales (relative to individual movements: Richardson et al. 2014), where divergent natural selection would need to be prohibitively strong in order to create divergence in the face of gene flow (Supplementary Fig. 5). In that sense, habitat choice is complementary to natural selection. Habitat choice may also play a similar role to natural selection in a range of major topics in ecology and evolution, such as the maintenance of genetic variation (directly by matching distinct genotypes to environments favorable to them, and indirectly by favoring positive assortative mating) or the evolution of reproductive isolation (Levene 1953, Hedrick 2006, Armsworth 2009, Webster et al. 2012, Bolnick and Otto 2013, Wang and Bradburd 2014, Berner and Thibert-Plante 2015, Jacob et al. 2015). However, habitat choice could do so much faster than natural selection, at finer spatial scales, and at a smaller demographic cost (Barton and Partridge 2000). Whether or not a local redistribution of genotypes across space should be seen as structuring populations or "just" as increasing local performance depends on one´s definition (or application thereof) of what constitutes a population (see Waples and Gaggiotti 2006). In our case, the observed small spatial scale of mate-searching (Fig. 5) increases the probability that a grasshopper will mate with an individual using the same type of pavement, and therefore with a similar color. This reduces gene flow among pavements, and increases homozygosity at the genes involved in coloration. As a result, grasshoppers on different urban pavements are arguably partly distinct genetic populations.

One of the attractive features of urban habitat is often the lower abundance of certain natural predators (Shochat et al. 2006, Sih et al. 2011). As an effect, urban populations often differ in behavior or other anti-predation traits (Carrete and Tella 2010, 2011, Lowry et al. 2013). In our study, predation risk also appears much weaker in urban than in natural habitat, which will benefit urban

colonization. Nonetheless, the grasshoppers still preferred those urban pavements that provided them with greater crypsis. Most likely they do so because adaptive habitat choice previously evolved in their natural habitats where selection for crypsis is stronger (Rowell 1972, Gillis 1982, Forsman et al. 2011), and this behavior persists in the urban environment even if it has lost most of its functional value. Although we did not quantify this, habitat choice may even be somewhat maladaptive, as the selective avoidance of specific habitats may restrict access to resources or impose other forms of stress. For example, dark individuals restricting themselves to using dark asphalt roads and avoiding the adjacent pale sidewalks may feel safer, but have to deal with higher surface temperatures (>50 degrees Celsius), have lower access to a novel food resource (dog faeces) which is found more on sidewalks than on asphalt roads, and have lower access to the scarce plants providing nutrients and water. Hence, this can be seen as a form of an ecological trap (which occurs when animals mistakenly prefer habitats where their fitness is lower than in other available habitats following rapid environmental change; Gilroy and Sutherland 2007; Hale and Swearer 2016), but then applied at the individual level instead of at the population/species level.

In conclusion, we report some of the first evidence that biased dispersal due to habitat choice can drive population divergence during the colonization of novel urban habitats. Moreover, in a rare comparison of alternative drivers, we even find it is a dominant force. While the process is often neglected, biased dispersal due to habitat choice can help explain why certain species move into urban environments and others do not, how divergence between urban and rural populations can arise, and even how divergence within the urban setting can originate and be maintained. More generally, this study reinforces that improved local performance and adaptive evolution can result from the active and adaptive spatial redistribution by genotypes, even when natural selection is currently not acting. This biased dispersal can even be the main driver, as shown here. We propose that eco-evolutionary studies (as do related fields: Child 1997; Johnson 2007) more fully incorporate the independent consequences that individuals are not only selected upon by the environment, but also are selectors of their own environments.

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Author contributions

P.E. planned and coordinated the study, and wrote the first and final draft of the paper. A.B.-V. and P.E. designed experiments, collected many of the data, and performed the simulations and most data analyses. D.Q.-C. and A.J.-A. designed a part of the experiments and collected and analyzed data. G.E. helped to design some experiments, took care of animals and logistics and collected part of the data. D.I.B. helped with project design, data interpretation, simulation of selective mortality, and writing. All authors participated in improving the manuscript.

References

Akcali, C. K. and Porter, C. K. 2017. Comment on Van Belleghem et al. 2016: Habitat choice mechanisms in speciation and other forms of diversification. - Evolution (N. Y). 71: 2754–2761.

Alberti, M., Correa, C., Marzluff, J. M., Hendry, A. P., Palkovacs, E. P., Gotanda, K. M., Hunt, V. M., Apgar, T. M. and Zhou, Y. 2017. Global urban signatures of phenotypic change in animal and plant populations. - Proc. Natl. Acad. Sci.: 201606034.

Armsworth, P. R. 2009. Conditional dispersal, clines, and the evolution of dispersiveness. - Theor. Ecol. 2: 105–117.

Baños-Villalba, A., Quevedo, D. P. and Edelaar, P. 2018. Positioning behavior according to individual color variation improves camouflage in novel habitats. - Behav. Ecol. in press.

Barton, N. and Partridge, L. 2000. Limits to natural selection. - BioEssays 22: 1075–1084.

Barton, N. H., Briggs, D. E. G., Eisen, J. A., Goldstein, D. B. and Patel, N. . 2007. Evolution. - Cold Spring Harbor Laboratory Press.

Berdahl, A., Torney, C. J., Schertzer, E. and Levin, S. A. 2015. On the evolutionary interplay between dispersal and local adaptation in heterogeneous environments. - Evolution (N. Y). 69: 1390–1405.

Berner, D. and Thibert-Plante, X. 2015. How mechanisms of habitat preference evolve and promote divergence with gene flow. - J. Evol. Biol. 28: 1641–1655.

Bolnick, D. I. and Otto, S. P. 2013. The magnitude of local adaptation under genotype-dependent dispersal. - Ecol. Evol. 3: 4722–4735.

Bonte, D., Van Dyck, H., Bullock, J. M., Coulon, A., Delgado, M., Gibbs, M., Lehouck, V., Matthysen, E., Mustin, K., Saastamoinen, M., Schtickzelle, N., Stevens, V. M., Vandewoestijne, S., Baguette, M., Barton, K., Benton, T. G., Chaput-Bardy, A., Clobert, J., Dytham, C., Hovestadt, T., Meier, C. M., Palmer, S. C. F., Turlure, C. and Travis, J. M. J. 2012. Costs of dispersal. - Biol. Rev. 87: 290–312.

Börger, L. and Fryxell, J. 2012. Quantifying individual differences in dispersal using net squared displacement. - In: Dispersal Ecology and Evolution. pp. 222–230.

Brans, K. I., Jansen, M., Vanoverbeke, J., Tüzün, N., Stoks, R. and De Meester, L. 2017. The heat is on: Genetic adaptation to urbanization mediated by thermal tolerance and body size. - Glob. Chang. Biol. in press.

Calderone, J. B. and Jacobs, G. H. 2003. Spectral properties and retinal distribution of ferret cones. - Vis. Neurosci. 20: 11–17.

Carrete, M. and Tella, J. L. 2010. Individual consistency in flight initiation distances in burrowing owls: a new hypothesis on disturbance-induced

habitat selection. - Biol. Lett. 6: 167–170.

Carrete, M. and Tella, J. L. J. 2011. Inter-individual variability in fear of humans and relative brain size of the species are related to contemporary urban invasion in birds (FR Adler, Ed.). - PLoS One 6: e18859.

Cheptou, P.-O., Carrue, O., Rouifed, S. and Cantarel, A. 2008. Rapid evolution of seed dispersal in an urban environment in the weed Crepis sancta. - Proc. Natl. Acad. Sci. 105: 3796–3799.

Child, J. 1997. Strategic choice in the analysis of action, structure, organizations and environment: Retrospect and prospect. - Organ. Stud. 18: 43–76.

Cox, G. W. and Cox, D. G. 1974. Substrate color matching in the grasshopper, Circotettix rabula (Orthoptera: Acrididae). - Gt. Basin Nat. 34: 60–70.

Darwin, C. 1859. On the origins of species by means of natural selection. - London: Murray: 247.

Davis, J. M. and Stamps, J. A. 2004. The effect of natal experience on habitat preferences. - Trends Ecol. Evol. 19: 411–416.

Edelaar, P. and Bolnick, D. I. 2012. Non-random gene flow: An underappreciated force in evolution and ecology. - Trends Ecol. Evol. 27: 659–665.

Edelaar, P., Siepielski, A. M. and Clobert, J. 2008. Matching habitat choice causes directed gene flow: A neglected dimension in evolution and ecology. - Evolution (N. Y). 62: 2462–2472.

Edelaar, P., Jovani, R. and Gomez-Mestre, I. 2017a. Should I Change or Should I Go? Phenotypic Plasticity and Matching Habitat Choice in the Adaptation to Environmental Heterogeneity. - Am. Nat.: 000–000.

Edelaar, P., Baños-Villalba, A., Escudero, G. and Rodríguez-Bernal, C. 2017b. Background colour matching increases with risk of predation in a colour-changing grasshopper. - Behav. Ecol. 20: 65–71.

Ellis, E. C., Goldewijk, K. K., Siebert, S., Lightman, D. and Ramankutty, N. 2010. Anthropogenic transformation of the biomes, 1700 to 2000. - Glob. Ecol. Biogeogr. 19: 589–606.

Estes, S. and Arnold, S. J. 2007. Resolving the Paradox of Stasis: Models with Stabilizing Selection Explain Evolutionary Divergence on All Timescales. - Am. Nat. 169: 227–244.

Faillace, C. A. and Morin, P. J. 2016. Evolution alters the consequences of invasions in experimental communities. - Nat. Ecol. Evol. 1: 13.

Forsman, A., Karlsson, M., Wennersten, L., Johansson, J. and Karpestam, E. 2011. Rapid evolution of fire melanism in replicated populations of pygmy grasshoppers. - Evolution (N. Y). 65: 2530–2540.

Gillis, J. E. E. 1982. Substrate colour-matching cues in the cryptic grasshopper Circotettix rabula rabula (Rehn & Hebard). - Anim. Behav. 30: 113–116.

Gilroy, J. J. and Sutherland, W. J. 2007. Beyond ecological traps: perceptual errors and undervalued resources. - Trends Ecol. Evol. 22: 351–356.

Hale, R. and Swearer, S. E. 2016. Ecological traps: current evidence and future directions. - Proc. R. Soc. London B Biol. Sci. 283: 20152647.

Hart, N. S. 2001. The Visual Ecology of Avian Photoreceptors. - Prog. Retin. Eye Res. 20: 675–703.

Hedrick, P. W. 2006. Genetic Polymorphism in Heterogeneous Environments: The Age of Genomics. - Annu. Rev. Ecol. Evol. Syst. 37: 67–93.

Holt, R. D. 1987. Population dynamics and evolutionary processes: the manifold roles of habitat selection. - Evol. Ecol. 1: 331–347.

Jacob, S., Bestion, E., Legrand, D., Clobert, J. and Cote, J. 2015. Habitat matching and spatial heterogeneity of phenotypes: implications for metapopulation and metacommunity functioning. - Evol. Ecol. 29: 851–871.

Jaenike, J. and Holt, R. D. 1991. Genetic variation for habitat preference: evidence and explanations. - Am. Nat. 137: S67–S90.

Johnson, W. 2007. Genetic and environmental influences on behavior: Capturing all the interplay. - Psychol. Rev. 114: 423–440.

Jones, J. S. and Probert, R. F. 1980. Habitat selection maintains a deleterious allele in a heterogeneous environment. - Nature 287: 632–633.

Karpestam, E., Wennersten, L. and Forsman, A. 2012. Matching habitat choice by experimentally mismatched phenotypes. - Evol. Ecol. 26: 893–907.

Kawecki, T. J. and Ebert, D. 2004. Conceptual issues in local adaptation. - Ecol. Lett. 7: 1225–1241.

Kerr, B. and Godfrey-Smith, P. 2009. Generalization of the price equation for

evolutionary change. - Evolution (N. Y). 63: 531–536.

Klein, C. A. 2013. Selection and adaptation during metastatic cancer progression. - Nature 501: 365–372.

Laland, K. N. and Sterelny, K. 2006. Perspective: seven reasons (not) to neglect niche construction. - Evolution 60: 1751–62.

Lebreton, J. D. and Pradel, R. 2002. Multistate recapture models: Modelling incomplete individual histories. - J. Appl. Stat. 29: 353–369.

Lenormand, T. 2002. Gene flow and the limits to natural selection. - Trends Ecol. Evol. 17: 183–189.

Levene, H. 1953. Genetic Equilibrium When More Than One Ecological Niche is Available. - Am. Nat. 87: 331–333.

Lovell, P. G., Ruxton, G. D., Langridge, K. V. and Spencer, K. A. 2013. Egg-laying substrate selection for optimal camouflage by quail. - Curr. Biol. 23: 260–264.

Lowry, H., Lill, A. and Wong, B. B. M. 2013. Behavioural responses of wildlife to urban environments. - Biol. Rev. 88: 537–549.

Maynard Smith, J. 1966. Sympatric speciation. - Am. Nat. 100: 637–650.

McKinney, M. L. 2002. Urbanization, Biodiversity, and Conservation. - Bioscience 52: 883.

Miranda, A. C., Schielzeth, H., Sonntag, T. and Partecke, J. 2013. Urbanization and its effects on personality traits: A result of microevolution or phenotypic plasticity? - Glob. Chang. Biol. 19: 2634–2644.

Peralta-Rincon, J. R., Escudero, G. and Edelaar, P. 2017. Phenotypic plasticity in color without molt in adult grasshoppers of the genus Sphingonotus (Acrididae: Oedipodinae). - J. Orthoptera Res. 26: 21–27.

R Core Team 2017. R: A Language and Environment for Statistical Computing. - R Found. Stat. Comput.: {ISBN} 3-900051-07-0, http://www.R-project.org.

Ravigné, V., Dieckmann, U. and Olivieri, I. 2009. Live Where You Thrive: Joint Evolution of Habitat Choice and Local Adaptation Facilitates Specialization and Promotes Diversity. - Am. Nat. 174: E141–E169.

Richardson, J. L., Urban, M. C., Bolnick, D. I. and Skelly, D. K. 2014. Microgeographic adaptation and the spatial scale of evolution. - Trends Ecol. Evol. 29: 165–176.

Ronce, O. 2007. How Does It Feel to Be Like a Rolling Stone? Ten Questions About Dispersal Evolution. - Annu. Rev. Ecol. Evol. Syst. 38: 231–253.

Rose, M. R. and Lauder, G. V 1996. Adaptation. - Academic Press.

Rowell, C. H. F. 1972. The Variable Coloration of the Acridoid Grasshoppers. - Adv. In Insect Phys. 8: 145–198.

Sala, O. E., Stuart Chapin, F., III, Armesto, J. J., Berlow, E., Bloomfield, J., Dirzo, R., Huber-Sanwald, E., Huenneke, L. F., Jackson, R. B., Kinzig, A., Leemans, R., Lodge, D. M., Mooney, H. A., Oesterheld, M., Poff, N. L., Sykes, M. T., Walker, B. H., Walker, M. and Wall, D. H. 2000. Global biodiversity scenarios for the year 2100. - Science (80-.). 287: 1770–1774.

Scheiner, S. M. 2016. Habitat Choice and Temporal Variation Alter the Balance between Adaptation by Genetic Differentiation, a Jack-of-All-Trades Strategy, and Phenotypic Plasticity. - Am. Nat. 187: 633–646.

Schlichting, C. D. and Pigliucci, M. 1998. Phenotypic Evolution: A Reaction Norm Perspective.

Schneider, C. A., Rasband, W. S. and Eliceiri, K. W. 2012. NIH Image to ImageJ: 25 years of image analysis. - Nat. Methods 9: 671–675.

Shochat, E., Warren, P. S., Faeth, S. H., McIntyre, N. E. and Hope, D. 2006. From patterns to emerging processes in mechanistic urban ecology. - Trends Ecol. Evol. 21: 186–191.

Siddiqi, A., Cronin, T. W., Loew, E. R., Vorobyev, M. and Summers, K. 2004. Interspecific and intraspecific views of color signals in the strawberry poison frog Dendrobates pumilio. - J. Exp. Biol. in press.

Sih, A., Ferrari, M. C. O. and Harris, D. J. 2011. Evolution and behavioural responses to human-induced rapid environmental change. - Evol. Appl. 4: 367–387.

Sol, D., Lapiedra, O. and González-Lagos, C. 2013. Behavioural adjustments for a life in the city. - Anim. Behav. 85: 1101–1112.

Troscianko, J. and Stevens, M. 2015. Image calibration and analysis toolbox - a

free software suite for objectively measuring reflectance, colour and pattern. - Methods Ecol. Evol. 6: 1320–1331.

Tuomainen, U. and Candolin, U. 2011. Behavioural responses to human-induced environmental change. - Biol. Rev. 86: 640–657.

Vorobyev, M. and Osorio, D. 1998. Receptor noise as a determinant of colour thresholds. - Proc. R. Soc. London B Biol. Sci. in press.

Vorobyev, M., Brandt, R., Peitsch, D., Laughlin, S. B. and Menzel, R. 2001. Colour thresholds and receptor noise: Behaviour and physiology compared. - Vision Res. 41: 639–653.

Wang, I. J. and Bradburd, G. S. 2014. Isolation by environment. - Mol. Ecol. 23: 5649–5662.

Waples, R. S. and Gaggiotti, O. 2006. What is a population? An empirical evaluation of some genetic methods for identifying the number of gene pools and their degree of connectivity. - Mol. Ecol. 15: 1419–1439.

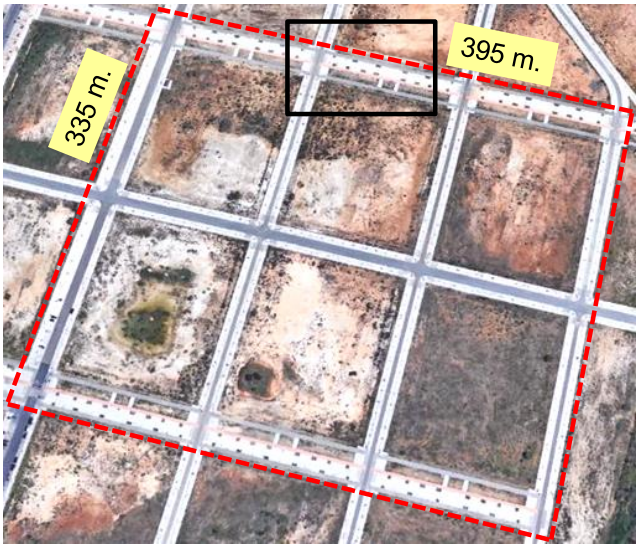
Webster, S. E., Galindo, J., Grahame, J. W. and Butlin, R. K. 2012. Habitat choice and speciation. - Int. J. Ecol. in press.

White, G. C. and Burnham, K. P. 1999. Program mark: Survival estimation from populations of marked animals. - Bird Study 46: S120–S139.

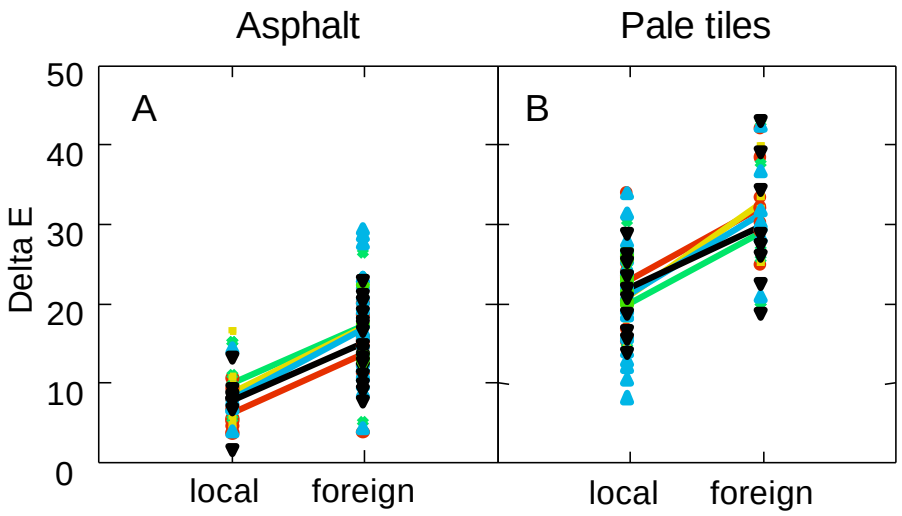
Yerushalmi, Y. and Pener, M. P. 2001. The response of a homochrome grasshopper, Oedipoda miniata, to the dark-colour-inducing neurohormone (DCIN) of locusts. - J. Insect Physiol. 47: 593–597.

