Received: 19 May 2017 | Revised: 30 August 2017 | Accepted: 5 September 2017

DOI: 10.1111/mec.14353

ORIGINAL ARTICLE

WILEY MOLECULAR ECOLOGY

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

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Funding information
Max Planck Society; the Spanish Ministry of Economy and Competitiveness, Grant/Award Number: RYC-2009-04860, RYC-2010-07120, RYC-2011-07889, CGL-2012-35232, CGL2013-49460-EXP, CGL2016-79483-P; Fundación Repsol; ICTS, Grant/Award Number: 38/2010

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

1 | 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 & 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, Carrete, Gallardo, & Tella, 2016; Edelaar et al., 2015; Ochocki & 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, Simmonds, & Wong, 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 pre-establishment 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, 2013; Biro & Dingemanse, 2009; Stuber et al., 2013) and relates to the exploration of novel food sources (Sol, Griffin, Bartomeus, & Boyce, 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 (Cote, Fogarty, Weinersmith, Brodin, & Sih, 2010; Duckworth & Badyaev, 2007; Reale, Reader, Sol, McDougall, & Dingemanse, 2007).

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 (Bird Life International 2016; Lever, 2005). Nowadays, the wildlife pet trade is a major source of biological invasion among vertebrates, in particular birds (Abellán, Carrete, Anadón, Cardador, & Tella, 2016; Dyer et al., 2017; Su, Cassey, & Blackburn, 2016). 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, Korsten, et al., 2013; Mueller, Partecke, Hatchwell, Gaston, & Evans, 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, Partecke, 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, Anderson, Braasch, Cang, & Gillette, 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 long-term 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.

2 | METHODS

2.1 | 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, Carrete, Edelaar, Potti, & Tella, 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, and 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.

2.2 | 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 ($ACCL_{yes}$, $N = 99$) or died ($ACCL_{no}$, $N = 44$; 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 $ACCL_{yes} - ACCL_{no}$ 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 ($SURV1_{yes}$, $N = 54$ vs. $SURV1_{no}$, $N = 45$; $SURV2_{yes}$, $N = 11$ vs. $SURV2_{no}$, $N = 43$). 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.

2.3 | 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 r^2 values below 0.5; the average r^2 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, Partecke, 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, Emlen, Rubenstein, & Bogdanowicz, 2005); WBSW7 (McRae & 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, and Dawson (1998).

2.4 | 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* (R Development Core Team 2012, Warnes, 2013). 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, Devillard, & Balloux, 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, Stephens, & Donnelly, 2000). The web tool STRUCTURE HARVESTER was used to combine the STRUCTURE output of 10 independent runs (Earl & von Holdt, 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, Muir, Wang, & Frasier, 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* (Goudet & Jombart, 2015; Jombart, 2008). 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 (Li & Ji, 2005; Nyholt, 2004). 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).

3 | RESULTS

3.1 | 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.

3.2 | 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 $ACCL_{yes}$, to $SURV1_{yes}$ and to $SURV2_{yes;}$ note that survival was assessed at two stages, whereby statistics were averaged because the final surviving group was small; see Section 2). Expected heterozygosity estimates did not differ significantly between the mistnet sample SEN and all other samples (all loci combined; SEN: $H_e = 0.239$, TRAP: $H_e = 0.237$, $ACCL_{yes}$: $H_e = 0.238$, $SURV1_{yes}$: $H_e = 0.237$, $SURV2_{yes}$: $H_e = 0.211$, permutation test: all $p > .05$).

Across all loci, the expected heterozygosity of the two invasive populations (SPA: $H_e = 0.235$, POR: $H_e = 0.238$) 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).

3.3 | 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 ($ACCL_{yes}$ vs $ACCL_{no}$) and survival in captivity ($SURV_{yes}$ vs $SURV_{no}$). 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). The likelihood that the observed extreme frequency shifts occurred in both candidate SNPs together by chance was very low ($p = 1.6 \times 10^{-5}$). 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