Supporting information

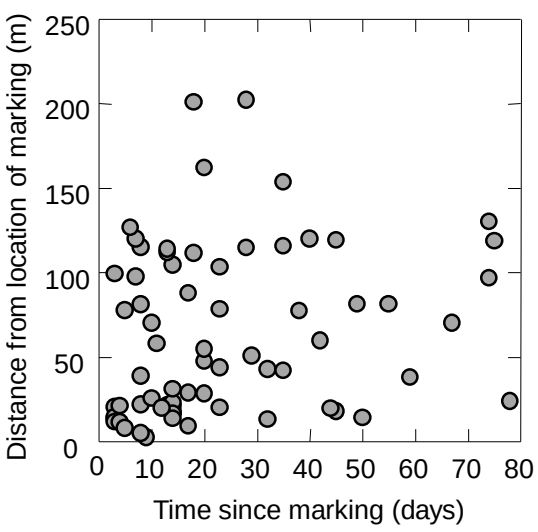
Supplementary Figure 1. A grasshopper in its novel urban habitat (side walk made of pale tiles). This illustrates its ground-perching behavior (red arrow), and the presence of some plants and dog faeces as food sources (black arrows).



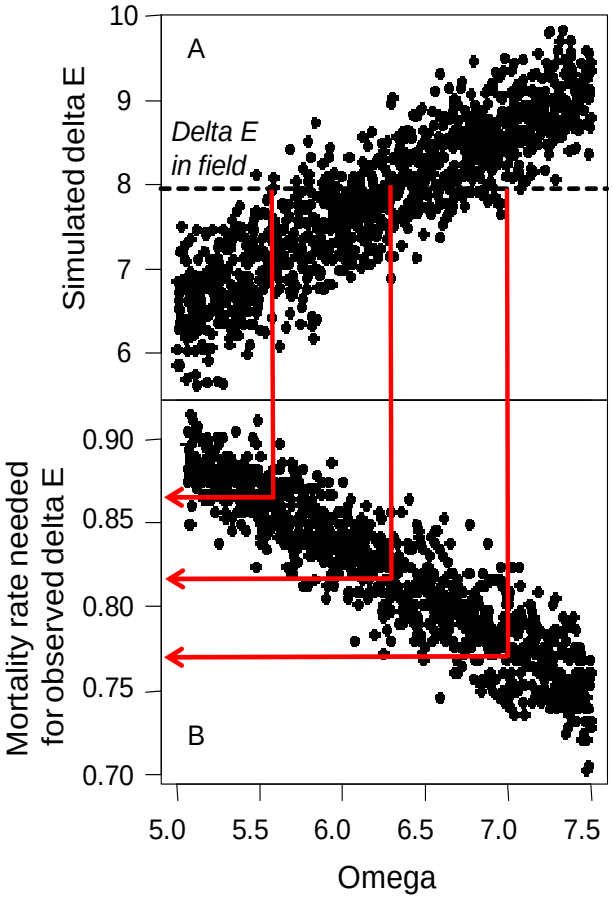
Supplementary Figure 2. Spatial configuration of the four different linear urban habitats (pavements). Pavements lie in-between large square, partly unvegetated areas containing natural brown, pale grey and some dark grey soils. Grasshoppers on the pavements present within the red dotted area have been photographed, marked and studied for movement and survival; the black rectangle is the area magnified for Fig. 2 of the main text.



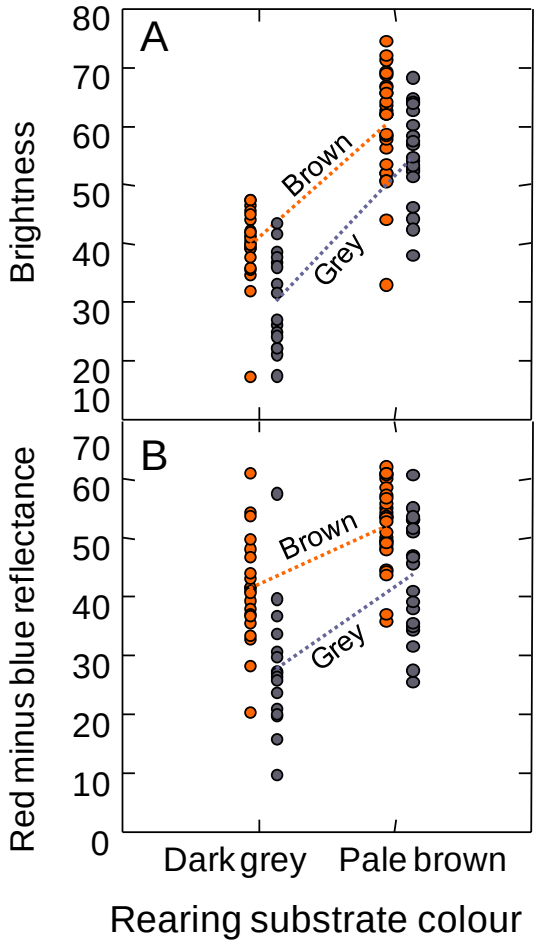
Supplementary Figure 3. Divergence in grasshopper color is replicated. In different streets (N=5) (each given a different color in the plot) with asphalt bordered by pale tiles (see Supplementary Figure 2, Fig. 6C), individuals are more cryptic in their local habitat than in the alternative habitat, just a few meters away.



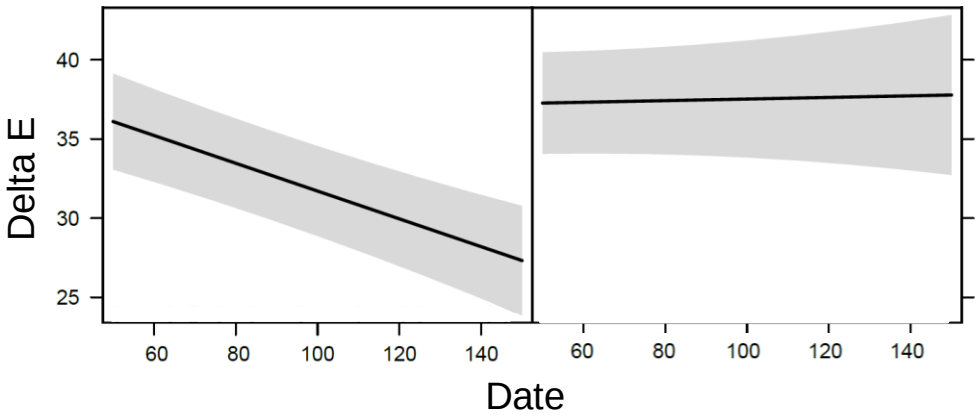
Supplementary Figure 4. Net distances moved by grasshoppers between consecutive sightings. Data (N=72 movements) were modeled as explained in the Methods using log transformation. In that model, the slope was significantly different from zero but not significantly different from one (0.88 ± 0.23 SE), meaning that individuals conform to the model assumption that they behave as freely moving Brownian particles without indications of having a home range or external boundaries to movement. Our estimate of a constant average movement of 12.3 meters/day (based on the log-log model) fits well with the untransformed distances as observed within the first days after capture as shown here.



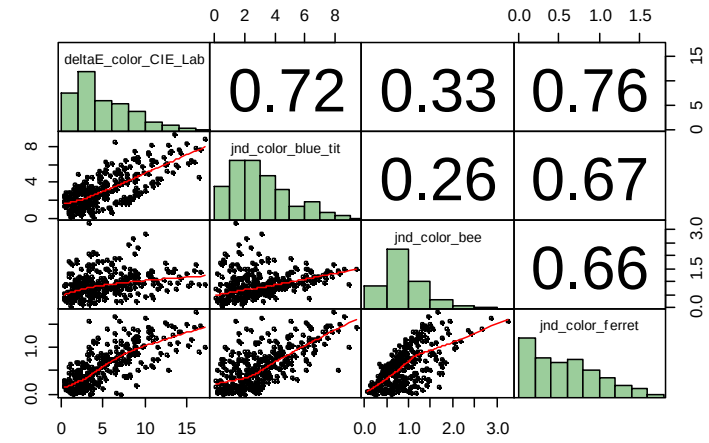
Supplementary Figure 5. Derivation of the rate of selective mortality needed to obtain an observed average delta E for a specific pavement type. We show the derivation for the asphalt pavement. (A) The obtained delta E is smaller for smaller values of omega (ω), which implies stronger stabilizing selection. The horizontal dotted line indicates the average delta E for grasshoppers observed on asphalt, which can be caused by a range of values of ω (vertical red lines). (B) Lower, average and upper ω values that could achieve the observed delta E in (A) are transformed into the expected mean mortality rate this would inflict on the population (horizontal red lines), here on average about 82% (and always larger than 70%). For each data point selective mortality acting on our entire sample of grasshoppers from the field (N=272) grasshoppers was simulated.



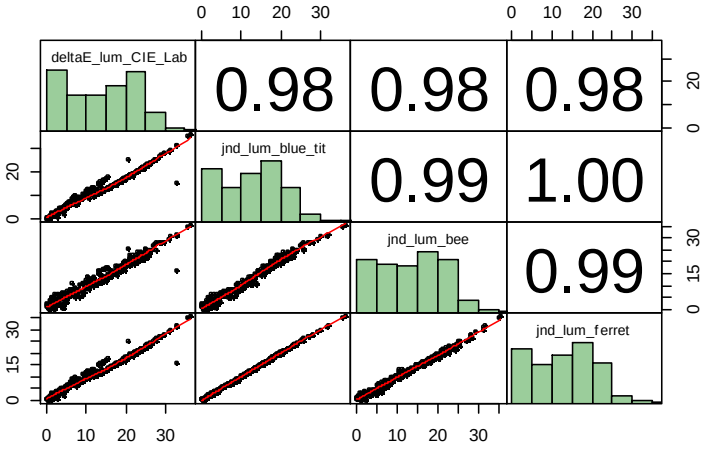
Supplementary Figure 6. Adult grasshopper coloration has both an environmental and a heritable component. Both for (A) overall brightness and (B) the difference between the amount of red and blue reflectance of the adult offspring (N=85) there is a strong effect of the color of the rearing substrate color (paler, and redder, when reared on pale reddish-brown substrate, which increases crypsis). However, there is also a large effect of the color of the parents: when the parents were pale reddish-brown, the offspring was also consistently paler (A: $\chi^2_1=10.5$, $p=0.0012$) and redder (B: $\chi^2_1=15.4$, $p < 0.0001$), in both environments. The most parsimonious interpretation is that coloration has a genetic basis (although non-genetic effects are not yet fully excluded). Shown are the values for each individual offspring. Dot color and labels in the figure indicates the color of the parents; dotted lines are least squares regression lines only fitted to help the eye.



Supplementary Figure 7. Degree and direction of plasticity in coloration for adults exposed to contrasting environments. Adults exposed to a black substrate (N=20) slowly reduced their visual distance to the background, at a rate of 0.088 delta E/day, whereas adults exposed to a white substrate (N=20) basically retained their visual distance with time (0.005 delta E/day increase). Hence, all adults became darker with age, almost imperceivably on white substrates, and a bit more but still very slowly on black substrates (to change one unit of delta E takes on average 11.4 days). Shown are the fitted regression lines with their 95% confidence intervals. The difference between treatments was significant (interaction of date by color treatment: $t_{25.2} = 3.51$, $p=0.002$, N = 264 observations on 40 individuals). The results of this experiment coincide with our general experience with hundreds of wild-caught individuals brought into the laboratory, which show only slow color change, mostly gradually turning darker and a bit bluer with age.



Supplementary Figure 8. Comparison between distance in color for human vision-based CIE Lab color space, and JND units for color between individuals and the background in which they were found based on the visual systems of the bird *Cyanistes caeruleus*, the insect *Apis mellifera,* and the mammal *Mustela putorius*. Numbers in bold are the Pearson correlation coefficients, and the red line represent a Loess smooth of the data.



Supplementary Figure 9. Comparison between distance in luminosity for human vision-based CIE Lab color space, and JND units for color between individuals and the background in which they were found based on the visual systems of the bird *Cyanistes caeruleus*, the insect *Apis mellifera,* and the mammal *Mustela putorius*. Numbers in bold are the Pearson correlation coefficients, and the red line represent a Loess smooth of the data.

Supplementary Appendix I – Detailed Materials and Methods

Study species

The Azure Sand Grasshopper (*Sphingonotus azurescens:* Fig. 2A) is a 2.5 to 4 cm large grasshopper from the subfamily Oedipodinae. It is a colonizing species that normally lives on open (disturbed) sand or clay soils that may be mixed with stones. Individuals do not perch on plants as many other grasshopper species do, but sit and walk on the soil (Fig. 2A, Supplementary Figure 2). Here they feed on the few low herbs and grasses present, and also on dead invertebrates. They mainly displace by walking slowly, but fly well when disturbed or dispersing. Activity is limited to the hotter hours of the day, but when temperature becomes too high (most of the days during summer) individuals seek shade: at this time we would stop collecting data on habitat use. There is a single generation per year. Nymphs start appearing in early spring and develop into adults after 6 molts in about 6 weeks. Reproduction is mainly recorded in September and October so adult grasshoppers need to survive several weeks to months as non-reproducing adults. This would select for a high daily survival rate, which is aided by their cryptic overall coloration. This can differ tremendously among individuals and populations, varying continuously in darkness from very pale to almost black, and from bluish-grey to orange-brown (Fig. 2A, 4A). Grasshopper coloration typically resembles that of the local substrate on which they occur (Fig. 2A), a striking phenomenon that has also been recorded in many other species of the subfamily and by many observers (Rowell 1972). This has typically been assigned to adaptive developmental plasticity during development (homochromic response (Rowell 1972)), but we report here that color is also heritable and that habitat choice as a function of the phenotype occurs in adults, and results in the same pattern.

Study area

We studied the colonization of urban pavements by grasshoppers in a deserted housing development site between the towns of Montequinto and Dos Hermanas (province of Seville, Spain; 37.306 ºN, 5.932 ºE). Here large blocks of little-vegetated natural soils are subdivided by roads composed of four different types of pavement: asphalt roads, a bike path made of brown bricks, a foot path made

of grey bricks, and other surfaces and sidewalks made of pale tiles (Figure 2B,C; Supplementary Figure 3). We combined the areas of pale cement parking spaces along the asphalt road with the pale tile sidewalks because of their very similar coloration (Figure 5C). The area is fenced off to cars so there is very little traffic, although there are some people biking or walking their dogs. Because of the low level of use and maintenance of the area, some plants are growing in-between the bricks and tiles, and also between the asphalt road and the side walk, which provide food, water and shade to grasshoppers (see Supplementary Figure 2). Dog faeces are also used as novel food source (observed in the field and confirmed in the lab: average survival time without food was only 6.3 days, but with dog faeces this increased five-fold to 32 days, N=6 for each treatment). Grasshoppers are relatively common on these pavements, and clearly regard and use them as suitable alternatives to natural soils: adult males are commonly displaying, we have seen copulations and egg deposition (in-between tiles and between asphalt and parking spaces), and nymphs are common in spring. Nonetheless, grasshoppers are also common on the natural soils in-between the roads, and undoubtedly there is frequent and continuous interchange between both types of habitat during the entire season, i.e. the colonization of the urban pavements is ongoing. For this study we restricted ourselves to monitor an area of roads of 390 by 335 meters, which included 5 transects of asphalt/pale tiles and two transects of brown bricks/pale tiles/grey bricks (see Supplementary Figure 3).

Catch, mark and recapture in the study area

We systematically surveyed all areas of pavements in the study area (Supplementary Figures 2,3) for grasshoppers. We searched for perched or disturbed grasshoppers by walking slowly while swinging a capture net from left to right, thereby passing the net over all paved areas which should disturb all individuals present. Any grasshopper detected was captured with a net, also (if possible) if it escaped to natural soils. It was individually marked with a combination of three letters on the posterior part of both fore wings (which already have some irregular dark markings), using a black permanent marker pen (Staedtler permanent Lumocolor, resistant to water and UV light), allowing for subsequent visual tracking using binoculars with minimal disturbance. After marking, individuals were photographed (see below for details on color

measurement) and then released at the location of first encounter. For each individual we recorded sex, time of capture, type of substrate on which it was found, and the GPS location (afterwards corrected where necessary to coincide with capture substrate). The entire study area was revised for marked and new, unmarked adult grasshoppers ten times from June to October, covering the time period when adults are common, noting down the location and identity of any marked grasshoppers, and catching and marking unmarked grasshoppers.

Calculation of daily grasshopper movement

We followed (Börger and Fryxell 2012) in using Net Squared Displacement as a synthetic measure of animal movement rate. The net displacement is simply the Euclidean distance from start to end point(s). In an individual moving randomly at a constant rate, this is expected to increase with time. Therefore, the Mean Net Squared Displacement (MSD) is an exponential function of time multiplied by a diffusion constant: $MSD = Dt^{\alpha}$. In its double logarithmic form, this is a simple linear equation: log(MSD) = log(D) + αlog(t). We fitted our data (see Supplementary Figure 4) in this form by a mixed model, including individual identity to deal with the repeated measures of some individuals (it was not necessary to include sex) (lme4 code: model <- lmer(log.NSD ~ log.days + (1|individual), data = data)). We then back-transformed the model estimate for the diffusion parameter D (raising 10 to power of the estimated mean and its upper and lower 95% confidence intervals, then taking their square root). We omitted the value of one individual that moved more than 200 meters within one day (statistically detected outlier). Together with the likely detection bias against individuals that have moved greater distances back to natural soils or out of the study area, this means that our estimate of daily movement is biased downwards, and conservative with respect to our inferences.

Simulation of population homogenization with increasing movement

Movement is expected to homogenize populations and reduce divergence if movement is random, with greater effects as individuals move greater distances (Lenormand 2002, Richardson et al. 2014)To test if the average movement of grasshoppers as observed in the field should homogenize populations across the

four pavements, resulting in higher mean delta E values (delta E is a measure of visual distance between the colors of a grasshopper and a substrate; see below for color measurement and calculation of delta E), we simulated the spatially explicit effects of movement if it were random (i.e. no habitat choice). We did this with a custom code written in the R environment (R Core Team 2017). In the simulations, each individual for which we have recapture data (N=72) is simulated as starting at its original observation site (coordinates and type of pavement as recorded in the field), then moving in a random direction and distance. The random distance is drawn from a flat distribution ranging from zero to a certain maximum distance. (We did not use the empirical dispersal kernel because if there is habitat choice, this kernel will be biased. Furthermore, long-distance dispersal is under recorded as individuals leave the study area, introducing further bias. A flat distribution is intuitively easy to interpret, e.g. a flat distribution with a maximum distance of 20 meters yields an average dispersal distance of 10 meters.) Based on the spatial lay-out of the study area, this random movement dictates on which pavement the grasshopper arrives (only pavement within the study area is acceptable, otherwise the initial movement is repeated until accepted), after which we calculate this individual´s new delta E value. This is repeated for all individuals, and a new delta E value for the population is calculated. This sequence was repeated 1000 times (independent, uncorrelated runs), after which the average population-wide new delta E was calculated. This was done for a range of maximum distances of movement up to 200 meters (the maximum movement recorded in the field). This allowed us to plot how the observed local similarity in color disappears with increasing movement distances (Figure 3), if these movements are random.

Measurement of color and color distances

To quantify background color matching, grasshoppers and pavements were photographed in situ under controlled lighting conditions inside a black box and with a dual lamp Mecablitz 15MS-1 diffuser flash mounted on the lens. Photographs were taken with a Canon 1200D camera mounting a 18-55 mm Canon lens (locked at 55mm) using as fixed camera settings f/12 aperture, 1/50 shutter speed, ISO200. Pictures were taken in RAW format and included an 18% grey standard card. Aligned and normalized mean values of L*a*b in the CIE-Lab

color space were extracted from RAW files using the software Image J (Schneider et al. 2012) and the Mica Toolbox version 1.11, following Troscianko and Stevens (2015). For grasshoppers we measured color of a defined diamond-shaped area in the dorsal part of the metazone of the pronotum, which is representative for the overall body color. For the pavements we calculated the average color over images of five different sites per pavement type. We then calculated chromatic differences between grasshoppers and each of the four pavements by measuring the Euclidean distances between their L*a*b values (called "delta E"). Ideally, chromatic differences should be calculated for the visual system of the relevant predator. However, our grasshoppers might be predated on by a wide range of visual predators with very different visual systems, and in unknown proportions. We therefore used the same parsimonious route to obtain conservative estimates of delta E values as Lovell et al. (2013). We also compared the obtained delta E distances from the CIE-Lab color space which is based on human vision with distances using the visual systems of three potential predators; a bird (based on the Blue tit *Cyanistes caeruleus* (Hart 2001)), an insect (based on the Honey bee *Apis mellifera* (Vorobyev et al. 2001)) and a mammal (based on the Ferret *Mustela putorius* (Calderone and Jacobs 2003)). Since grasshoppers and backgrounds do not reflect ultraviolet radiation (as checked by spectrophotometry) we did not include the UV cone types of these predators. We quantified color contrasts between photon catches of grasshoppers and photon catches of backgrounds according to a log-linear form of the color discrimination model, which assumes that visual discrimination is limited by receptor noise (Vorobyev and Osorio 1998), and using a Weber fraction value of 0.05 for the most frequent cone type. We also quantified luminance contrasts using a version of the model based only on achromatic differences (based on Blue tit double cones and medium-wave cones for Honey bee and Ferret). These color and luminance contrasts were expressed in "just-noticeable-differences" (JND) whereby values between 1.0 and 3.0 indicate difficult discrimination, while values increasing above 3.0 indicate increasingly improved discrimination (Siddiqi et al. 2004). These JND values were compared with the color and luminosity distances of the CIE-Lab color space, using the L axis to calculate the luminosity distance, and the *a* and *b* axes for the color distance (Figures 8 and 9).

Testing for local adaptation in color

Following (Kawecki and Ebert 2004), we tested in two complementary ways for local adaptation. The home-away contrast (Figure 4B) tests for local adaptation from the view point of the individuals, whether the current habitat is the one providing a better match for them compared to other nearby habitats. This contrast is particularly suited to test if individuals have selected their personal best environment, thus representing a test of optimization. For this we compared for all individuals the delta E value for the habitat in which we first encountered them (observed delta E at home) with the average delta E value each individual would have if it used the other three available habitats in proportion to their availability (predicted delta E away). We tested in a mixed model the effect of the comparison class (home versus away) on delta E values, correcting for the mean differences between pavement types, and the interaction pavement type by comparison class (lme4 code: model <- lmer(deltaE ~ original.habitat + comparison + original.habitat*comparison + (1|individual), data = firstdata)). We included individual identity as a random effect to account for the two non-independent measurements we have of each individual (its delta E at home and its delta E away). We also tested with a mixed model for the comparison effect for each pavement separately (model <- lmer(deltaE ~ comparison + (1|individual), data = subset.habitat)). All residuals were approximately normally distributed. The resident-immigrant contrast (Figure 4C) tests for local adaptation from the view point of the local environment, whether the current individuals are the ones that match best with it. For this we compared the delta E values of all individuals first encountered on a focal pavement (observed delta E residents) with the delta E values that foreign individuals (first encountered on the other three pavements) would have if they were on the focal pavement (expected delta E immigrants). As above, we tested in a mixed model the effect of the comparison class (resident versus immigrant) on delta E values, correcting for the mean differences between pavement types, and the interaction pavement type by comparison class (model <- lmer(deltaE ~ focal.habitat + comparison + focal.habitat*comparison + (1|individual), data = firstdata)). We included individual identity as a random effect because for all individuals we have four observations (its delta E as a resident plus three times as an immigrant to the other three pavements). We also tested with a linear model for the comparison

effect for each pavement separately (model <- lm(deltaE ~ comparison, data = subset.habitat)) (without the random effect of individual as in those subsets there are no repeated measures). All residuals were approximately normally distributed.

Simulation of necessary mortality rates to obtain observed color similarity

To simulate similarity in color by natural selection alone (e.g. through selective predation on less cryptic individuals), we assumed that there is no adaptive plasticity, and no habitat choice. Initially, all marked and phenotyped grasshoppers from across the four pavement types are introduced onto a single focal pavement, as if settlement is completely random by phenotype. Next, stabilizing selection is exerted on these individuals, with fitness distributed normally around the optimum (delta E = 0), according to the standard function (see e.g. *53*): fitness = $\exp(-(\text{delta E})^2/(2\omega^2))$, where ω^2 is the variance of the fitness function (inversely related to the strength of stabilizing selection) and delta E is an individual´s measure of nonmatching in coloration with the focal pavement. Actual death or survival of each individual was subsequently stochastically determined by a draw from the binomial distribution, with a probability of survival equal to its relative fitness as calculated with the fitness function. By stepwise decreasing ω as the parameter of selection strength (N=1000 steps in the relevant range), we can increase the amount of selective mortality, which brings the population composed of surviving individuals closer to the optimum. We visually determined the range of probable values for selection strength ω that could have resulted in the observed mean delta E value for the focal habitat (see Supplementary Figure 6A), and then derived which mortality rates this implied (Supplementary Figure 6B).

Measuring predation rate with decoy grasshoppers

One way to assess the presence and impact of predators is to measure the removal rate of food items. Here we used dried, dead grasshoppers. These were kept in place on the street pavements with the aid of some Blue Tag poster fixing material. The next day we counted how many individuals were removed, presumably by predators. To differentiate removal by ants (which are not able to capture live grasshoppers) from removal by other, larger types of predators, we

attached a small metal ball to each grasshopper. Since ants dissemble their prey into smaller pieces for transport, these metal balls then stay behind (as tested with some dead grasshoppers placed next to ant colonies), whereas larger predators move the ball at least some distance during their attack. Indeed in a few cases of removed grasshoppers these balls were retrieved at the exact same spot, indicating predation by ants, and these cases were omitted from the total sample size. The experiment was repeated in a natural area elsewhere (where, amongst others, a high density of lizards was observed, in contrast to our urban pavements) to validate that visual predators indeed attack such fixed dead grasshoppers.

Measuring survival with multistate capture-recapture modeling

Capture-recapture models provide estimates of survival that take into account that detection probability for surviving individuals may not be 100%. We were interested to test whether natural selection (selective mortality) acts on crypsis. We therefore fitted multi-state capture-recapture models to our data on the individually marked but otherwise unmanipulated live grasshoppers using program Mark (White and Burnham 1999). Contrary to a one-state model, in a multi-state model any alive individual can move between occasions from one state (of a finite set) to another (Lebreton and Pradel 2002). Here we used as relevant two-state variable whether a grasshopper used the pavement on which it was most cryptic (lowest delta E value) or not. These models fit three types of parameters: recapture probability, probability to switch states, and survival probability. For each type of parameter we fitted a model where the parameter was either state-dependent or not, which combines into (2^3=) 8 possible models. We did not fit time-dependence for any parameter since we had no hypotheses for this and did not want to overparameterize the models, but we did correct for the unequal number of days between each capture occasion. Based on preliminary analyses we also did not include sex-dependence, except for recapture probability. This resulted in 16 possible models which were all fitted to the data. We did not select a preferred model, partly because several models had a high likelihood. Instead, we combined the insights provided by each model by calculating an average, model-weighted estimate for each parameter, summing the multiplications of the

estimate by each model with its AIC model weight. The same was done to construct model-weighted lower and upper 95% confidence limits. Note that grasshoppers could move from the pavements in our study area to the natural soils within the study area (Supplementary Figure 3) that we did not check, or leave the study area altogether, where they would remain alive yet undetected. This will lead to a reduction of the perceived recapture probability and survival probability. Our survival estimates are therefore conservative with respect to the hypothesis that natural selection (a high mortality rate) could drive population divergence.

Measurement of rate of phenotypic plasticity in adult coloration

We maintained 40 young adults reared in the laboratory in individual boxes. Twenty boxes were painted black on the inside, and twenty were painted white on the inside. A small plastic vial with food and a vial with water in the form of gel were provided; the vials were painted in the same color as the box. In grasshopper nymphs these distinct colors of the rearing environment result in the development of matching colors which increase crypsis (Rowell 1972, Edelaar et al. 2017b) (see also Supplementary Figure 7), so a similar response could be expected from adults. We took pictures of each adult at regular intervals until it died, up to 162 days later. From these pictures we measured its visual distance to the type of box it used (delta E), as explained elsewhere. If there is phenotypic plasticity, this distance should diminish over time. We therefore estimated how the visual distance changed over time for each color treatment separately (see Supplementary Figure 8) by fitting the interaction between time and color treatment, while allowing for random intercepts and random slopes for each individual (this also takes into account the repeated measurements of each individual). Sex was provisionally included but was not significant ($p=0.98$) and therefore not included in the final model (model <- lmer(deltaE.box ~ date + treatment + date*treatment + (1+date|box), data = data)). Residuals were close to normal; a few larger residuals were not removed since they had very low leverage and hardly influenced the results.

Heritability of adult coloration

We collected two groups of adult grasshoppers in the wild determined visually to be differently colored: pale reddish-brown grasshoppers from reddish-brown natural soils (10 females, 7 males), and dark blue-grey grasshoppers from dark grey artificial substrates (11 females, 9 males). These were placed in large transparent plastic breeding boxes with a thick layer of dry sand and allowed to mate freely. Egg pods (clutches) deposited in the sand were collected and maintained in individual Petri dishes, where they were moistened each week. They were first kept at 25 ºC for about 2 months, and then stored at 7 ºC for several months to induce diapause. Development was finished by placing them back at 25 ºC for an additional 6 weeks. After hatching we placed the nymphs of each clutch in a transparent plastic box (Fauna Box, 11.7 * 17.8 cm floor surface). They were raised on a mixture of dried red mosquito larvae (45%), wheat bran (45%) and infant formula milk powder (10%). Water was provided in upside-down test tubes filled with bottled mineral water with a closing cotton plug which stayed moist, allowing the nymphs to obtain water by chewing the cotton. We provided heat with terrarium heating mats placed below the boxes, obtaining temperatures between 35-40 ºC. Nymphs which had reached 3rd or 4th stage (out of 6 stages) were placed in new rearing boxes (same type) with either a substrate of small pale reddish-brown stones and white walls or a substrate of small dark blue-grey stones and black walls. Water (in the form of a gel) and food were provided in small plastic horizontally placed vials which were painted the same color as the walls. Up to ten nymphs of a single clutch were equally divided over each type of rearing environment (N= 169 nymphs from 19 clutches, grouped in time in two batches), and each rearing box received nymphs from two different clutches. We marked the nymphs of one clutch by cutting one antenna: these do not regenerate completely when adult, and cutting the antenna does not affect survival or coloration (data not shown). Nymphs were raised to adulthood (N=85), and pictures were taken once the adults were a few days old and color was fully developed. Coloration was measured as described above. We tested statistically if adult coloration is influenced by the color of the rearing environment during the last nymphal stages, and by the coloration of the parent population (Supplementary Figure 7). In the model we allowed for any effects of sex and batch (fixed effects), and clutch and rearing box identity (random effects). ((For

luminosity: model <- lmer(luminosity ~ rearing environment + parent population + sex + batch + (1|rearbox) + (1|clutch), data = adults), for red minus blue reflection: model <- lmer(Red_minus_Blue_Standardised ~ rearing environment + parent population + sex + batch + (1|rearbox) + (1|clutch), data = adults)).

Manipulation of grasshopper color

We used two different techniques to change the color of individuals. (i) We applied pigments externally by using pale or dark aquarelle paint (for Fig. 5A). These dry quickly, give a very natural final look, are not UV reflecting (just like unmanipulated grasshoppers and the test substrates), and do not contain solvents which might harm the condition and behavior of the grasshopper. We painted the area immediately surrounding the eye (avoiding contact with the antenna and ocelli), top and sides of the pronotum, the tegmens (covering front wings), and femur and tibia of all legs. These are the areas likely visible to the grasshoppers. In test trials and experiments we have noted no additional mortality or unusual behavior in painted individuals. (ii) We injected individuals with corazonin to induce the deposition of dark pigments into the cuticle by the individuals themselves (for Fig. 5B and 5C). This hormone triggers darkening in a wide range of species, including ours. We used a micro-syringe to inject nymphs in their abdomen with synthetically produced corazonin diluted in purified olive oil, following (Yerushalmi and Pener 2001),which invariably resulted in much darker adults (Figure 5B). Control individuals were not injected.

Laboratory habitat use experiment

Habitat use as a function of individual coloration was measured in a rectangular transparent plastic box (32.0 by 17.3 cm) where each side was filled with a thin layer of a pale or a dark substrate, thus creating two long rectangular contrasting habitat patches. To prevent grasshoppers from looking outside the box and to reinforce the contrast between the patches, the sides of the box were covered with a five cm high strip of paper, black on the side of the dark habitat and white on the side of the pale habitat. The top of the box was covered by nylon panty material to allow air flow and prevent damage to jumping grasshoppers. No food or water was provided during the choice trials in order to prevent these from

influencing habitat use. Heat was provided from below using heat mats, and boxes were placed at a height of two meters to avoid disturbance, under high performance daylight fluorescent tubes **(Philips TL-D 90 De Luxe** Master). Grasshoppers were placed individually in the centre of a box, and left for about 30 minutes to habituate. We next recorded the position of the grasshopper every 15 minutes, until 20 data points were collected. (For any individuals sitting on the boundary between habitats, we assigned it to the habitat above which the (greatest proportion of the) head was placed). Individuals were made to jump after each moment of data collection in order to obtain more independent measures of habitat use. For the painted grasshoppers (see above), we modeled the use of the dark habitat (as a binomial response variable) with a generalized linear mixed model, with color manipulation and rearing substrate (the substrate of collection in the field) as fixed effects, and the identity of the individual (replicated data from different days) and the rearing box in the lab as a random effect, and tested for the effect of color manipulation on habitat use by a log-likelihood ratio test (Figure 5A) (model <- glmer(y ~ color painted + rearing substrate + rearing substrate * color painted + (1|individual) + (1|expbox), family = binomial, data = data)). As the average use of dark habitat by darkened individuals is still below parity, it does not seem likely that darkening just results in random habitat use. Habitat use of grasshoppers injected with corazonin (see above) was modeled and tested the same way, except that we modeled corazonin injection (yes/no) and date as fixed effects (all were reared on the same substrate), and the rearing box and experimental box as random effects (Figure 5B) (model <- glmer(y ~ corazonin + date + (1|exp.box) +(1|origin.box), data = data, family= binomial)).

Habitat selection in the field after manipulating grasshopper color

We tested habitat selection of color-manipulated grasshoppers in the field by releasing them in a 115-metre long street composed of two pavement types: a 7 meter wide central area of dark asphalt that was very similar to the color of grasshoppers made darker by injection with corazonin (mean delta E < 10), and a strip of pale cement parking space and pale tile sidewalk of 5.5 meters on either side that was very similar to the color of control, pale grasshoppers (mean delta E < 10). We released groups of mixed individuals at four locations on the border of the two pavements in the morning. The next morning we surveyed the entire

street, and recorded the type of pavement selected by recaptured individuals. By keeping the interval between release and recapture short we minimize the effect of any selective predation. Moreover, the experiment with dead grasshoppers (see "Measuring predation rate with decoy grasshoppers" above) was done on the same day and showed very low predation pressure (see main text). This experiment was repeated in two different years. In year 1 we released N = 30 manipulated and N = 20 control individuals (N = 14 and N = 3 recaptured, respectively); in year 2 we released N = 40 manipulated and N = 22 control individuals (N = 13 and N = 11 recaptured, respectively). We modeled habitat use (binomial response variable) with a generalized linear mixed model, with color manipulation, sex and year as fixed effects, and the identity of the rearing box in the lab as a random effect, and tested for the effect of color manipulation on habitat use by a log-likelihood ratio test (Figure 5C) (model <- glmer(habitat ~ color manipulation + sex + year + (1|origin.box), data = data, family = binomial)). As the average use of dark habitat by darkened individuals in the field is above parity, it again does not seem that darkening just results in random habitat use.

Male-female mating interactions

From observations in the field and the lab, it appears that females have a lower drive to mate than males. Females can fertilize several clutches with a single mating, although tend to mate again after depositing a clutch (about once a week). Since females are much bigger than males, females can prevent mating by kicking males away with their hind legs. Males have a short display flight, in which they jump and fly about one meter into the air and land nearby while making a clicking sound with their wings, possibly to attract females or to deter other males. Males respond towards movement of other grasshoppers (including males) by approach and investigation, after which attempts of mating follow in case it is a female. Therefore, a successful mating typically starts with the detection of a female by a male. In order to determine over which distances males can detect females, we looked for a perched female on a pavement, and made her to jump and fly a few meters. As soon as she landed, we looked for any males that responded to her, either by reorientation towards the female, or by walking or jumping into her direction. This would be complicated to do on a natural soil, but on the flat and

nearly unvegetated pavements it is straightforward to detect even small male movements. When a moving male was detected, we classified its initial distance to the female in one-meter categories. This flushing and observing was repeated with the same female until we had data from a few different males, after which a new female was located and the sequence was repeated. There was no effect of female identity on the male response distances (as tested by a random effect in a mixed model), so we treated all observations as equal. With the recorded male response distances we constructed a generalized response curve (see Figure 6) using as an index the number of responses/relative number of males available, taking into account the surface of each one-meter wide ring-shaped area around the female, and assuming that initial male density was independent of female landing position. This curve probably overestimates the probability of a mating as a function of initial male-female distance, since flying females are easier to detect than females just walking around and females fly little unless disturbed, hence our estimate for the spatial scale of assortative mating is probably conservatively large with respect to our interpretations.

Chapter IV

Positioning behavior according to individual color variation improves camouflage in novel habitats

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Abstract

Behavior can play a key role in adaptation, especially in novel environments. Here we study how ground-perching grasshoppers that colonized street pavements as novel habitats behaviorally manage their detection rates by predators. We found that grasshoppers positioned themselves aligned with the spaces between adjacent bricks more than expected by chance. By performing a virtual predation experiment, we confirmed that this positioning behavior decreases the predation rate. Surprisingly, individuals with a poorer cryptic coloration made greater use of this positioning behavior, whereas individuals with a better cryptic coloration relied more on background color matching. Additionally, positioning behavior interacted with other anti-predation behaviors, individuals that were positioned on the space between bricks allowed potential predators to get closer before fleeing. These results indicate that these grasshoppers showed adaptive flexibility in camouflage and escape behaviors as a function of both individual and environmental variation. Such behavioral flexibility should allow organisms to cope better with novel environments, which deserves more study especially in the current context of global change.

Keywords

urban adaptation, behavior, camouflage, background matching, novel environment, behavioral flexibility

Introduction

How organisms adapt to novel environments has become a key question due the increasing rate of rapid human-induced changes to natural habitats (Sih et al. 2011). Urbanization is one of such changes, causing major habitat transformation. Even though urban expansion into natural areas generally has a negative effect on biodiversity , for some species it promotes new opportunities to exploit new ecological niches (Diamond 1986). However, it is still unclear why and how these species are able to adapt to urban settings (Carrete et al. 2011). Behavioral changes can allow organisms to benefit from the new opportunities arising (Sol et al. 2011), including the decision to move to novel habitats when these provide a better match between phenotype and environment (Edelaar et al. 2008,

Duckworth 2009, Carrete and Tella 2010, Karpestam et al. 2011). Thus behavior may play an important role in how organisms cope with novel conditions, often being an essential component of the rapid responses necessary to deal with environmental changes or novel habitats (Holway and Suarez 1999).

Adaptation to a certain habitat through camouflage is a common strategy in nature. The prevention of detection, called crypsis, is probably the most studied camouflage strategy with numerous examples across taxa and ecosystems. There are numerous strategies to achieve crypsis like background matching (matching the color, lightness and/or pattern of a background), disruptive coloration (creating the appearance of false edges), countershading (showing dark colors on body parts exposed to light and light colors on parts usually shaded), and several others (Stevens and Merilaita 2009). There are also forms of camouflage that are different from crypsis, such as masquerade (ensuring organisms are misidentified once they have been detected)(Skelhorn et al. 2010) or motion dazzle (markings that hinder the estimation of speed and trajectories)(Stevens and Merilaita 2009, Hogan et al. 2016). Animal behavior can interact with all these strategies and forms of camouflage and could therefore be very important in their optimization. There are several studies that have investigated the relationship between camouflage and behavior for crypsis (De Ruiter 1956, Edmunds and Grayson 1991, Wilkens 1993, Webster et al. 2009, Kang et al. 2012, Lovell et al. 2013, Wilson-Aggarwal et al. 2016) or other forms of camouflage like masquerade (Skelhorn et al. 2011, Skelhorn and Ruxton 2013). Nonetheless, in general we are just starting to appreciate how important animal behavior is in enhancing camouflage strategy so more research effort is needed in this area (Hensley et al. 2015, Wilson-Aggarwal et al. 2016), especially in the context of rapid environmental change.

In this study, we focus on a natural colonization of a recently urbanized area by ground-perching grasshoppers, and on the individual responses to this novel habitat in the context of behavior camouflage interactions. These grasshoppers appear to enhance camouflage through background color matching as well as by a positioning behavior involving perching site choice and body orientation. There is however a trade-off between background matching and this positioning behavior after movement (e.g. a short escape flight): background matching

requires immobility to avoid detection by movement, whereas behavioral positioning requires some adjusting local movement. We might therefore expect that individuals make different choices depending on their variation in color. Specifically, we test: 1) if a positioning behavior strategy improves survival, 2) if grasshoppers use the positioning behavior strategy more than that would be expected at random, 3) if a greater level of camouflage provided by background matching (in color and luminosity) reduces the use of a positioning behavior, and 4) how the camouflage strategy used affects the escape behavior of individuals.