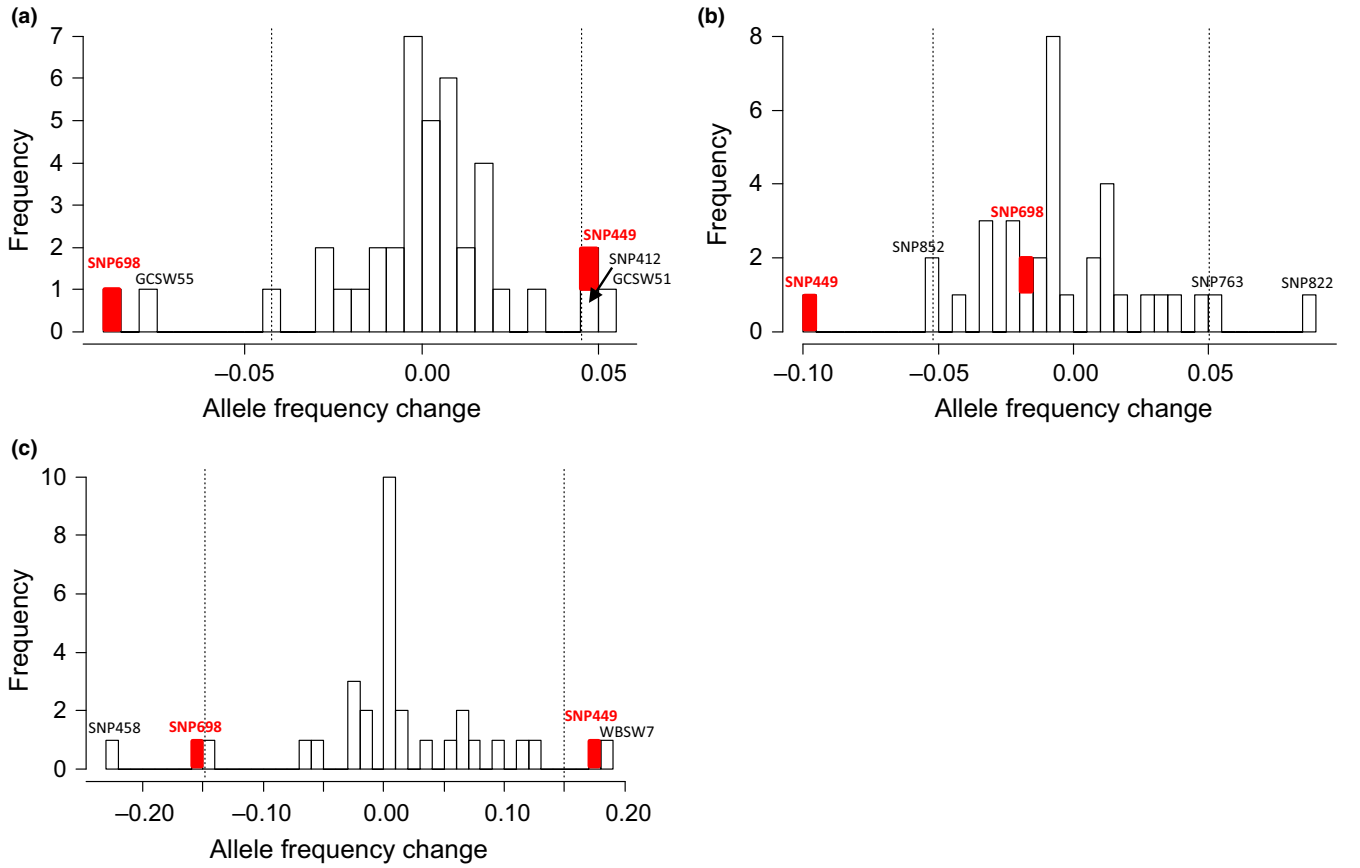

FIGURE 1 Frequency changes of the major alleles in all polymorphic loci (subset of 31 *DRD4* SNPs, and one *SERT* and nine random microsatellites) for the comparisons of (a) the trapping filter (TRAP − SEN), (b) the acclimation filter ($ACCL_{yes}$ − $ACCL_{no}$) and (c) the survival filter ($SURV_{yes}$ − $SURV_{no}$). 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

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.

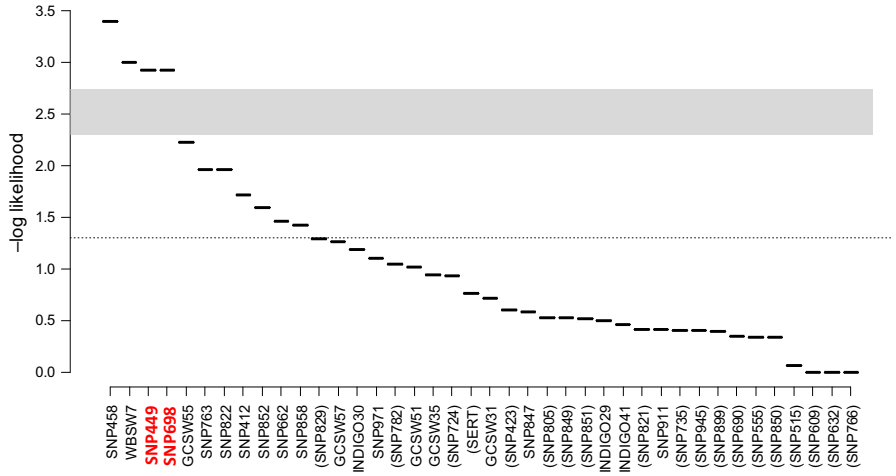

FIGURE 2 Permutation likelihood (-log) of observed (or more extreme) absolute changes in allele frequency for each locus along the three filter comparisons combined. Candidate *DRD4* SNPs are marked in red. Loci monomorphic in all subsamples of the filter comparisons are excluded. Loci with total minor 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