Methods

Study system

We studied the adaptation of the Azure Sand Grasshopper (*Sphingonotus azurescens*). This is a ground-perching grasshopper that normally lives on natural open soils and doesn´t climb into plants. We recently found it colonizing novel urban-like habitats at Dos Hermanas (province of Seville, Spain; 37.306º N, 5.932º E). These novel habitats are pavements (streets) in an abandoned housing area that is closed off to traffic. The streets are composed of four different types of pavement: dark asphalt, paths made of brown bricks, paths of grey bricks, and sidewalks of pale tiles. The streets surround large blocks of little-vegetated natural soils, where grasshoppers are common. Because of the low level of use and maintenance of the pavements, some colonizing food plants are growing in-between the bricks and tiles, allowing grasshoppers in turn to colonize these streets as alternatives to natural soils. The fact that these pavements are acceptable habitat is confirmed by the presence of many individuals, adult males that are displaying, recaptures of marked individuals, observations of copulations and egg deposition, and the presence of nymphs in spring.

Individual grasshoppers vary in a continuous manner in body coloration from very pale to almost black, and from bluish-grey to orange-brown. Their coloration normally resembles that of the local substrate on which they occur. This color match thus provides camouflage via the background color matching strategy. However, , initial observations suggested that on the urban pavements

grasshoppers sometimes align their body with the lines that arise where two tiles or bricks meet (Figure 1).

Data collection

To test for the differential use of positioning behavior by grasshoppers in urban habitats we searched for individuals perched on grey bricks (Figure 1). Each individual (n=35) was disturbed 10 times such that it jumped up and flew a few meters away to another spot. We noted their position after each escape (aligned/not aligned with lines, i.e. use of positioning behavior or not), 10 s after each landing to allow individuals to move a bit in order to better align their body with the lines, if they wanted to. At the end, we caught the grasshopper using a net to determine sex, take a photograph (see below for details on color measurement) and measure the length of the individual.

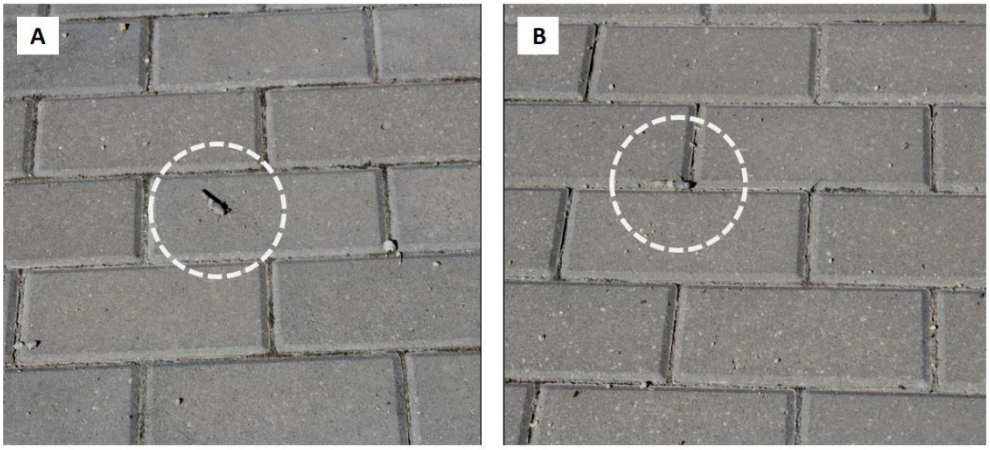

Figure 1. Camouflage strategies used by grasshoppers in urban habitats (white circles show the position of individuals). (A) Background color matching. (B) Positioning behavior by the alignment of the body with the line between two bricks.

In addition, we systematically surveyed all four pavements types in the study area for grasshoppers. Any grasshopper detected was captured and was individually marked with a combination of three letters on the posterior part of both fore wings (which already have some irregular dark markings), using a black permanent marker pen (Staedtler permanent Lumocolor, resistant to water and

UV light). All individuals were photographed and then released at the location of first encounter. For each individual we recorded sex, day of capture, type of substrate on which it was found, the initial perching position (on a line or not), the flight initiation distance and the distance flown. The flight initiation distance (FID) is the distance at which an organism begins to flee an approaching threat; it is an important component of the antipredatory behavior and thought to be an indicator of an animal's perception of threat (Blumstein et al. 2003, Gotanda et al. 2009, Carrete and Tella 2010). The entire study area was searched regularly for marked and new, unmarked adult grasshoppers from June to October, covering the entire period when adults are common.

Virtual predation experiment

We tested the survival rate of grasshoppers according to their camouflage strategy. We performed a predation experiment using humans as predators. Humans adopt a similar search pattern to birds (one of the more commonly observed potential predators of the Azure Sand Grasshopper at our study site) when looking for prey on a computer screen (Ruxton et al. 2005), have comparable information processing capabilities (Dukas and Ellner 1993, Dukas and Kamil 2001, Dukas 2002, Xiao and Cuthill 2016) and the results obtained using humans are comparable to those of analogous studies using birds (Cooper 1984, Beatty et al. 2005, Fraser et al. 2007, Knill and Allen 2010, Karpestam et al. 2013, Stevens et al. 2013, Xiao and Cuthill 2016). For this virtual predation experiment we used photos of 16 different backgrounds in our study area (10 of pavements and 6 of natural open soils), taken at a distance of 1.5 m perpendicular to the ground. We also used photos of 40 grasshoppers (26 males and 14 females) in which we removed the background of the photo (see "Image taking and processing" for details on image capture and color measurement). We developed a computer program written in JavaScript in which the 16 different backgrounds appear on screen in a random order. On each of these background images, we placed the images of 2 to 4 random grasshoppers (out of the set of 40) that appeared in a random location with a random orientation. On the same background, we also placed another 2 to 4 random grasshoppers, but these appeared randomly within a finite set of previously fixed locations and

orientations such that they aligned with the elements of the background (sticks for natural soils and lines between bricks for pavements). We presented these different combinations on a touch screen with a resolution of 1920×1080 pixels as a computer game to 261 human participants. Participants were instructed to find and touch ("capture") as many grasshoppers as possible in a total of 160 s for the 16 different screens. An example screen was provided before the start for instruction and training, and people could move to the next screen when they wanted (i.e. when no more grasshoppers were seen by them). The program recorded the following data: the identity (self-created nickname), age and gender of the human participant, the identity of the background used, the number, identity and sex of grasshoppers placed, the position of each grasshopper (aligned or not), and if the grasshopper was captured or not.

Image taking and processing

To quantify background color matching in the field, grasshoppers and backgrounds were photographed *in situ* with a Canon 1200D camera mounting a 18-55 mm Canon lens (locked at 55mm) using fixed camera settings of f/12 aperture, 1/50 shutter speed, ISO 200. Pictures were taken in RAW format and included an 18% reflectance grey standard. Following Troscianko and Stevens (2015), we linearized the images and converted these from camera color space to the relative photon catches of the relevant predator. The grasshoppers might be predated on by a wide range of visual predators (mammals, birds, lizards, insects and spiders) with very different visual systems, but we used the spectral sensitivity of the blue tit, *Cyanistes caeruleus* (cone ratios from Hart (2001)), because birds appear to be the most abundant visual predators in the area (all author's pers. observation). Since grasshoppers and backgrounds do not reflect ultraviolet radiation (as checked by spectrophotometry) we did not include the UV cone types into the analysis, performing a trichromatic color analysis (Stevens et al. 2007). For the virtual predation experiment involving human predators we instead used the spectral sensitivity of humans (Hofer et al. 2005). The color measures in grasshoppers were made on a pre-defined diamond-shaped area in the dorsal part of the metazone of the pronotum, which is representative for the overall body

color. Next we quantified color contrasts between photon catches of grasshoppers and photon catches of backgrounds according to a log-linear form of color discrimination model, which assumes that visual discrimination is limited by receptor noise (Vorobyev and Osorio 1998), and using a Weber fraction value of 0.05 for the most frequent cone type. We also quantified luminance contrasts using a version of the model based on achromatic differences (based on blue tit double cones and human luminance, i.e. perceived lightness, respectively). These color and luminance contrasts were expressed in "just-noticeable-differences" (JND) whereby values between 1.0 and 3.0 indicate difficult discrimination, whereas values increasing above 3.0 indicate increasingly improved discrimination (Siddiqi et al. 2004).

Statistical methods

Analyses were performed in *R* version 3.2.3 (R Core Team 2017). As a partial test of whether grasshoppers use positioning behavior as a camouflage enhancement technique on urban pavements, we tested if they perched on the lines between two bricks more often than expected by random placement. To obtain this random expectation, we first determined that the available proportion of a brick that could be considered part of the line between two bricks is 16.4% (i.e. the surface area close to the edges of a brick). Since the pavement has a regular pattern, this value is the same for all bricks. We then used a binomial process to determine the percentage of times that an individual would be perched on a line if it was positioned randomly 10 times (the number of data per individual in the field), repeated this for 35 hypothetical individuals (our sample size in the field), and calculated the average (population) percentage of line use. Finally, we repeated this procedure 100,000 times to obtain a distribution of this percentage for the population. Then, we compared the observed average value of the use of lines in the field with the expected distribution for random space use.

Using this same data set of field observations, we tested if their color might influence the differential use of positioning behavior by grasshopper individuals (background color matching versus positioning). We fitted a generalized linear model, modelling the use of positioning with background lines as the dependent

variable (scored as yes or no, modelled using a binomial error structure;,10 observations for each individual, individual identity included as random effect). Fixed effects were fitted for differences between grasshopper and whole area background in color and luminosity (for the blue tit visual model, in JND units), grasshopper sex (male/female) and length, and day of observation (two different days). We also tested if perching on lines could actually be explained as a micro site choice to achieve greater background matching in the color and luminosity components (because these components could be different between the central surface of the brick and the lines due to the presence of lichens, moss, dirt, etc.). For this, the same model structure was fitted, but using the differences in color and luminosity between the grasshopper and the lines between bricks instead of the surface of the grey brick, as measured from the images.

To test if there was an effect of grasshopper position on its escape behavior when a potential predator is approaching, we analyzed the escape data by fitting a Bayesian generalized linear bivariate mixed model using the *MCMCglmm* R-package (Hadfield 2010). This approach allowed us to fit a bivariate mixed model, which is better than fitting two separate models for FID and distance flown since these variables were correlated (r =0.31). We used the flight initiation distance (FID) and the distance flown as response variables (n=345), using a Gaussian family error distribution. Fixed effects were fitted for use of positioning behavior (aligned with lines: yes/no), type of habitat (four different types of pavement), sex (male/female) and color and luminosity differences between grasshopper and background (for the blue tit visual model, in JND units). We also included day (33 different days) and individual identity (211 individuals) as random effects. The joint posterior distribution for the model was estimated from 1,100,000 Markov Chain Monte Carlo iterations sampled at 1,000 iteration intervals after an initial burn-in period of 100,000 iterations (leaving 1,000 uncorrelated effective samples), using weakly informative parameter-expanded priors for the variance components; the degree of belief parameter (nu) was 2 for the random effects and 0.002 for the residuals. Convergence of models was verified by visually inspecting output plots following Hadfield (2015) and model convergence diagnostics (autocorrelation, Gelman and Rubin 1992).

The data from the virtual predation experiment was analyzed to test what determines the probability of survival of the grasshoppers. For this we used generalized linear mixed-effects models specified in the *lme4* R-package (Bates et al. 2014). We used the capture of the grasshopper individual (captured or not, n=14,910) as dependent variable. Fixed effects were fitted for sex of the grasshopper (male/female), gender of the observer (male/female), type of background (natural soil/ pavement – binary), alignment with items (aligned or not, i.e. a test for positioning behaviour), density of grasshoppers (number of grasshoppers on the screen, ranging from 2 to 8) and color and luminosity differences between grasshopper and background (for human visual model, in JND units) (i.e. a test for background matching on their color and luminosity components). As random effects we fitted the identity of the observer, the identity of the grasshopper and the identity of the background photo since we had repeated data for each of these.

Results

Grasshoppers clearly use the positioning behavior by perching more often on the line that arises where two grey bricks meet than expected by random chance (Figure 2). On average, grasshoppers were about twice as likely to perch on a line as expected. Moreover, there was a striking effect of the grasshopper-background color difference on this probability: individuals with a poorer cryptic coloration were more likely to perch on a line (Figure 3, Table 1). Use of the line for perching was independent of grasshopper sex or size, and it did not depend on the luminance difference (Table 1). The same effects were found when we used the line instead of the surface of the brick to calculate background-grasshopper color and luminance differences (Table S1).

The virtual predation experiment confirmed that positioning behavior (perching near a line) significantly increases survival by 39% (36.7% survival rate for aligned locations versus only 26.4% for random locations across backgrounds). The mixed model yielded significant effects for alignment with items (Estimate (aligned yes) = -0.421 ± 0.097 SE, $P < 0.0001$) and JND difference in color (Estimate= 0.120 ± 0.051 SE, $P = 0.019$) confirming that

background matching (in color) and positioning behavior decrease predation (the effect for background matching in luminosity was very weak and non-significant: P = 0.092). We also found a mild but significant effect of the density of grasshoppers decreasing predation rate (Estimate = -0.061 ± 0.019 SE, P= 0.002). The rest of the fixed effects (sex of grasshopper and gender of observer, type of background) were not significant (P > 0.05) whereas all random effects (identities of observers, grasshoppers and backgrounds) were significant (P <0.0001 for each one) (Table S2).

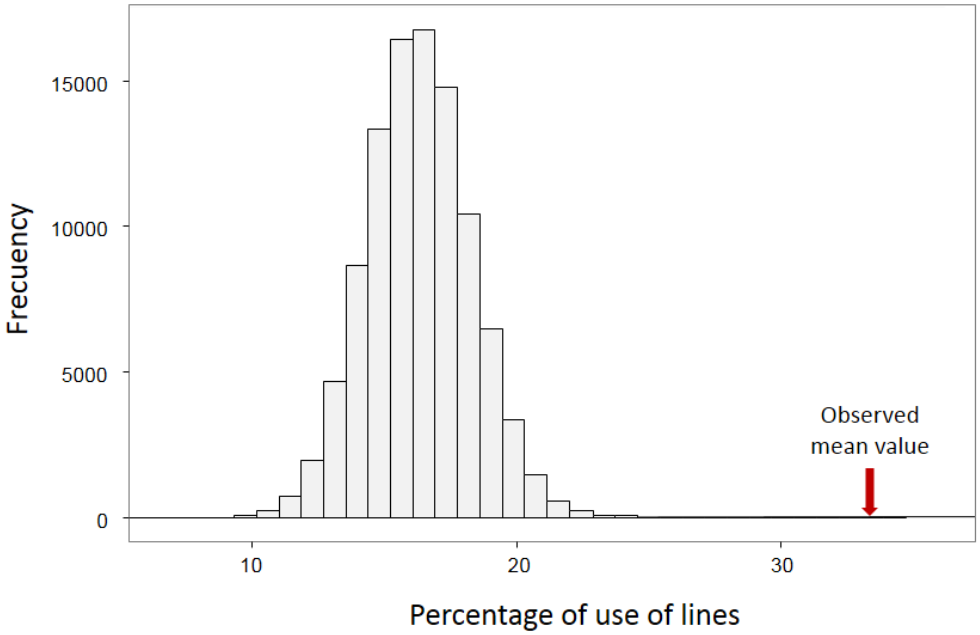

Figure 2. Grasshoppers perch on lines more often than expected. The distribution is the expected mean percentage of use of lines between two bricks if the usage of the pavement were random (based on 100,000 simulations of a random binomial distribution involving 10 trials for 35 individuals each, random probability of line use 16.4%). The observed mean value in the field (red arrow, based on 10 observations for 35 individuals each) does not overlap with 95% of the distribution for random space use.

When grasshoppers are already aligned with lines upon first approach by an observer, they have a shorter flight initiation distance (posterior mean= -0.58, Credible Interval = -1.03 to -0.15) (Table 2, Table S3). Females flew a greater distance than males, but for the other fixed effects the posterior 95% credible

intervals overlapped with 0. Both random effects (individual identity and day) had clear effects (Table 2, Table S3).

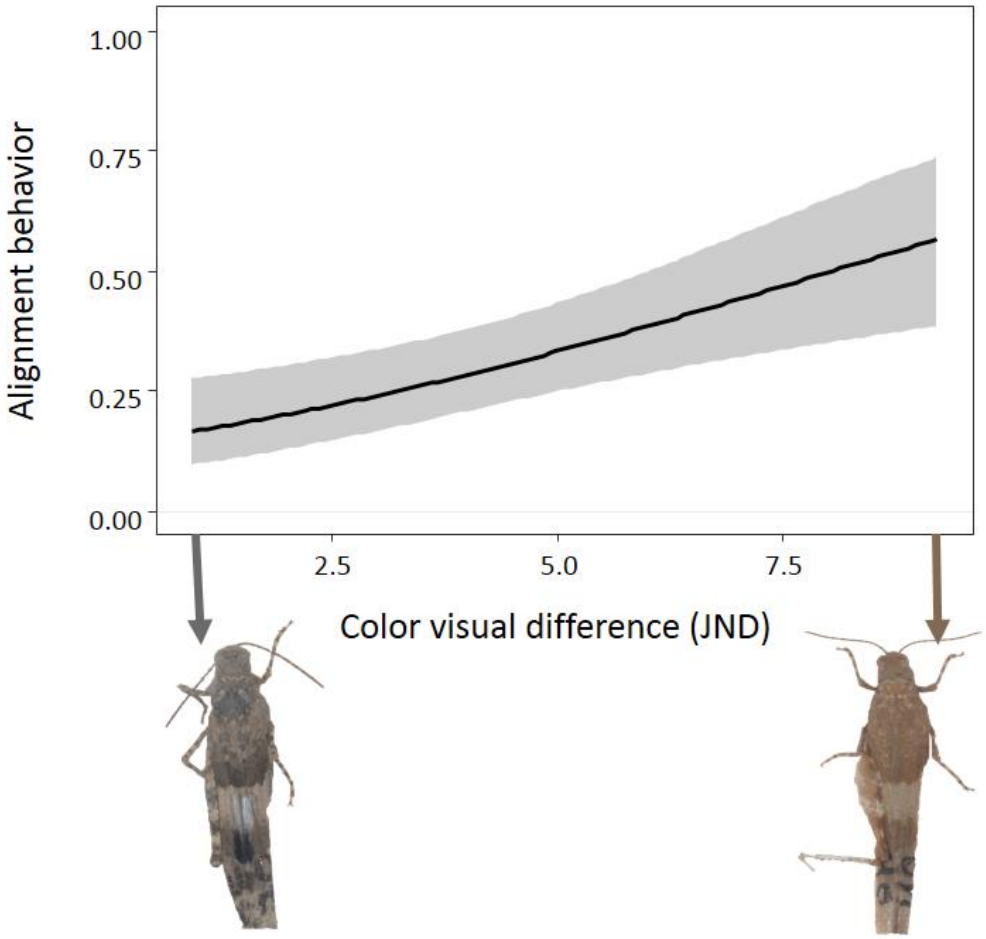

Figure 3. Cryptically coloured grasshoppers mostly use background color matching, whereas less cryptically coloured grasshoppers increasingly use the positioning behavior. Shown is the relationship between the colour difference (between grasshoppers and grey brick urban pavement for the Blue tit visual model, in JND units) and the alignment with the lines between bricks for perching. The black line is the model prediction and the grey shadow is its 95% confidence level. Also shown are the images of the grasshoppers with the best (0.95 JND units) and the worst (9.21 JND units) background matching in color, with respect to grey bricks (shown in Figure 1).

Table 1. Overview of effects on the probability to perch on a line and their statistical support (generalized linear model, binomial family). The coefficient for the reference categories (not listed) is always zero. Significant effects ($p<0.05$) are highlighted in bold.

	Estimate	Std. Error	Z value	p-value
(Intercept)	-0.929	3.076	-0.302	0.763
JND Background - grasshoppers colour difference	0.204	0.068	3.000	**0.003**
JND Background - grasshoppers luminance difference	-0.046	0.055	-0.836	0.403
Sex (male)	-0.370	0.633	-0.585	0.559
Day (2nd day)	-0.190	0.239	-0.794	0.427
Size	-0.001	0.012	-0.074	0.941

Discussion

We found in our virtual predation experiment that grasshoppers that exhibit a positioning behavior (perching close to objects such as sticks or lines between bricks) have an increased survival (Table S2). In the urban study site, grasshoppers perch on lines between bricks more often than expected (Fig. 2). They do so especially when their degree of background matching (in color) is worse (Fig. 3 and Table 1), and this effect is not because the lines provide better background matching in color (Table S1). Finally, when they are perched on a line, they allow a potential predator to approach more closely before fleeing (Table 2). All these results support that grasshoppers are actively using positioning behavior to increase camouflage and thereby reduce predation risk. In general, individuals who exhibit an alignment behavior benefit from an improvement in their camouflage. This may be due to several mechanisms. The first one could be background matching in pattern since jointly, lines between bricks make up a regular pattern in the background, so by aligning with this pattern the individual resemble a scene's overall pattern more than if the grasshopper is perched out in the open, away from the lines. We also have to note that background complexity increases dramatically around the lines, which is known to interfere with detection

and therefore improve camouflage (Xiao and Cuthill 2016). Other effects also could explain this camouflage improvement, like self-shadow concealment (Thayer 1896, Cott 1940, Kiltie 1988) or the concealing of three-dimensional surface disruption, since the area between two bricks is a bit lower than the surface of the bricks (Stevens and Merilaita 2009). Masquerade could also be an explanation of this improvement of camouflage, with aligned grasshoppers masquerading as a line between bricks and being initially detected but subsequently misclassified by predators. Confirming masquerading requires a focus on the responses of predators, by manipulating their experience with putative models and prey (Skelhorn et al. 2010).

Table 2. Posterior distributions for fixed effects (mean and its 95% Credible Interval) and random effects (mean for the variance and its 95% credible interval) on flight initiation distance (FID) and distance flown. Effects with 95% Credible Intervals overlapping zero are not shown (but provided in Table S3).

FID	Posterior mean	95% CrI
Fixed effects		
Aligned with lines (yes)	-0.580	-1.026 to -0.147
Random effects		
Individual	0.511	0.147 to 0.897
Day	0.026	$3.845*10^{-08}$ to 0.096
Distance flown		
Fixed effects		
Sex (male)	-0.579	-1.095 to -0.058
Random effects		
Individual	0.988	0.344 to 1.733
Day	0.110	$2.296*10^{-06}$ to 0.363

Irrespective of how exactly camouflage is increased, individual grasshoppers face a trade-off: positioning behavior requires small-scale movements to align with other objects (like brick lines), whereas crypsis benefits from immobility in order to prevent detection by movement. Figure 3 indicates that variation among

individuals in color and therefore in the relative benefit of background color matching results in a shifting balance between camouflage strategies: positioning behavior is used more frequently when background color matching is lower. Evaluation of to what extent these results may vary with predator characteristics like visual system, foraging behavior (we assumed aerial views by an avian predator) or viewing distance (Skelhorn and Ruxton 2014) would need further testing.

Camouflage by crypsis implies a match between phenotype and environment, but environments can exhibit a great variation in color, brightness or pattern in space and time. One of the solutions to environmental variation in general is the evolution of genetic polymorphisms via divergent natural selection (Bond and Kamil 2006) (for which we have some evidence in our system (Edelaar et al. 2017)), but in the absence of habitat choice this has a large demographic cost (selective mortality) and does not deal well with rapid changes or very heterogeneous habitats. Improving the organism's appearance through phenotypic plasticity is a more flexible strategy (well-developed in our immature grasshoppers (Edelaar et al. 2017)). However, the changes in the environment with which an organism has to match (because of environmental changes and/or individual movements across different environments) could be faster than the ability of individuals to change their appearance. Even though some organisms like cephalopods or chameleons have the ability to develop rapid color changes and patterns, in general slow color changers (which need from days to months to change, like our grasshoppers (Peralta-Rincon et al. 2017, Edelaar et al. 2017) are likely to be more widespread in nature (Stevens 2015). In these cases, only adaptive behavior that tries to match the environment to the phenotype can provide a rapid response to environmental heterogeneity in time or space. In the absence of the ability of grasshoppers to change the local environment (e.g. its color) where they currently are, they can only increase this match by selecting and if necessary moving to environments that provide them with greater camouflage (a form of non-random dispersal (Edelaar et al. 2008, Karpestam et al. 2011, Edelaar and Bolnick 2012). Here we have demonstrated how indeed grasshoppers respond behaviorally to local environments depending on the match between their phenotype and the environment: if the color match is good they stay on the

grey bricks which enhances background matching in color, but if the color match is poorer they move and adjust their body orientation with the lines between bricks which also enhances camouflage. Such a flexible behavior and adaptive selection of their environment at a small scale in general increases performance, and here would still allow grasshoppers with a less-matching color to successfully colonize novel habitats.

The grasshoppers change their anti-predatory behavior, in this case the flight initiation distance, in a flexible way depending on the camouflage strategy used. They also showed behavioral flexibility in the use of different camouflage strategies depending on their level of camouflage provided by background matching in color. This could imply a level of cognition by the individuals in a broad sense, perceiving the environment, learning, classifying and making decisions (Shettleworth 2001, 2010, Rowe and Healy 2014, Skelhorn and Rowe 2016) that enable them to evaluate their degree of phenotypic matching to the environment (in this case camouflage). In this way, organisms can have a beneficial behavioral response to environmental changes, which provides a better adjustment to the environment very quickly, almost instantly. Due the important ecological and evolutionary implications that these interactions between cognition, behavior and camouflage could have in our study system and presumably in many other ecological systems, more research on this topic is necessary (Stevens 2015, Skelhorn and Rowe 2016). A few recent studies have explored this, like FID in ground nesting birds depending on the level of camouflage (Wilson-Aggarwal et al. 2016), moths that select a resting position to improve their camouflage (Kang et al. 2012, 2015), or cuttlefish that change between camouflage strategies (Buresch et al. 2011). Overall, such behavioral interactions can provide a rapid adaptive response and might be key in understanding how individuals can cope with natural or human-caused rapid changes in the environment, or how native and non-native, invasive organisms can colonize new habitats.

Conclusion

We found that Azure sand grasshoppers, adapting to a novel urban environment, use the lines between bricks more than expected by chance. A virtual predation experiment suggests that such a positioning behavior results in a reduction in predation rate. However, individuals use different camouflage strategy depending on their cryptic coloration, since less cryptic individuals made greater use of positioning behavior whereas more cryptic individuals relied more on background color matching. Additionally, individuals using positioning behavior showed shorter flight initiation distances. Together, our results support an adaptive flexibility in camouflage and escape behaviors as a function of individual and environmental variation, allowing grasshoppers to cope better with traditional as well as novel environments.

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Author Contributions

Conception and design: ABV and DPQ. Field work: ABV and DPQ. Virtual predation experiment design: ABV and PE. Data analyses: ABV. First manuscript drafting: ABV. All authors participated in improving the manuscript.

References

Bates, D., Maechler, M., Bolker, B. and Walker, S. 2014. lme4: Linear mixed-effects models using Eigen and S4.

Beatty, C., Bain, R. and Sherratt, T. 2005. The evolution of aggregation in profitable and unprofitable prey. - Anim. Behav.

Blumstein, D. T., Anthony, L. L., Harcourt, R. and Ross, G. 2003. Testing a key assumption of wildlife buffer zones: is flight initiation distance a species-specific trait? - Biol. Conserv. 110: 97–100.

Bond, A. B. and Kamil, A. C. 2006. Spatial heterogeneity, predator cognition, and the evolution of color polymorphism in virtual prey. - Proc. Natl. Acad. Sci. U. S. A. 103: 3214–9.

Buresch, K. C., Mäthger, L. M., Allen, J. J., Bennice, C., Smith, N., Schram, J., Chiao, C. C., Chubb, C. and Hanlon, R. T. 2011. The use of background matching vs. masquerade for camouflage in cuttlefish Sepia officinalis. - Vision Res. 51: 2362–2368.

Carrete, M. and Tella, J. L. 2010. Individual consistency in flight initiation distances in burrowing owls: a new hypothesis on disturbance-induced habitat selection. - Biol. Lett. 6: 167–170.

Carrete, M., Tella, J. L. 2011. Inter-Individual Variability in Fear of Humans and Relative Brain Size of the Species Are Related to Contemporary Urban Invasion in Birds (FR Adler, Ed.). - PLoS One 6: e18859.

Cooper, J. M. 1984. Apostatic selection on prey that match the background. - Biol. J. Linn. Soc. 23: 221–228.

Cott, H. B. 1940. Adaptive Coloration in Animals. - Methuen, London: 1–602.

De Ruiter, L. 1956. Countershading in Caterpillars. - Arch. Néerlandaises Zool. 11: 285–341.

Diamond, J. M. 1986. Natural selection: Rapid evolution of urban birds. - Nature 324: 107–108.

Duckworth, R. A. 2009. The role of behavior in evolution: a search for mechanism. - Evol. Ecol. 23: 513–531.

Dukas, R. 2002. Behavioural and ecological consequences of limited attention. - Philos. Trans. R. Soc. London B Biol. Sci. in press.

Dukas, R. and Ellner, S. 1993. Information Processing and Prey Detection. - Ecology 74: 1337–1346.

Dukas, R. and Kamil, A. C. 2001. Limited attention: the constraint underlying search image. - Behav. Ecol. 12: 192–199.

Edelaar, P. and Bolnick, D. I. 2012. Non-random gene flow: An underappreciated force in evolution and ecology. - Trends Ecol. Evol. 27: 659–665.

Edelaar, P., Siepielski, A. M. and Clobert, J. 2008. Matching habitat choice causes directed gene flow: a neglected dimension in evolution and ecology. - Evolution (N. Y). 62: 2462–72.

Edelaar, P., Baños-Villalba, A., Escudero, G. and Rodríguez-Bernal, C. 2017. Background colour matching increases with risk of predation in a colour-changing grasshopper. - Behav. Ecol. 20: 65–71.

Edmunds, M. and Grayson, J. 1991. Camouflage and selective predation in caterpillars of the poplar and eyed hawkmoths (Laothoe populi and Smerinthus ocellata). - Biol. J. Linn. Soc. 42: 467–480.

Fraser, S., Callahan, A., Klassen, D. and Sherratt, T. N. 2007. Empirical tests of the role of disruptive coloration in reducing detectability. - Proc. R. Soc. London B Biol. Sci. in press.

Gelman, A. and Rubin, D. B. 1992. Inference from Iterative Simulation Using Multiple Sequences. - Stat. Sci. 7: 457–472.

Gotanda, K. M., Turgeon, K. and Kramer, D. L. 2009. Body size and reserve protection affect flight initiation distance in parrotfishes. - Behav. Ecol. Sociobiol. 63: 1563–1572.

Hadfield, J. D. 2010. MCMC Methods for Multi-Response Generalized Linear Mixed Models: The MCMCglmm *R* Package. - J. Stat. Softw. 33: 1–22.

Hadfield, J. 2015. MCMCglmm course notes. - Available cran. us. r-project. org/web/packages/MCMCglmm/vignettes/CourseNotes. pdf. Accessed Oct. 13: 2014.

Hart, N. S. 2001. The Visual Ecology of Avian Photoreceptors. - Prog. Retin. Eye Res. 20: 675–703.

Hensley, N. M., Drury, J. P., Garland, T. and Blumstein, D. T. 2015. Vivid birds do not initiate flight sooner despite their potential conspicuousness. - Curr. Zool. in press.

Hofer, H., Carroll, J., Neitz, J., Neitz, M. and Williams, D. R. 2005. Organization of the Human Trichromatic Cone Mosaic. - J. Neurosci. in press.

Hogan, B. G., Cuthill, I. C. and Scott-Samuel, N. E. 2016. Dazzle camouflage, target tracking, and the confusion effect. - Behav. Ecol. 27: 1547–1551.

Holway, D. A. and Suarez, A. V 1999. Animal behavior: an essential component of invasion biology. - Trends Ecol. Evol. 14: 328–330.

Kang, C.-K., Moon, J.-Y., Lee, S.-I. and Jablonski, P. G. 2012. Camouflage through an active choice of a resting spot and body orientation in moths. - J. Evol. Biol. 25: 1695–702.

Kang, C., Stevens, M., Moon, J. Y. J. -y., Lee, S.-I. S. I. and Jablonski, P. G. 2015. Camouflage through behavior in moths: The role of background matching and disruptive coloration. - Behav. Ecol. 26: 45–54.

Karpestam, E., Wennersten, L. and Forsman, A. 2011. Matching habitat choice by experimentally mismatched phenotypes. - Evol. Ecol. 26: 893–907.

Karpestam, E., Merilaita, S. and Forsman, A. 2013. Detection experiments with humans implicate visual predation as a driver of colour polymorphism dynamics in pygmy grasshoppers. - BMC Ecol. 13: 17.

Kiltie, R. A. 1988. Countershading: Universally deceptive or deceptively universal? - Trends Ecol. Evol. 3: 21–23.

Knill, R. and Allen, J. A. 2010. Does Polymorphism Protect? An Experiment with Human "Predators." - Ethology 99: 127–138.

Lovell, P. G., Ruxton, G. D., Langridge, K. V. and Spencer, K. a. 2013. Egg-laying substrate selection for optimal camouflage by quail. - Curr. Biol. 23: 260–264.

Peralta-Rincon, J. R., Escudero, G. and Edelaar, P. 2017. Phenotypic plasticity in color without molt in adult grasshoppers of the genus Sphingonotus (Acrididae: Oedipodinae). - J. Orthoptera Res. 26: 21–27.

R Core Team 2017. R: A Language and Environment for Statistical Computing. - R Found. Stat. Comput.: {ISBN} 3-900051-07-0, http://www.R-

project.org.

Rowe, C. and Healy, S. D. 2014. Measuring variation in cognition. - Behav. Ecol. 25: 1287–1292.

Ruxton, G., Brown, S., Sherratt, T. and Jackson, A. 2005. The effects of group size, shape and composition on ease of detection of cryptic prey. - Behaviour 142: 811–826.

Shettleworth, S. J. 2001. Animal cognition and animal behaviour. - Anim. Behav. 61: 277–286.

Shettleworth, S. J. 2010. Cognition, evolution, and behavior. - Oxford University Press.

Siddiqi, A., Cronin, T. W., Loew, E. R., Vorobyev, M. and Summers, K. 2004. Interspecific and intraspecific views of color signals in the strawberry poison frog Dendrobates pumilio. - J. Exp. Biol. in press.

Sih, A., Ferrari, M. C. O. and Harris, D. J. 2011. Evolution and behavioural responses to human-induced rapid environmental change. - Evol. Appl. 4: 367–387.

Skelhorn, J. and Ruxton, G. D. 2013. Size-dependent microhabitat selection by masquerading prey. - Behav. Ecol. 24: 89–97.

Skelhorn, J. and Ruxton, G. D. 2014. Viewing distance affects how the presence of inedible models influence the benefit of masquerade. - Evol. Ecol. 28: 441–455.

Skelhorn, J. and Rowe, C. 2016. Cognition and the evolution of camouflage. - Proc. R. Soc. B Biol. Sci. 283: 20152890.

Skelhorn, J., Rowland, H. M., Speed, M. P. and Ruxton, G. D. 2010. Masquerade: camouflage without crypsis. - Science (80-.). 327: 51.

Skelhorn, J., Rowland, H. M., Delf, J., Speed, M. P. and Ruxton, G. D. 2011. Density-dependent predation influences the evolution and behavior of masquerading prey. - Proc. Natl. Acad. Sci. U. S. A. 108: 6532–6.

Sol, D., Griffin, A. S., Bartomeus, I., Boyce, H., Mayr, E., Lefebvre, L., Duncan, R., Blackburn, T., Cassey, P., Lefebvre, L., Sol, D., Bacher, S., Reader, S., Lefebvre, L., Greenberg, R., Mettke-Hofmann, C., Reale, D., Reader, S., McDougall, P., Dingemanse, N., Shochat, E., Warren, P., Faeth, S.,

McIntyre, N., Hope, D., Liker, A., Bókony, V., Møller, A., Kark, S., Iwaniuk, A., Schalimtzek, A., Banker, E., Case, T., Lever, C., Tideman, C., Griffin, A., Boyce, H., MacFarlane, G., Biro, P., Dingemanse, N., Bouchard, J., Goodyer, W., Lefebvre, L., Reader, S., Laland, K., Boogert, N., Reader, S., Laland, K., Crawley, M., Arbuckle, J., Laland, K., Reader, S., Reader, S., Laland, K., Boogert, N., Reader, S., Hoppitt, W., Laland, K., II, L. M., Fitzgerald, L., Hone, J., Overington, S., Morand-Ferron, J., Boogert, N., Lefebvre, L., Gosling, S. and Griffin, A. 2011. Exploring or Avoiding Novel Food Resources? The Novelty Conflict in an Invasive Bird (A Iwaniuk, Ed.). - PLoS One 6: e19535.

Stevens, M. 2015. Anti-predator coloration and behaviour : a longstanding topic with many outstanding questions. - Curr. Zool. 61: 702–707.

Stevens, M. and Merilaita, S. 2009. Animal camouflage: current issues and new perspectives. - Philos. Trans. R. Soc. Lond. B. Biol. Sci. 364: 423–7.

Stevens, M., Párraga, C. A., Cuthill, I. C., Partridge, J. C., Troscianko, T. O. M. S., Psychology, E., Road, W. and Bs, B. 2007. Using digital photography to study animal coloration. - Biol. J. Linn. Soc. 90: 211–237.

Stevens, M., Marshall, K. L. A., Troscianko, J., Finlay, S., Burnand, D. and Chadwick, S. L. 2013. Revealed by conspicuousness: distractive markings reduce camouflage. - Behav. Ecol. 24: 213–222.

Thayer, A. H. 1896. The Law Which Underlies Protective Coloration. - Source Auk 13: 124–129.

Troscianko, J. and Stevens, M. 2015. Image Calibration and Analysis Toolbox - a free software suite for objectively measuring reflectance, colour and pattern (S Rands, Ed.). - Methods Ecol. Evol. 6: 1320–1331.

Vorobyev, M. and Osorio, D. 1998. Receptor noise as a determinant of colour thresholds. - Proc. R. Soc. London B Biol. Sci. in press.

Webster, R. J., Callahan, A., Godin, J.-G. J., Sherratt, T. N., Bond, A. B., Kamil, A. C., Bond, A. B., Kamil, A. C., Cott, H. B., Cuthill, I. C., Székely, A., Cuthill, I. C., Partridge, J. C., Bennett, A. T. D., Church, S. C., Hart, N. S., Hunt, S., Cuthill, I. C., Stevens, M., Sheppard, J., Maddocks, T., Parraga, C. A., Troscianko, T. S., Endler, J. A., Fraser, S., Callahan, A., Klassen, D., Sherratt, T. N., Hart, N. S., Hunt, D. M., Kelman, E. J., Baddeley, R. J., Shohet, A. J., Osorio, D., Kettlewell, H. B. D., Kingsland, S., Merilaita, S.,

Merilaita, S., Merilaita, S., Lind, J., Moss, R., Jackson, R. R., Pollard, S. D., Pietrewicz, A. T., Kamil, A. C., Ray, S., Rensink, R. A., Oregan, J. K., Clark, J. J., Ruxton, G. D., Sherratt, T. N., Speed, M., Sargent, T. D., Sargent, T. D., Sargent, T. D., Sargent, T. D., Sargent, T. D., Sargent, T. D., Keiper, R. R., Schaefer, H. M., Stobbe, N., Stevens, M., Stevens, M., Merilaita, S., Stevens, M., Cuthill, I. C., Párraga, C. A., Troscianko, T., Stevens, M., Cuthill, I. C., Windsor, A. M. M., Walker, H. J., Thayer, G. H., Thomas, R. J., Bartlett, L. A., Marples, N. M., Kelly, D. J., Cuthill, I. C., Wilkinson, D. M., Sherratt, T. N. and Zar, J. H. 2009. Behaviourally mediated crypsis in two nocturnal moths with contrasting appearance. - Philos. Trans. R. Soc. Lond. B. Biol. Sci. 364: 503–10.

Wilkens, R. T. 1993. On the cryptic side of life: being unapparent to enemies and the consequences for foraging and growth of caterpillars (NE (Binghamton U Stamp Binghamton, NY), Ed.). - Chapman and Hall.

Wilson-Aggarwal, J. K., Troscianko, J. T., Stevens, M. and Spottiswoode, C. N. 2016. Escape Distance in Ground-Nesting Birds Differs with Individual Level of Camouflage. - Am. Nat. 188: 000–000.

Xiao, F. and Cuthill, I. C. 2016. Background complexity and the detectability of camouflaged targets by birds and humans. - Proc. R. Soc. London B Biol. Sci. in press.

Supporting information

Table S1. Overview of effects on the probability to perch on a line and their statistical support, using the specific background of the lines to calculate the background-grasshopper differences in colour and luminance (generalized linear model, binomial family). The coefficient for the reference categories (not listed) is always zero. Significant effects ($p<0.05$) are highlighted in bold.

	Estimate	Std. Error	z value	p-value
(Intercept)	-0.274	3.034	-0.090	0.928
JND Lines - grasshoppers colour difference	**0.211**	**0.072**	**2.914**	**0.004**
JND Lines - grasshoppers luminance difference	0.028	0.041	0.693	0.489
Sex (male)	-0.482	0.630	-0.765	0.444
Day (2nd day)	-0.227	0.238	-0.955	0.339
Size	-0.005	0.012	-0.373	0.709

Table S2. Overview of effects on the probability of predation of the grasshoppers and their statistical support (generalized linear model, binomial family). The coefficient for the reference categories (not listed) is always zero. Significant effects ($p<0.05$) are highlighted in bold.