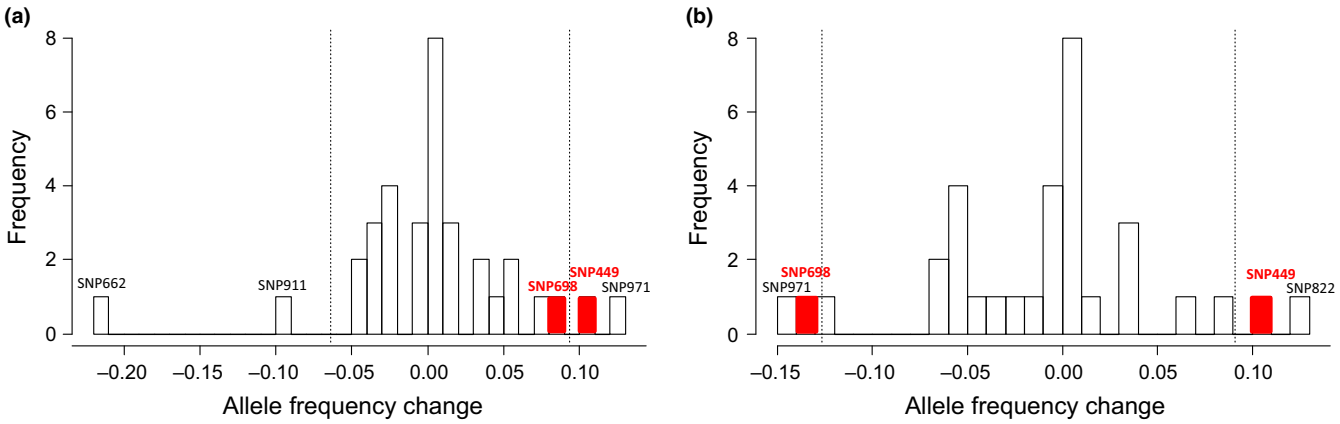

FIGURE 3 Frequency changes of the major alleles in all polymorphic loci (subset of 31 *DRD4* SNPs, and one *SERT* and nine random microsatellites) for the comparison between the Senegalese native population and (a) the Spanish invasive population (SPA − SEN) and (b) the Portuguese invasive population (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

3.4 | 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). 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 $p = .23$).

4 | DISCUSSION

We analysed allelic changes in behaviour-related genes as well as presumably neutral microsatellite loci during the earliest stages of a

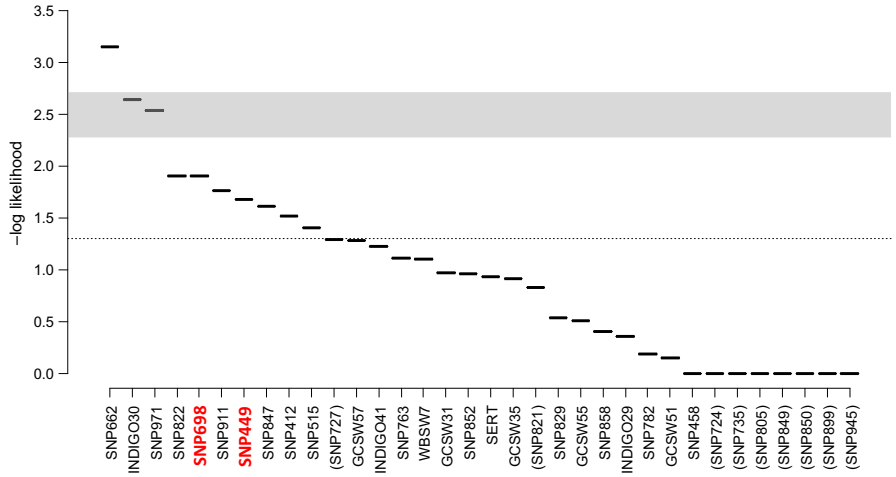

FIGURE 4 Permutation likelihood (-log) of observed (or more extreme) absolute changes in allele frequency for each locus along the two invasive-native comparisons combined. Candidate *DRD4* SNPs are marked in red. Loci monomorphic in all subsamples are excluded. Loci with total minor allele count ≤ 2 in both subsamples combined (see Section 2) 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