	Estimate	Std. Error	z value	p-value
(Intercept)	**1.583**	**0.419**	**3.779**	**<0.002**
Gender of the observer (woman)	0.003	0.072	0.039	0.969
Sex of the grasshopper (female)	0.010	0.174	0.057	0.954
Type of background (natural soil)	0.402	0.591	0.681	0.496
Aligned with lines (yes)	**-0.421**	**0.097**	**-4.323**	**<0.002**
JND Background - grasshoppers colour difference	**0.120**	**0.051**	**2.341**	**0.019**
JND Background - grasshoppers luminance difference	-0.016	0.010	-1.680	0.093
Density of grasshoppers	**-0.061**	**0.019**	**-3.139**	**0.002**

Chapter V

Background colour matching increases with risk of predation in a colour-changing grasshopper

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Abstract

Cryptic colouration can be adjusted to the local environment by physiological (rapid) change, and/or by morphological (slow) change. The threat-sensitivity hypothesis predicts that the degree of crypsis should respond to the risk of predation (assuming some cost to crypsis). This has not been studied for morphological colour changers, so we manipulated the colour of the rearing substrate (black versus white) and the perceived risk of predation (higher versus lower) for the grasshopper Sphingonotus azurescens. Over a period of several weeks, both nymphs and adults greatly adjusted the brightness of their body towards that of the substrate. Moreover, when individuals were exposed to a greater simulated predation risk (disturbance by hand), they became even more similar in brightness to their substrates, apparently augmenting their degree of crypsis. This study on a morphological colour changer shows that the degree of cryptic colouration (body brightness) is under individual control and appears to change adaptively in response to increased predation risk. In addition, based on analyses of systematic differences in colour in lab-reared offspring, we found indications that even in colour changers there is genetic variation in colouration among individuals, and that populations have diverged adaptively. Such integration of factors determining the cryptic phenotype improves our understanding of the natural selection and constraints imposed on crypsis, which influence both its optimization and evolution.

Keywords

camouflage, crypsis, environmental variability, heritability, morphological colour change, Oedipodinae, phenotypic plasticity, threat-sensitivity hypothesis

Introduction

Improving crypsis (the ability to avoid detection when potentially perceivable by an observer) is an obvious example of adaptation, widely known and easily recognized even by the general public. For visual concealment, it does not just depend on individual characteristics: the level of crypsis is an interaction between the colouration of an individual and that of the environment (e.g. Manríquez et al. 2009). Hence, when environments are variable in space or time, a single

phenotype may not be cryptic everywhere or always. Therefore organisms may have been selected for responsiveness to such environmental variation. Indeed, in several species changes in cryptic colouration within individuals are observed (Stevens and Merilaita 2009, Stuart-Fox and Moussalli 2009, Umbers et al. 2014)

This ability to change colour is often divided into two kinds, physiological and morphological colour change (Stuart-Fox and Moussalli 2009, Umbers et al. 2014) Physiological colour change such as in chameleons and cephalopods occurs by movement (dispersion or concentration) of pigments within the skin, taking place over a time scale of milliseconds up to hours. As such, it can be highly dynamic and responsive to changes in the environment, and indeed has been recorded to respond to environmental factors relevant for crypsis, like background colouration (e.g., Ramachandran et al. 1996; Manríquez et al. 2009). In contrast, morphological colour change occurs by changes in the number and proportion of pigment-containing cells and the amount and quality of pigments deposited in them, and normally takes place over a time scale of days to months (Stuart-Fox and Moussalli 2009, Umbers et al. 2014).

Almost without exception, studies of slow colour change have focused on the ability and benefits of organisms to adapt to the colour of the environment alone (Umbers et al. 2014). However, for any anti-predation trait in general, the threat-sensitivity hypothesis (Helfman 1989) states that if there are costs to an anti-predation trait (e.g. production costs, or interference with other functions), the expression of the anti-predation trait should be adjusted to the predation risk. There are a few examples of rapid (physiological) colour changers becoming more cryptic in the presence of predators (Hemmi et al. 2006; Stuart-Fox, Moussalli and Whiting 2008; Stuart-Fox and Moussalli 2009). However, other studies could not fully confirm such an effect (e.g., Garcia and Sih 2003; Segev 2009; Garcia, Paoletti and Blaustein 2009). Moreover, there are no such examples for morphological or slow physiological colour changers.

The grasshopper *Sphingonotus azurescens* (Rambur, 1838) is a member of the subfamily Oedipodinae. The species is found on soils of sand or clay with a variable degree of stones in a Mediterranean climate (hot summers) (Husemann et al. 2013). When active (only during warm and sunny days), it is almost exclusively found on sparsely or unvegetated soils, and it does not climb or perch

on (vertical) plants. In the wild, populations typically match the colour of the substrate on which they are found, e.g. individuals on a reddish-brown clay soil are also reddish-brown and individuals on white sand are very pale grey (Vosseler 1903, Eisentraut 1927). This strong colour matching may be necessary because individuals mainly are born in May but reproduce in September, so high daily survival rates might be required. Predators on nymphs and adults range from ants, wasps and jumping spiders (P Edelaar, personal observations) to lizards, mammals and birds, the majority of which are (at least partly) visual hunters.

The typical match between the colour of grasshoppers and the soil on which they are found could be explained by natural selection favoring more cryptic genotypes (classical local adaptation, assuming colour is heritable), or because individuals preferentially disperse to and settle on soils on which they are more cryptic given their own colour (matching habitat choice: Edelaar et al. 2008; Edelaar and Bolnick 2012). However, it has also been experimentally confirmed that several species of the Oedipodinae are able to change colour during their development to match that of the whitish-yellow, reddish-brown or bluish-grey soils on which they live, a form of phenotypic plasticity called homochromy (reviewed by Rowell 1971). Homochromy is typically thought to reduce the risk of predation (Rowell 1971; Yerushalmi and Pener 2001; Hochkirch et al. 2008; see also Discussion). This is a slow, morphological colour change that occurs when the nymph molts into the next stage or into the final adult stage (Rowell 1971). In our species there are six nymphal stages which each take about one week, and adaptive colour change seems most pronounced in the last three stages. As is the case in some other grasshopper species (Tanaka 2000), adults can become darker as well even though they cannot molt anymore, but this is a slow process taking several weeks or even months (P Edelaar, unpublished data). In addition, adults are larger and can fly, and live for several months, so compared to nymphs they are exposed to much more spatial and/or temporal environmental variability.

In view of these considerations, we test here for this morphological colour changer whether its colouration is influenced by the risk of predation, and we do so both for nymphs and adults. Additionally we test if average colouration and plasticity in colouration are heritable, such that they can evolve through selection.

To test for signatures of selection, we compare the colouration and plasticity of three populations from the wild.

Material and methods

Experimental design

The design is summarized in Figure 1. We caught adult grasshoppers at three locations in the south of Spain (province of Seville). These three locations have different soil characteristics: population 1 is intermediate in soil brightness (pale clay mixed with pale and dark stones), population 2 has the brightest soil (only pale clay), and population 3 has the darkest soil (mostly a dense cover of relatively dark stones). Each sample was placed in its own communal breeding box in the laboratory where individuals freely mated and laid clutches of eggs. Subsequently all clutches were collected and stored individually. Hence, nymphs originating from each clutch can be assigned to one of the three field locations and are known to share at least the same mother and possibly the same father, but we do not know their parents individually. After hatching, we reared the nymphs of a given clutch in a single transparent plastic box (Fauna Box, 11.7*17.8 cm floor surface) under identical conditions. Briefly, water was obtained by chewing a moist cotton plug closing a plastic laboratory test tube filled with mineral water that was placed upside down. As the species is an omnivore, *ad libitum* food was a mixture of dried wheat bran (45%), dried mosquito larvae (45%), and infant formula milk powder (10%). Heat was provided from below the boxes by electric terrarium heating mats, resulting in cage temperatures between 35 and 40 degrees Celsius. Light was provided by normal office fluorescent ceiling tubes.

To trigger environmental effects in the development of colour, after reaching the third nymphal stage we moved nymphs to differently coloured rearing boxes (Figure 1). We did not move younger stages because these seem to have little plasticity and are too sensitive to the handling. Boxes were either painted black or

white on the inside, and as part of another investigation half of the boxes had a layer of small stones of the same colour as the paint on the bottom. We moved 10 nymphs per family to 32 experimental rearing boxes (n=32 families, n=320 individuals). We divided the 10 nymphs of the same clutch equally over a white and a black box to distinguish between consistent clutch (potentially genetic) effects and induced environmental (box colour) effects on the resulting colour of grasshoppers (Figure 1). At the same time, to better control for box effects, each box received 5 nymphs from two different clutches (marked by clipping the distal part of the left or right tarsus of the second leg, which is normally not recovered in subsequent molts; Hagler and Jackson 2001). Using clutches from parents collected at three different field sites (populations) allowed us to test for an effect of population identity on colour, assumed to reflect population genetic differentiation. Families and populations may also differ in plasticity, i.e. may respond stronger or weaker to the manipulation of environmental colour. Since we divided the nymphs of all clutches over black and white boxes, genetic variation in plasticity in response to box colour among families and populations could therefore also be tested.

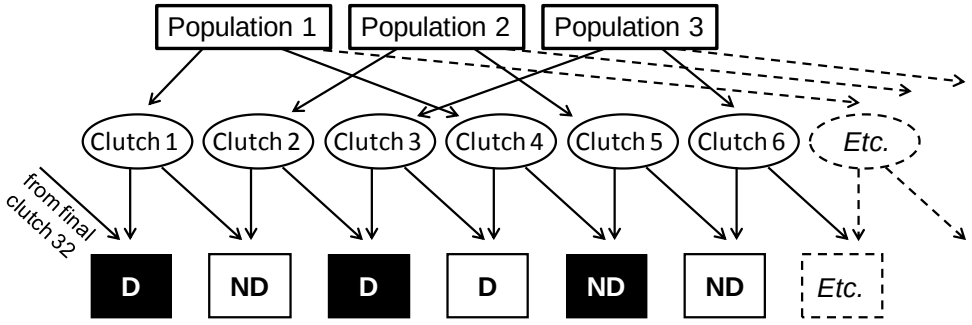

Figure 1. Overview of the experimental design. Grasshoppers from three different populations produced clutches in captivity. Nymphs from each clutch were split over two distinct environments: a black box or a white box. Each box received nymphs from two different clutches. Half of the boxes was exposed to a disturbance treatment (D), or acted as a control with no additional disturbance (ND): this treatment was balanced for white vs. black boxes, but necessarily unbalanced for clutch identity.

Finally, to simulate a higher risk of predation, in half of the boxes of each colour (Figure 1) all grasshoppers were intentionally disturbed twice per day by flushing and (if necessary) touching all individuals until they responded with the typical jumping escape behavior.

Generally, the disturbance only had to be applied just for a few seconds, and during the course of the experiment it became easier to disturb treated individuals: just scratching the roof of the cage resulted in vigorous and repeated jumping until the scratching stopped. The same happened when changing food and water, whereas individuals in the control treatment were much calmer: this confirms that the individuals in some way responded to the treatment. (In a subsequent experiment we also found that individuals prefer the habitat in which they are not disturbed; Edelaar et al. unpubl. data). This disturbance treatment was maintained until all individuals had reached the adult stage (on average after 44.3 days for the white boxes and 45.1 days for the black boxes, with less than 0.1 day difference between disturbance treatments). Even though only about 43% of the individuals reached the adult stage (mostly due to failed molting and cannibalism), this proportion was identical for the two disturbance treatments.

Data collection

To measure the colour of individuals we took digital photographs of last stage nymphs and first-week adult grasshoppers (approximately 10 days between these measures). These images were taken with a Pentax K-r camera mounted on a tripod at a constant height with a Pentax 18-55mm zoom lens and a dual flash with diffusers, using fixed camera settings (55mm zoom, f=14, shutter speed=1/50, ISO=200), fixed flash settings, and a constant ambient lighting. Following Hochkirch et al. (2008), individuals were immobilized by pressing them down with a clean and transparent plastic lid into a Petri dish filled with cotton wool, such that the dorsal part of their pronotum was parallel to the front of the camera lens (i.e. "flat"). Petri dishes were placed on a white sheet of paper on which we wrote identifying information which was included in the image (Figure 2).

To quantify grasshopper colouration, we defined a diamond-shaped polygon representative for the global body colour in the metazone of the pronotum (Figure 2). Since the grasshoppers were reared in white and black boxes, we measured their brightness as the percentage of reflectance (based on the grey layer). To do this we extracted the RGB values of the images using the software ImageJ (Schneider et al. 2012). Next, we followed Stevens et al. (2007) on how to linearize these RGB values (i.e. how to correct for camera-specific spectral sensitivities). We obtained images of a set of reflectance standards (an X-rite ColorChecker Passport) taken with the same camera and settings, and determined a calibration curve for the camera response to changes in light intensity. This was used to derive a linearization equation, which we applied to linearize our original RGB values. We also determined the ratio between the camera's response in the R, G, and B channels with respect to the reflectance standards, and equalized the response of the different colour channels. In spite of the constant environmental lighting and fixed camera and flash settings, we detected variation in lighting across pictures. We corrected for this in our data values by including the brightness value of a grey standard in each picture. For this we used the white background paper as a 82.87% reflectance standard (which was found to be consistent in its reflectance values, without fluorescence and with a flat reflectance spectrum across all measured wavelengths, as checked with a spectrophotometer Konica-Minolta CM-2600d).

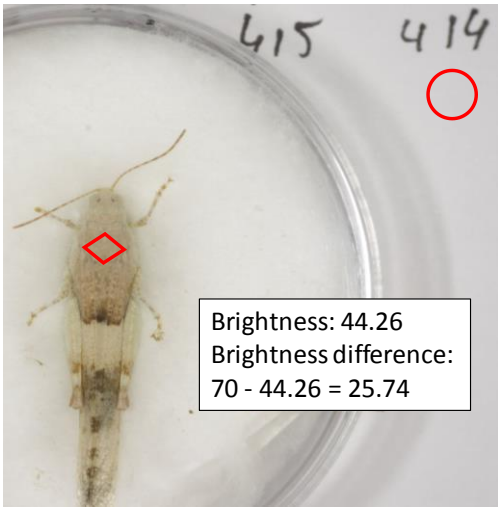

Figure 2. Example of an image to measure brightness. The grasshopper was held in place by the transparent lid of the Petri dish to obtain a correct position. Brightness was measured in the red diamond-shaped part of the thorax. The brightness of a small area of the white background paper was also measured (red circle). This was later used in our models to statistically correct for differences in illumination between images.

Data analysis

We omitted a very small percentage of individuals for which we did not know their exact clutch identity (due to loss of the opposite tarsus as well). Since we could not apply individual marks to nymphs and therefore did not track individuals from nymph to adult stage, nymphs (n=177) and adults (n=138) are analyzed separately to avoid pseudo-replication (i.e. including the same individual multiple times without correcting for this).

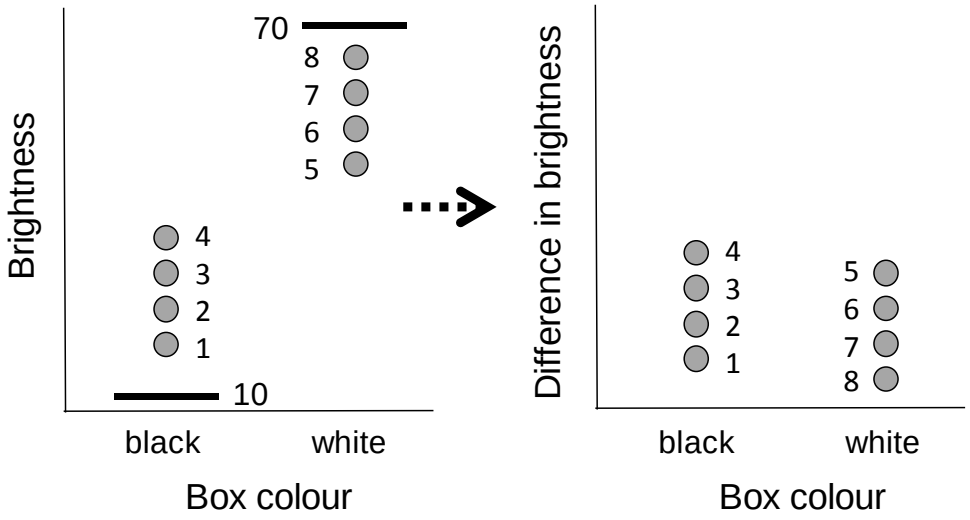

Figure 3. Converting brightness measures into absolute differences in brightness. The brightness of each individual (# 1-8) was contrasted with the fixed brightness level of its environment, depending on the colour of box it was reared in (70 for white boxes, 10 for black boxes).

As we are interested in testing how closely the grasshoppers resembled their environments, we used as dependent variable the difference in brightness between each grasshopper and its box (Figure 3). None of the grasshoppers was as dark as the black boxes, or as pale as the white boxes. We therefore simply calculated the absolute difference with an arbitrary high brightness value (70) if reared in a white box or low brightness value (10) if reared in black box, such that the differences between grasshopper and rearing background were roughly

comparable between colour treatments (convenient for graphical reasons; Figure 3).

We modeled these brightness differences with linear mixed models using *R* software (R Core Team 2017) and the package *lme4* (Bates et al. 2014). Fixed effects were fitted for disturbance (yes/no), sex (male/female; nymphs were not sexed), and population (three field locations). Population was fitted as a fixed effect because we have only three levels, and because we are interested in each level, also in interactions. Note that these variables test whether individuals of a certain category (e.g. disturbed grasshoppers) are better than individuals of another category (e.g. undisturbed grasshoppers) at approximating the brightness across *both* environments. We furthermore statistically controlled for the effects of box colour (black/white), for the presence of a layer of stones in the boxes (yes/no), and for potential spatial effects of location of the boxes (expressed as rank order on the shelves). We also fitted the biologically more interesting or likely interactions: box colour*disturbance, box colour*sex, box colour*population, and sex*disturbance. We did not fit all possible interactions to avoid increasing type 1 error and obtaining spurious results. Finally, we included family (clutch ID) as a random (hierarchical) design variable since we measured several related individuals of the same clutch and therefore need to correct for their likely non-independence.

To test in more detail for potential genetic effects on grasshopper colouration in the presence of variation in the environment, we modeled the brightness (not the difference in brightness) of all individuals as a function of the same variables as mentioned above. An effect of population (fixed effect) indicates consistent differences in brightness between populations (different intercepts), while the interaction population*box colour indicates variation in the degree of plasticity between populations (different slopes in the response to box colour). Similarly, an effect of clutch when fitted as a random intercept indicates consistent differences among clutches in brightness, while an effect of the random slopes of clutches indicates variation in plasticity among clutches (i.e. different slopes in the response to box colour).

To evaluate statistical support for a certain effect we used the Akaike Information Criterion (AIC), where a lower AIC value for a given model indicates

greater statistical support for it (Burnham and Anderson 2002). To determine support for a focal interaction we compared the full model with the model without the focal interaction. To determine support for a focal main effect, we first removed all interactions it was involved in and then compared this model with the model without the focal main effect (since interactions should not be left in a model without its main effects, and since removing a main effect together with its interactions at the same time gives inconclusive results with respect to which effect one is really testing). Similarly and for comparison, we also obtained p-values by doing the same model comparisons using a log-likelihood ratio test. Following Bolker et al. (2009), to evaluate fixed effects we used maximum likelihood to fit the mixed models, whereas for random effects we used restricted maximum likelihood.

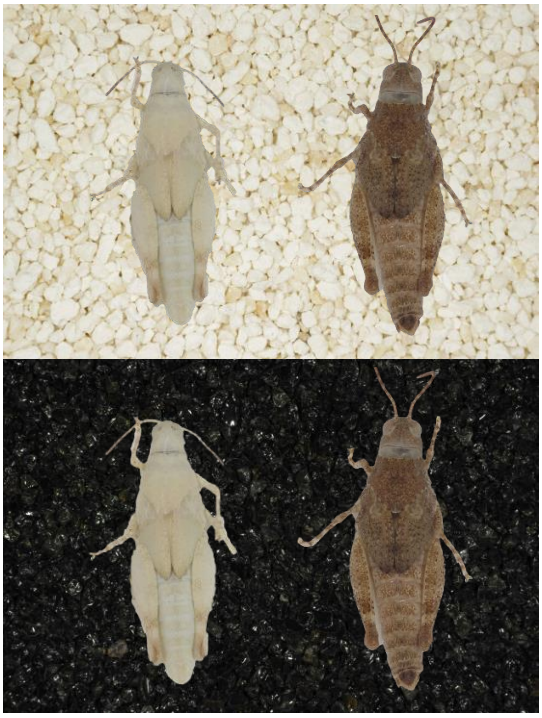

Figure 4. A grasshopper nymph reared on a white background (left images) is less conspicuous on this white background (top background) whereas a nymph reared on a black background (right images) is less conspicuous on a black background (bottom background). (Image of each nymph pasted on top of either background). Note that they are never as pale or as dark as the respective backgrounds on which they were reared, such that the plasticity response remains imperfect.

Results

The grasshoppers strongly adjusted their colouration to that of the environment: grasshoppers in white boxes were much paler than those in black boxes (Figure 4). To put the observed differences into context, the difference between nymphs reared in black vs. white boxes was 15.6 points on the 100 point CIELab scale from pure black to pure white, and adults differed somewhat less at 10.0 points between these colour treatments.

There was good statistical support for an effect of disturbance on the brightness difference (Table 1): grasshoppers exposed to disturbance were more similar to the environment (Figure 5). In other words, compared to the control treatment, disturbed grasshoppers in white boxes became even paler whereas those in black boxes became even darker. This was true both in nymphs and in adults (Table 1, Figure 5).

There was little support for differences between the sexes or populations in brightness difference, indicating that across environments these are equally similar to their environment (Table 1). However, in adults there was good support for an interaction between box colour and population: specifically, individuals from Population 2 (originating from the brightest soil) are a bit paler and therefore were more similar to the environment in the white treatment, whereas individuals from Population 3 (originating from the darkest soil) are a bit darker and therefore were more similar to the environment in the black treatment (Table 1). In adults there was also good support for an interaction between box colour and sex: females are a bit brighter so were more similar to the environment in the white treatment, whereas males are a bit darker so were more similar to the environment in the black treatment (Table 1). There was also strong support for an effect of box colour in adults: this effect simply depends on our specific (yet subjective) choice of reference brightness of the black and whites boxes (Figure 3) to plot the brightness difference of Figure 5, but was included in table 1 for completeness.

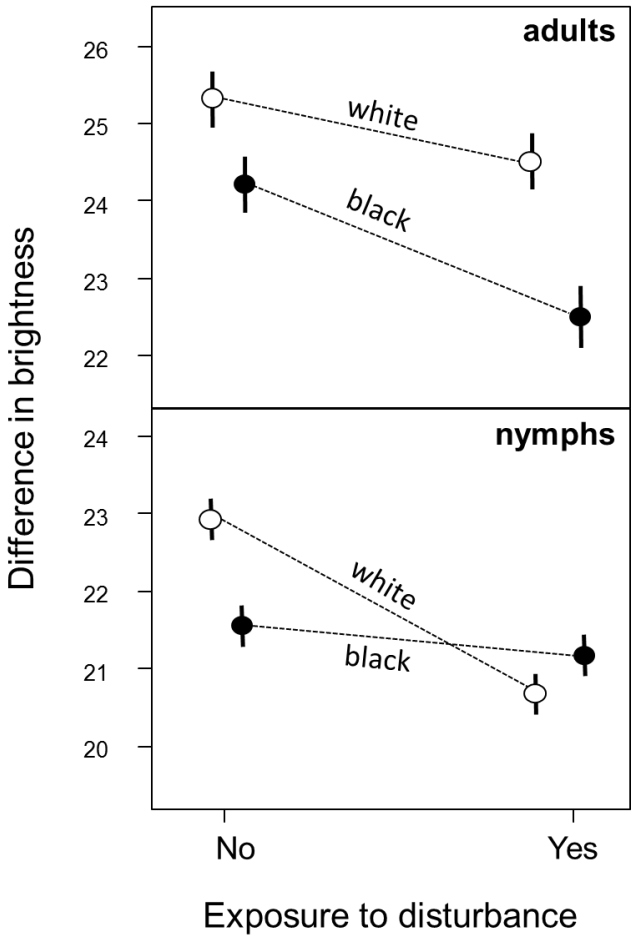

Figure 5. Grasshopper adults (top panel) and nymphs (bottom panel) reduce their difference in brightness with the environment (model estimated means ± SE) when they are exposed to disturbance (simulated predation risk). Results are plotted separately for individuals reared in black or in white boxes.

Table 1. Overview of statistical support for effects on nymph and adult grasshopper colouration (expressed as difference in brightness between grashopper and rearing environment). The coefficient for the reference categories (not listed) is always zero. Changes in AIC values and p-values were obtained by comparing models with versus without the variable of interest (see text). AIC changes that to us indicate meaningful statistical support are highlighted in bold.

Tested effect *(+ category of provided coefficient)*	**Nymphs**			**Adults**		
	coefficient	***ΔAIC***	***p-value***	***coefficient***	***ΔAIC***	***p-value***
Disturbance (yes)	-0.38	**-1.52**	**0.060**	-1.92	**-3.0**	**0.030**
Box colour (white)	-0.54	1.4	0.45	-2.27	**-6.47**	**0.003**
Population (2)	0.51	2.8	0.54	0.10	2.8	0.56
Population (3)	-0.23			-1.85		
Sex (male)	*sex of nymphs unknown*			-1.83	3.3	0.72
Box colour * Disturbance (white*yes)	-1.87	0.07	0.16	0.91	1.1	0.35
Box colour * Population (white*2)	0.73	3.7	0.87	-0.40	**-3.7**	**0.021**
Box colour * Population (white*3)	0.72			2.69		
Box colour * Sex (white*male)	*sex of nymphs unknown*			3.88	**-13.9**	**< 0.001**
Disturbance * Sex (yes*male)	*sex of nymphs unknown*			0.36	1.8	0.69

We also obtained some evidence for heritable effects on brightness. In nymphs there was a well-supported family effect (change in AIC = -3.2, p = 0.0428), although this effect is quite modest in comparison with the strong effect of box colour (Figure 6). As already indicated in the results above, in adults an effect of population of origin was strongly supported (change in AIC = -7.4, p = 0.003), with parents of Population 2 (originating from the brightest soil) producing paler adult offspring and parents of Population 3 (originating from the darkest soil) producing darker adult offspring. We found virtually no support for heritable differences in plasticity in nymphs or adults, neither at the family nor at the population level (all p-values > 0.54).

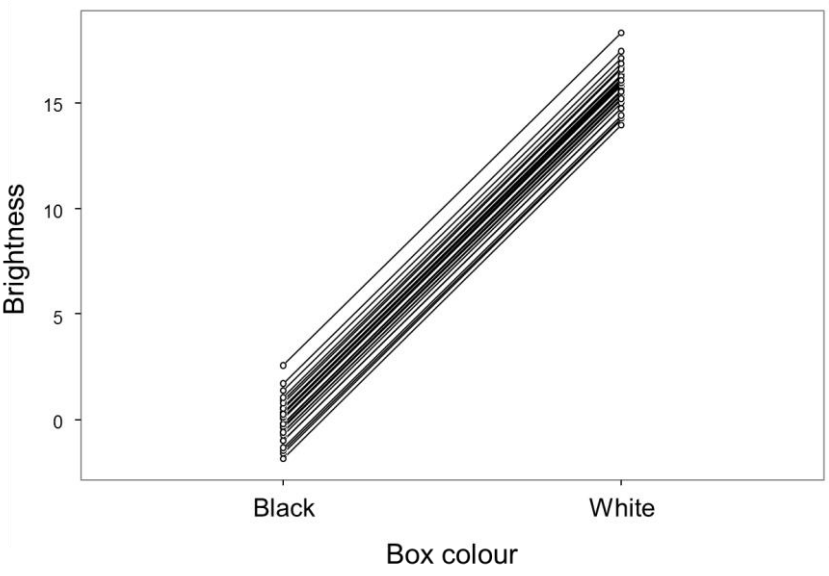

Figure 6. Grasshopper families differ consistently in their colouration (brightness) as nymph across environments. Lines depict the differences in average brightness among families (N=32) whose members are exposed to either a black (reference value here) or a white coloured rearing box, as estimated by a mixed model random intercept effect. Slopes are drawn parallel in view of the lack of support for variation among families in slopes (see text).

Discussion

Interpretation of results

Our main result is that grasshoppers exposed to a greater simulated risk of predation (disturbance by hand) show a greater similarity between their own brightness and that of the environment, and hence appear to be more cryptic (Table 1, Figure 5). An effect of risk of predation on change in cryptic colouration has been little studied and has met with mixed results. As far as we know, this is the first time that such threat-sensitivity has been shown by experimental manipulation in a morphological colour changer. However, before discussing the implications of this result, other potential explanations for the variation in colouration among our grasshoppers need to be evaluated.

Two other main explanations for animal colouration are thermoregulation, and signaling. Grasshoppers can indeed obtain a greater heating from environmental radiation if they are darker (e.g. Forsman et al. 2002; Ahnesjö and Forsman 2006). However, if the brightness of grasshoppers changed in our experiment in response to variation in environmental temperature, we would expect them to be darker on the paler (colder) substrate, while we observed the reverse. In addition, we cannot conceive how additional disturbance could have an interactive effect on thermoregulation, with individuals on pale substrates getting even paler and individual on dark substrates getting even darker, so we discard this alternative explanation. With respect to signaling, sexual dimorphism in colouration is also very restricted in this species (the dimorphism in brightness we observed here is very minor) and males and females responded similarly to the disturbance treatment. In addition, we again cannot conceive why individuals would change their colours so that they are more similar to their environment (instead of more distinctive), and even more so when disturbance is higher, if these effects would be due to a signaling function of colouration only.

Finally, disturbance may have had an effect on colouration that has nothing to do with the perception of predation risk. For example, mechanical stimulation is used in the lab to simulate population density in the grasshopper *Schistocerca*

gregaria, which can cause the development of a dark, gregarious morph (Pener and Simpson 2009).

However, in our experiment (i) we did not find much support that disturbance decreases overall brightness (no interaction between disturbance and box colour on brightness difference), (ii) this effect cannot explain the observed effect of disturbance on overall brightness similarity (i.e. both for pale and dark backgrounds), and (iii) we did not obtain statistical support for an effect of population density on nymphal or adult colouration (results not shown, even though final density after developmental mortality ranged from 1 to 10 individuals per box). Similar arguments are valid for other interpretations of what the effect of the manual disturbance might have been, such as reduced energy available for pigment production or some other generalized stress response. In none of these cases would we predict that grasshoppers in black environments produce *more* pigment when exposed to disturbance, while grasshoppers in white environments produce *less* pigment when disturbed. These considerations lead us to the most parsimonious conclusions that (i) the grasshoppers change their brightness during development in order to reduce their difference with the brightness of the environmental background, and that (ii) they change it towards an even smaller difference when the risk of predation is greater. In other words, we interpret our results to mean that individuals generally try to be cryptically coloured, but even more so when the risk of predation is higher.

Whether the observed adjustments in brightness of the grasshoppers truly provide any protection against predation due to crypsis remains to be formally tested. The degree of relative crypsis can be measured directly in experimental predation trials. Alternatively, an indirect assessment of crypsis can be done via a comparison of prey and background colouration in the predator´s visual space (Théry and Gomez 2010). However, grasshoppers are exposed to a range of vertebrate and invertebrate visual predators (e.g. spiders, wasps, lizards, birds) with very different visual systems, and of unknown numerical importance for mortality in wild populations. Hence, obtaining an inclusive numerical assessment of crypsis is very challenging (and probably therefore hardly ever done). Nonetheless, given the seemingly clear differences in how individuals stand out against one versus the other background (Figure 4), we do believe that it is

reasonable to assume that the observed reduced difference in brightness between grasshopper and background would overall result in a lower detection and predation risk (due to greater crypsis), as many potential grasshopper predators are visual hunters. We therefore interpret our results to indicate that grasshoppers influence the development of their colouration to become more cryptic, and especially so when the risk of predation is greater.

Implications of greater crypsis under risk of predation

As far as we know, this is the first time that an effect of predation risk on cryptic colouration has been found by experimental manipulation in a morphological colour changer. This has some interesting implications. First, it suggests that apart from the temporal scale and physiological mechanism by which their colours change, morphological and physiological colour changers in general might both respond adaptively to relative predation risk (but more studies on morphological colour changers are needed for this). Second, it fits the classical interpretation(Rowell 1971) that the homochrome response of grasshopper colour to the colour of the environment functions to enhance crypsis, instead of e.g. intraspecific signaling or thermoregulation. Third, it suggests that individuals do not always aim for maximal crypsis in the current environment, and may opt for a more intermediate and less cryptic phenotype when the risk of predation is lower. Since crypsis generally is expected to provide benefits in terms of greater survival probability, this result implies that there must also be costs to crypsis. This observation is in line with the threat-sensitivity hypothesis (Helfman 1989), which states that anti-predation behavior should be adjusted to the risk of predation when anti-predation behavior comes with a cost.

Such costs might include costs related to production ((True 2003, Kemp and Rutowski 2007, Wittkopp and Beldade 2009, Nijhout 2010, Bergstrom et al. 2012, Galván et al. 2015), interference with other functions of colouration (Ahnesjö and Forsman 2006; Stevens and Merilaita 2009; Stuart-Fox and Moussalli 2009; Kronstadt et al. 2013; Civantos et al. 2004; Ahnesjö and Forsman 2006; Karpestam et al. 2011), or interference with future crypsis as the environment

changes or because the individual moves between environments (e.g., Sorensen and Lindberg 1991).

Change in environmental conditions in space or time are known to select for the evolution of a more intermediate, generalist phenotype which might still be moderately cryptic in any environment (Merilaita et al. 1999, Houston et al. 2007, Nilsson and Ripa 2010). This could be an explanation for the reduced response to the environmental colour manipulation seen in adults (56% greater in nymphs than in adults), as adults are more likely to encounter temporal or spatial variation in environments (in view of their greater life span and mobility) and are less plastic than nymphs (Rowell 1971; P Edelaar, unpublished data). Nonetheless, the response to the disturbance treatment seems to be greater in adults than in nymphs, so the results are equivocal in this respect. In general, the various costs of cryptic colouration and their implications have been little studied and deserve more attention.

Environmental versus genetic effects on crypsis

Studies decomposing environmental and genetic contributions to crypsis are relatively scarce, as most focus on only one of these components (but see e.g., Wente and Phillips 2003; Karlsson et al. 2009; Bergstrom et al. 2012). Despite a great degree of plasticity in colouration, we also found evidence for heritable contributions to colouration. We found that members of the same clutch were more similar in colouration than random individuals (Figure 6). While non-genetic maternal effects cannot be excluded by our design, this result suggests that the females we used to produce the clutches held genetic variation for colouration which is expressed in their offspring, independently of the specific rearing environment (Figure 6). If so, natural selection (selective predation) may act on this genetic variation, favoring individuals that are locally more cryptic. Indeed, we also found some support that offspring produced by parents from different populations differed consistently and thus likely genetically in average colouration (a similar result was also obtained when comparing additional populations from reddish brown and dark grey soils, Edelaar et al. unpubl. results). Further confidence that this population differentiation is due to selection on genetic

variation comes from the observation that the population with the palest soil type (Population 2) produced consistently paler individuals in the laboratory environments, while the population with the darkest soil type (Population 3) produced consistently darker individuals, i.e. favoring crypsis. Hence, we conclude that the local adaptation in cryptic colouration seen in field populations is not only due to homochromy (plasticity) as is typically assumed but also partly due to genetic differentiation.

We found no support for heritable differences in plasticity among individual females or among populations. This could be an issue of lack of statistical power (with finite sample sizes, slopes have more stochastic variation then intercepts), but it may also have an unknown biological explanation.

Conclusion

While studies on physiological (rapid) colour changing organisms have provided detailed insights into our understanding of crypsis (Stevens and Merilaita 2009, Stuart-Fox and Moussalli 2009), both from proximate and ultimate perspectives, much less detail has been obtained using morphological (slow) colour changers. This study on a morphological colour changer shows that the degree of crypsis is under individual control and appears to depend on relative costs and benefits of crypsis under different circumstances, such as the colour of the environment and the risk of predation. In addition, genetic effects also seem to contribute to cryptic colouration. Such integration of factors determining the cryptic phenotype improves our understanding of the natural selection and constraints imposed on crypsis, which both influence its optimization and evolution (Stevens and Merilaita 2009, Stuart-Fox and Moussalli 2009).

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Author contributions

P.E. conceived of the study; P.E., G.E., and C.R.-B. designed the study; C.R.-B., G.E., and P.E. made the images; A.B.-V. analysed the images; A.B.-V. and P.E. and carried out the statistical analyses; P.E. and A.B.-V. drafted the manuscript; and all authors revised the manuscript. All authors gave final approval for publication.

References

Ahnesjö, J. and Forsman, A. 2006. Differential Habitat Selection by Pygmy Grasshopper Color Morphs; Interactive Effects of Temperature and Predator Avoidance. - Evol. Ecol. 20: 235–257.

Bates, D., Maechler, M., Bolker, B. and Walker, S. 2014. lme4: Linear mixed-effects models using Eigen and S4.

Bergstrom, C., Whiteley, R. and Tallmon, D. 2012. The heritable basis and cost of colour plasticity in coastrange sculpins. - J. Evol. Biol. 25: 2526–36.

Bolker, B. M., Brooks, M. E., Clark, C. J., Geange, S. W., Poulsen, J. R., Stevens, M. H. H. and White, J.-S. S. 2009. Generalized linear mixed models: a practical guide for ecology and evolution. - Trends Ecol. Evol. 24: 127–35.

Burnham, K. P. and Anderson, D. R. 2002. Model Selection and Multi-Model Inference: A Practical Information-Theoretic Approach. - Springer.

Civantos, E. and Ahnesjö, J. 2004. Indirect effects of prey coloration on predation risk: pygmy grasshoppers versus lizards. - Evol. Ecol.: 201–213.

Edelaar, P. and Bolnick, D. I. 2012. Non-random gene flow: An underappreciated force in evolution and ecology. - Trends Ecol. Evol. 27: 659–665.

Edelaar, P., Siepielski, A. M. and Clobert, J. 2008. Matching habitat choice causes directed gene flow: A neglected dimension in evolution and ecology. - Evolution (N. Y). 62: 2462–2472.

Eisentraut, M. 1927. Beitrag zum Frage der Farbanpassung der Orthopteren an die Färbung der Umgebung. I. Die Farbvariationen von Oedipoda coerulescens und Ihre Beziehung zur Bodenfarbe. - Z. Morph. u. Ökol. Tiere 7: 609–642.

Forsman, A., Ringblom, K., Civantos, E. and Ahnesjö, J. 2002. Coevolution of color pattern and thermoregulatory behavior in polymorphic pygmy grasshoppers Tetrix undulata. - Evolution 56: 349–360.

Galván, I., Jorge, A., Edelaar, P. and Wakamatsu, K. 2015. Insects synthesize pheomelanin. - Pigment Cell Melanoma Res. 28: 599–602.

Garcia, T. S. and Sih, A. 2003. Color change and color-dependent behavior in response to predation risk in the salamander sister species Ambystoma barbouri and Ambystoma texanum. - Oecologia 137: 131–9.

Garcia, T. S., Paoletti, D. J. and Blaustein, A. R. 2009. Correlated trait responses to multiple selection pressures in larval amphibians reveal conflict avoidance strategies. - Freshw. Biol. 54: 1066–1077.

Hagler, J. R. and Jackson, C. G. 2001. Methods for marking insects: current techniques and future prospects. - Annu. Rev. Entomol. 46: 511–43.

Helfman, G. S. 1989. Threat-sensitive predator avoidance in damselfish-

trumpetfish interactions. - Behav. Ecol. Sociobiol. 24: 47–58.

Hemmi, J. M., Marshall, J., Pix, W., Vorobyev, M. and Zeil, J. 2006. The variable colours of the fiddler crab Uca vomeris and their relation to background and predation. - J. Exp. Biol. 209: 4140–53.

Hochkirch, A., Deppermann, J. and Gröning, J. 2008. Phenotypic plasticity in insects: the effects of substrate color on the coloration of two ground-hopper species. - Evol. Dev. 10: 350–9.

Houston, A. I., Stevens, M., Cuthill, I. C. and Street, D. 2007. Animal camouflage: compromise or specialize in a 2 patch-type environment? - Behav. Ecol. 18: 769–775.

Husemann, M., Llucià-Pomares, D. and Hochkirch, A. 2013. A review of the Iberian Sphingonotini with description of two novel species (Orthoptera: Acrididae: Oedipodinae). - Zool. J. Linn. Soc. 168: 29–60.

Karlsson, M., Johansson, J., Caesar, S. and Forsman, a. 2009. No evidence for developmental plasticity of color patterns in response to rearing substrate in pygmy grasshoppers. - Can. J. Zool. 87: 1044–1051.

Karpestam, E., Wennersten, L. and Forsman, A. 2011. Matching habitat choice by experimentally mismatched phenotypes. - Evol. Ecol. 26: 893–907.