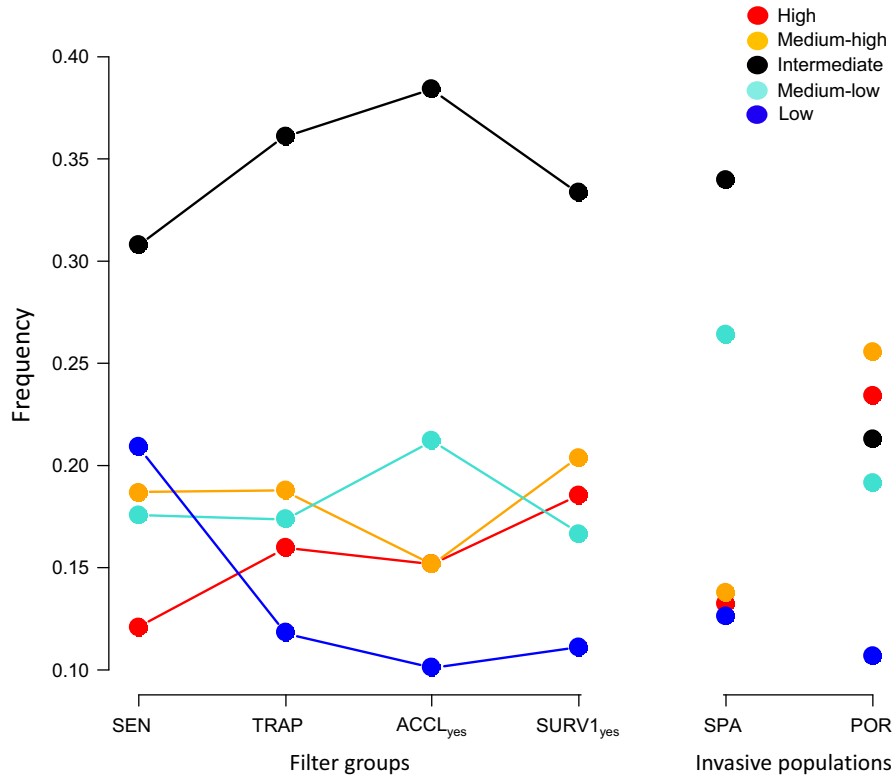

FIGURE 5 Frequencies of SNP449-SNP698 genotype combinations divided into five categories of predicted activity (according to Mueller et al., 2014) for the Senegal reference population (SEN), the different filtered groups (trapped, acclimation survivors and captivity survivors) and the two invasive populations (Spain and Portugal). The frequency in SURV2$_{yes}$ group is not plotted due to its small sample size

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 Chapple et al. (2012) and Carrete 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 and P. Edelaar, 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 frequency-dependent selection; van Oers & Mueller, 2010) with occasional adaptive shifts has been suggested for great tits *Parus major* (Mueller, Korsten, 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, Templeton, Zarmi, & Bar-David, 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, O'Hara, Cano, & Merila, 2008), it might be more informative to investigate specific trait-related 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 & Lesch, 2007; Murphy & Moya, 2011), aggression (Craig & Halton, 2009), distractibility (Maejima et al., 2007), dominance (Miller-Butterworth, Kaplan, Barmada, Manuck, & Ferrell, 2007) and vigilance and cognitive functions (Canli & Lesch, 2007; Homberg & 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, Partecke, 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, Partecke, 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.

ACKNOWLEDGEMENTS

Birds from the invasive populations were caught/purchased and kept in captivity under permit SGYB/FOA/AFR from the Consejería de Medio Ambiente, Junta de Andalucía (Spain), in the authorized centre for experimental avian research SE/16/U (REGA ES410910008016). Permits for capture and captivity in Senegal were obtained from the Ministry of the Environment and the Protection of Nature. The research was approved by the bioethical subcommittee of CSIC, Spain. We thank David Serrano and Markus Hanft for help with data collection; Pepe Ayala, Alberto Jurado, Carlos Camacho and Sebastián Palacios for field work in Senegal; Moussa Séga Diop and Graciela Escudero for essential help with organizing and conducting field work in Senegal; the people of the town Colonat for their warm welcome; and Christine Baumgartner for help in the laboratory. We also thank Yann Bourgeois and three anonymous reviewers for their constructive comments. This work was supported by the Max Planck Society (to B.K.), by the Spanish Ministry of Economy and Competitiveness grant number "BES-2013–062905 to A.B-V." (projects RYC-2009-04860 to M.C.; RYC-2010-07120 to J.B.; RYC-2011-07889, CGL-2012-35232, CGL2013-49460-EXP and CGL2016-79483-P to P.E., with support from the European Regional Development Fund), by Fundación Repsol and grant 38/2010 of the ICTS (Infraestructura Científica y Tecnológica Singular) program to J.L.T.

DATA ACCESSIBILITY

The data that support the findings of this study are available in the supplementary materials.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

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.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Mueller JC, Edelaar P, Baños-Villalba A, et al. Selection on a behaviour-related gene during the first stages of the biological invasion pathway. *Mol Ecol*. 2017;26:6110–6121. https://doi.org/10.1111/mec.14353