Kemp, D. J. and Rutowski, R. L. 2007. Condition dependence, quantitative genetics, and the potential signal content of iridescent ultraviolet butterfly coloration. - Evolution (N. Y). 61: 168–183.

Kronstadt, S. M., Darnell, M. Z. and Munguia, P. 2013. Background and temperature effects on Uca panacea color change. - Mar. Biol. 160: 1373–1381.

Manríquez, P. H., Lagos, N. A., Jara, M. E. and Castilla, J. C. 2009. Adaptive shell color plasticity during the early ontogeny of an intertidal keystone snail. - Proc. Natl. Acad. Sci. U. S. A. 106: 16298–303.

Merilaita, S., Tuomi, J. and Jormalainen, V. 1999. Optimization of cryptic coloration in. - Biol. J. Linn. Soc. 67: 151–161.

Nijhout, H. F. 2010. Molecular and physiological basis of colour pattern formation. - Adv. In Insect Phys. 38: 219–265.

Nilsson, J. and Ripa, J. 2010. The origin of polymorphic crypsis in a

heterogeneous environment. - Evolution (N. Y). 64: 1386–94.

Pener, M. P. and Simpson, S. J. 2009. Locust Phase Polyphenism: An Update. - Adv. In Insect Phys. 36: 1–272.

R Core Team 2017. R: A Language and Environment for Statistical Computing. - R Found. Stat. Comput.: {ISBN} 3-900051-07-0, http://www.R-project.org.

Ramachandran, V. S., Tyler, C. W., Gregory, R. L., Rogers-Ramachandran, D., Duensing, S., Pillsbury, C. and Ramachandran, C. 1996. Rapid adaptive camouflage in tropical flounders. - Nature 379: 815–8.

Rowell, C. 1971. The Variable Coloration of the Acridoid Grasshoppers. - In: Advances in Insect Physiology. pp. 145–198.

Schneider, C. A., Rasband, W. S. and Eliceiri, K. W. 2012. NIH Image to ImageJ: 25 years of image analysis. - Nat. Methods 9: 671–675.

Segev, O. 2009. Effects of background color and predation risk on color change in Fire salamander larvae. - Isr. J. Ecol. Evol. 55: 359–367.

Sorensen, F. E. and Lindberg, D. R. 1991. Preferential predation by American black oystercatchers on transitional ecophenotypes of the limpet Lottia pelta (Rathke). - J. Exp. Mar. Bio. Ecol. 154: 123–136.

Stevens, M. and Merilaita, S. 2009. Animal camouflage: current issues and new perspectives. - Philos. Trans. R. Soc. Lond. B. Biol. Sci. 364: 423–7.

Stevens, M., Párraga, C. A., Cuthill, I. C., Partridge, J. C., Troscianko, T. O. M. S., Psychology, E., Road, W. and Bs, B. 2007. Using digital photography to study animal coloration. - Biol. J. Linn. Soc. 90: 211–237.

Stuart-Fox, D. and Moussalli, A. 2009. Camouflage, communication and thermoregulation: lessons from colour changing organisms. - Philos. Trans. R. Soc. Lond. B. Biol. Sci. 364: 463–70.

Stuart-Fox, D., Moussalli, A. and Whiting, M. J. 2008. Predator-specific camouflage in chameleons. - Biol. Lett. 4: 326–9.

Tanaka, S. 2000. Induction of darkening by corazonins in several species of Orthoptera and their possible presence in ten insect orders. - Appl. Entomol. Zool. 35: 509–517.

Théry, M. and Gomez, D. 2010. Insect Colours and Visual Appearance in the

Eyes of Their Predators. - In: Advances in Insect Physiology. pp. 267–353.

True, J. R. 2003. Insect melanism: the molecules matter. - Trends Ecol. Evol. 18: 640–647.

Umbers, K. D. L., Fabricant, S. A., Gawryszewski, F. M., Seago, A. E. and Herberstein, M. E. 2014. Reversible colour change in Arthropoda. - Biol. Rev. 89: 820–848.

Vosseler, J. 1903. Beiträge zur Faunistik und Biologie der Orthopteren Algeriens und Tunesiens. II. Theil. - In: Zoologische Jahrbücher: Systematik, Geographie und Biologie. pp. 1–98.

Wente, W. H. and Phillips, J. B. 2003. Fixed green and brown color morphs and a novel color-changing morph of the Pacific tree frog Hyla regilla. - Am. Nat. 162: 461–73.

Wittkopp, P. J. and Beldade, P. 2009. Development and evolution of insect pigmentation: genetic mechanisms and the potential consequences of pleiotropy. - Semin. Cell Dev. Biol. 20: 65–71.

Yerushalmi, Y. and Pener, M. P. 2001. The response of a homochrome grasshopper, Oedipoda miniata, to the dark-colour-inducing neurohormone (DCIN) of locusts. - J. Insect Physiol. 47: 593–597.

GENERAL DISCUSSION AND SYNTHESIS

This thesis demonstrates the existence, importance and functioning of two largely overlooked mechanisms of adaptation in the context of environmental changes. Given that each of the five chapters of this thesis already incorporates an exhaustive discussion of the relevant results, this final section represents a synthesis of the most important topics addressed.

The existence of selection on individual variation during the early stages of biological invasion can have as a consequence that what happens in these early stages may impact the success of the introduced populations. Individual variation eliminated by natural selection will not be available in the subsequent stages. **Chapter I** confirms that selection indeed acts during the pre-establishment stages, in this case on genetic variation related to behaviour. We also find that the effects of this selection appear to be reflected in a differentiation between the native population of origin and the invasive populations established in non-native areas. Therefore, mortality of individuals, and so a process that resembles natural selection, does not act in a random way. Instead it is selecting individuals with a specific genotype, as previously predicted (Carrete et al. 2012; Chapple et al. 2012). Behaviour plays a key role in the success of invasions because of its importance in how individuals cope with the new situations that a new environment entails. Hence selection on a behaviour-related gene might influence range expansion into non-native areas (Liebl and Martin 2012) or the exploration of novel food resources (Sol et al. 2011). As far as we know this is the first time that selection on individual variation during the neglected pre-establishment stages of a biological invasion is investigated. Our positive results will hopefully trigger follow-up studies in other systems and in other types in invasions (e.g. unintentional transport).

In **Chapter II** we show that the results of Chapter I are not unusual, but that selection acts on many phenotypic traits during the first stages of the biological invasion. The investigated traits are important traits for survival and reproductive success, so they could therefore be important for the success of the potential invasive population. The ubiquitous presence of pre-establishment selection has

not been previously taken into account, but may be of great importance in the management of invasive species. If the conditions of these first stages shape the invasive potential of the future potentially introduced population, then hopefully we can act on these early stages to change the selective pressures towards individuals with less invasive potential. This would reduce the chances that individuals who finally reach a non-native area will succeed, establish a population, expand and become invasive.

In **Chapter III** we presented an extensive empirical study that estimates the relative importance of the four possible mechanisms of adaptation. We found a significant spatial structure in grasshopper coloration across the four different urban pavements, leading to local crypsis. Grasshoppers overall were more cryptic on their home pavement than they would be on other pavements, even in those cases after we experimentally manipulated the phenotype (body colouration). Surprisingly, two classically studied and assumed dominant mechanisms of adaptation - natural selection and phenotypic plasticity – turn out to play a role of virtually zero importance. Moreover, adjustment of the environment is discarded because of its infeasibility for this system, as there appears to be no manner that grasshoppers can manipulate soil colouration. Instead, our measures, simulations and experiments support that grasshoppers disperse on purpose to those substrates that provide them with greater crypsis. Hence, we show that an often-neglected process (habitat choice) may in fact be a dominant factor in improving ecological performance. In addition, we confirm that this selective, biased dispersal between substrates can create an adaptive population genetic structure, a capacity that is typically believed to be unique to natural selection. We even provide evidence that supports a certain degree of reproductive isolation between grasshoppers living on adjacent substrates, as an indirect effect of habitat choice.

As we manipulated artificially the colouration of grasshoppers and these changed their substrate use accordingly, it indicates that in our study system habitat choice is self-referential. Individuals apparently can evaluate their ecological performance (crypsis) in a certain habitat (type of substrate) and decide to disperse or not accordingly. This type of performance-based habitat choice has been named "matching habitat choice". In this type of habitat choice individuals

have the capacity to assess their degree of phenotype-environment matching (to maximize fitness). However, other forms of non-random dispersal might also occur in the system, and these were not extensively assessed. Habitat selection can be genetically linked to an individual trait that maximizes its fitness for this habitat (e.g. reddish grasshoppers may have an innate genetic preference to disperse to and settle on reddish soils). Alternatively, there may be imprinting effects on the selection of habitat (e.g. grasshoppers raised on dark soils later prefer dark soils). In any case, all these types of habitat choice represent a form of non-random dispersal, and increase the match between the phenotype and the environment, and thereby performance.

These different processes of habitat choice have not been taken into account in most studies of adaptation to new habitats in general and to urban habitats in particular. Yet our results confirm that habitat choice not only is a mechanism that contributes to adaptation, but that it can also be the main mechanism by which the population has adapted to the changes produced by urbanization. In this sense, the divergence between rural and urban populations that many studies seek to explain via natural selection and/or plasticity could actually be due to habitat choice. If so, by means of a non-random distribution across habitats of the individuals with the characteristics that best fit the new habitat, the spatial structure of the population is explained without the need for the intervention of natural selection or phenotypic plasticity.

The results of **Chapter IV** shown that individuals modulate their habitat choice depending on how good their phenotype-environment matching is (crypsis in this case). In this sense, this study investigates habitat choice very similar to **Chapter III**, but on a finer spatial scale. Here, after disturbance by a potential predator, individuals that are not very cryptic move and position themselves aligned with the lines between bricks (which decreases detectability), instead of sitting still on the substrate and trusting on their crypsis. This constitutes a trade-off between positioning behaviour (which requires small-scale movements which increases detection) and crypsis (which benefits from immobility in order to prevent detection by movement). The fact that less-cryptic individuals are more likely to move towards lines confirms the results of chapter III that grasshoppers know their local degree of crypsis: both on a larger scale (substrates) and smaller

scale (lines versus substrate surfaces) their choices are affected by their individual degree of matching between phenotype and environment.

Other important mechanism of adaptation to environmental variation is phenotypic plasticity. In **Chapter V** our grasshoppers exhibited body colouration change along the successive moults, and individuals adjusted their colouration to generally resemble that of the background. Moreover, this adaptive plastic response is also influenced by other aspects of the environment, as individuals with a higher perceived risk of predation adjusted their colouration better with the background. This result, together with the results of the previous **Chapter III**, demonstrates the flexibility in the mechanisms of adaptation, since individuals adaptively modulate their phenotypes as well as their habitats in response to several environmental conditions.

Overall, by studying in detail how individual organisms adapt to the changes produced by global change, we have provided compelling evidence for two mechanisms of adaptation that were neglected before. These two mechanisms share that they are based on taking into account the non-randomness of events that were previously assumed to be random. The selective capture and survival during the initial invasion stages has as a consequence that the introduced populations are not a random subset of individuals of the native population of origin; instead individuals that are finally introduced have particular characteristics that are different from those of source populations. As next steps we need to determine if this is a common and predictable effect, and what the consequences are for invasive potential and impact. In the same way, the non-randomness of movement and dispersal of individuals makes the consequences of the dispersal radically different compared to if it were random (Edelaar and Bolnick 2012). The dispersal of individuals has been typically assumed to be random with respect to genotypes, introducing maladaptive gene flow, and thus limiting the geographic ranges and ecological niches of species (Holt and Gomulkiewicz 1997, Kirkpatrick and Barton 1997). However, non-random dispersal due to habitat choice can deterministically drive adaptive evolution and population structuring.

This can therefore favour colonization and invasion of novel and changing habitats by pre-adapted individuals, increase viability of populations (larger population sizes, greater survival and reproductive rates), maintain greater genetic diversity and even facilitate speciation, if mating occurs within habitats. Future studies will need to determine how common phenotype-dependent habitat choice is, and how important its consequences are compared to other mechanisms driving phenotype-environment matching.

REFERENCES

Abellán, P., Tella, J. L., Carrete, M., Cardador, L. and Anadón, J. D. 2017. Climate matching drives spread rate but not establishment success in recent unintentional bird introductions. - Proc. Natl. Acad. Sci.: 201704815.

Alberti, M., Correa, C., Marzluff, J. M., Hendry, A. P., Palkovacs, E. P., Gotanda, K. M., Hunt, V. M., Apgar, T. M. and Zhou, Y. 2017. Global urban signatures of phenotypic change in animal and plant populations. - Proc. Natl. Acad. Sci.: 201606034.

Barnosky, A. D., Matzke, N., Tomiya, S., Wogan, G. O. U., Swartz, B., Quental, T. B., Marshall, C., McGuire, J. L., Lindsey, E. L., Maguire, K. C., Mersey, B. and Ferrer, E. A. 2011. Has the Earth's sixth mass extinction already arrived? - Nature 471: 51–57.

Barton, N. H., Briggs, D. E. G., Eisen, J. A., Goldstein, D. B. and Patel, N. . 2007. Evolution. - Cold Spring Harbor Laboratory Press.

Berdahl, A., Torney, C. J., Schertzer, E. and Levin, S. A. 2015. On the evolutionary interplay between dispersal and local adaptation in heterogeneous environments. - Evolution (N. Y). 69: 1390–1405.

Berner, D. and Thibert-Plante, X. 2015. How mechanisms of habitat preference evolve and promote divergence with gene flow. - J. Evol. Biol. 28: 1641–1655.

Blackburn, T. M., Lockwood, J. L. and Cassey, P. 2009. Avian Invasions: The Ecology and Evolution of Exotic Birds.

Blackburn, T. M., Pyšek, P., Bacher, S., Carlton, J. T., Duncan, R. P., Jarošík, V., Wilson, J. R. U. and Richardson, D. M. 2011. A proposed unified framework for biological invasions. - Trends Ecol. Evol. 26: 333–339.

Bolnick, D. I. and Otto, S. P. 2013. The magnitude of local adaptation under genotype-dependent dispersal. - Ecol. Evol. 3: 4722–4735.

Brans, K. I., Jansen, M., Vanoverbeke, J., Tüzün, N., Stoks, R. and De Meester, L. 2017. The heat is on: Genetic adaptation to urbanization mediated by thermal tolerance and body size. - Glob. Chang. Biol. in press.

Carrete, M. and Tella, J. L. 2010. Individual consistency in flight initiation

distances in burrowing owls: a new hypothesis on disturbance-induced habitat selection. - Biol. Lett. 6: 167–170.

Carrete, M. and Tella, J. L. J. 2011. Inter-individual variability in fear of humans and relative brain size of the species are related to contemporary urban invasion in birds (FR Adler, Ed.). - PLoS One 6: e18859.

Carrete, M., Edelaar, P., Blas, J., Serrano, D., Potti, J., Dingemanse, N. J. and Tella, J. L. 2012. Don't neglect pre-establishment individual selection in deliberate introductions. - Trends Ecol. Evol. 27: 67–68.

Catford, J. A., Jansson, R. and Nilsson, C. 2009. Reducing redundancy in invasion ecology by integrating hypotheses into a single theoretical framework. - Divers. Distrib. 15: 22–40.

Chapple, D. G., Simmonds, S. M. and Wong, B. B. M. 2012. Can behavioral and personality traits influence the success of unintentional species introductions? - Trends Ecol. Evol. 27: 57–62.

Cheptou, P.-O., Carrue, O., Rouifed, S. and Cantarel, A. 2008. Rapid evolution of seed dispersal in an urban environment in the weed Crepis sancta. - Proc. Natl. Acad. Sci. 105: 3796–3799.

Darwin, C. 1859. On the origins of species by means of natural selection. - London: Murray: 247.

Edelaar, P. and Bolnick, D. I. 2012. Non-random gene flow: An underappreciated force in evolution and ecology. - Trends Ecol. Evol. 27: 659–665.

Edelaar, P., Siepielski, A. M. and Clobert, J. 2008. Matching habitat choice causes directed gene flow: a neglected dimension in evolution and ecology. - Evolution (N. Y). 62: 2462–72.

Ellis, E. C., Goldewijk, K. K., Siebert, S., Lightman, D. and Ramankutty, N. 2010. Anthropogenic transformation of the biomes, 1700 to 2000. - Glob. Ecol. Biogeogr. 19: 589–606.

Holt, R. D. and Gomulkiewicz, R. 1997. How Does Immigration Influence Local Adaptation? A Reexamination of a Familiar Paradigm. - Am. Nat. 149: 563–572.

Husemann, M., Llucià-Pomares, D. and Hochkirch, A. 2013. A review of the Iberian Sphingonotini with description of two novel species (Orthoptera: Acrididae: Oedipodinae). - Zool. J. Linn. Soc. 168: 29–60.

Kirkpatrick, M. and Barton, N. H. 1997. Evolution of a species' range. - Am. Nat. 150: 1–23.

Kolar, C. S. and Lodge, D. M. 2001. Progress in invasion biology: Predicting invaders. - Trends Ecol. Evol. 16: 199–204.

Laland, K. N. and Sterelny, K. 2006. Perspective: seven reasons (not) to neglect niche construction. - Evolution 60: 1751–62.

Liebl, A. L. and Martin, L. B. 2012. Exploratory behaviour and stressor hyper-responsiveness facilitate range expansion of an introduced songbird. - Proc. R. Soc. B Biol. Sci. 279: 4375–4381.

Lowry, H., Lill, A. and Wong, B. B. M. 2013. Behavioural responses of wildlife to urban environments. - Biol. Rev. 88: 537–549.

Maynard Smith, J. 1966. Sympatric speciation. - Am. Nat. 100: 637–650.

McKinney, M. L. 2002. Urbanization, Biodiversity, and Conservation. - Bioscience 52: 883.

Miranda, A. C., Schielzeth, H., Sonntag, T. and Partecke, J. 2013. Urbanization and its effects on personality traits: A result of microevolution or phenotypic plasticity? - Glob. Chang. Biol. 19: 2634–2644.

Puth, L. M. and Post, D. M. 2005. Studying invasion: Have we missed the boat? - Ecol. Lett. 8: 715–721.

Ravigné, V., Dieckmann, U. and Olivieri, I. 2009. Live Where You Thrive: Joint Evolution of Habitat Choice and Local Adaptation Facilitates Specialization and Promotes Diversity. - Am. Nat. 174: E141–E169.

Reaser, J. K. 2008. Non-native wildlife risk assessment: a call for scientific inquiry. - Front. Ecol. Environ. 6: 466.

Ronce, O. 2007. How Does It Feel to Be Like a Rolling Stone? Ten Questions About Dispersal Evolution. - Annu. Rev. Ecol. Evol. Syst. 38: 231–253.

Rose, M. R. and Lauder, G. V 1996. Adaptation. - Academic Press.

Rowell, C. H. F. 1972. The Variable Coloration of the Acridoid Grasshoppers. - Adv. In Insect Phys. 8: 145–198.

Sala, O. E., Stuart Chapin, F., III, Armesto, J. J., Berlow, E., Bloomfield, J., Dirzo, R., Huber-Sanwald, E., Huenneke, L. F., Jackson, R. B., Kinzig, A.,

Leemans, R., Lodge, D. M., Mooney, H. A., Oesterheld, M., Poff, N. L., Sykes, M. T., Walker, B. H., Walker, M. and Wall, D. H. 2000. Global biodiversity scenarios for the year 2100. - Science (80-.). 287: 1770–1774.

Schlichting, C. D. and Pigliucci, M. 1998. Phenotypic Evolution: A Reaction Norm Perspective.

Shochat, E., Warren, P. S., Faeth, S. H., McIntyre, N. E. and Hope, D. 2006. From patterns to emerging processes in mechanistic urban ecology. - Trends Ecol. Evol. 21: 186–191.

Sih, A., Ferrari, M. C. O. and Harris, D. J. 2011. Evolution and behavioural responses to human-induced rapid environmental change. - Evol. Appl. 4: 367–387.

Sol, D., Griffin, A. S., Bartomeus, I., Boyce, H., Mayr, E., Lefebvre, L., Duncan, R., Blackburn, T., Cassey, P.. and Griffin, A. 2011. Exploring or Avoiding Novel Food Resources? The Novelty Conflict in an Invasive Bird (A Iwaniuk, Ed.). - PLoS One 6: e19535.

Sol, D., Lapiedra, O. and González-Lagos, C. 2013. Behavioural adjustments for a life in the city. - Anim. Behav. 85: 1101–1112.

Tuomainen, U. and Candolin, U. 2011. Behavioural responses to human-induced environmental change. - Biol. Rev. 86: 640–657.

Wolf, M., van Doorn, G. S., Leimar, O. and Weissing, F. J. 2007. Life-history trade-offs favour the evolution of animal personalities. - Nature 447: 581–584.

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