Impact of game restocking on common quail populations

Impacto de las sueltas cinegéticas en las poblaciones de codorniz común

Inés Sánchez Donoso

- Are farm-reared quails for game restocking really common quails (*Coturnix coturnix*)?: a genetic approach
- Decreased fitness of restocked hybrid quails prevents fast admixture with wild common quails
- Postcopulatory sexual selection favors fertilization success of restocking hybrid quails over native common quails
- Detecting slow introgression of invasive alleles in an extensively restocked game bird

Inés Sánchez Donoso
PhD thesis

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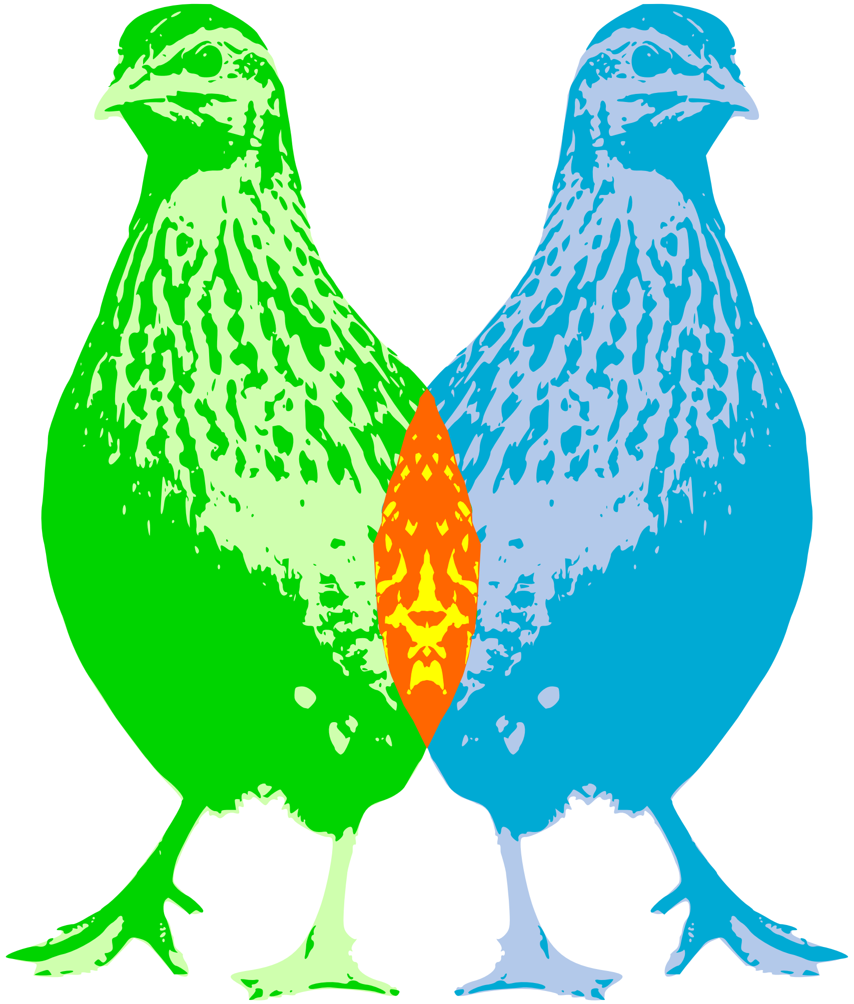

PhD Thesis

Inés Sánchez Donoso

IMPACT OF GAME RESTOCKING ON COMMON QUAIL POPULATIONS

Impacto de las sueltas cinegéticas en las poblaciones de codorniz común

Memoria presentada por

Inés Sánchez Donoso

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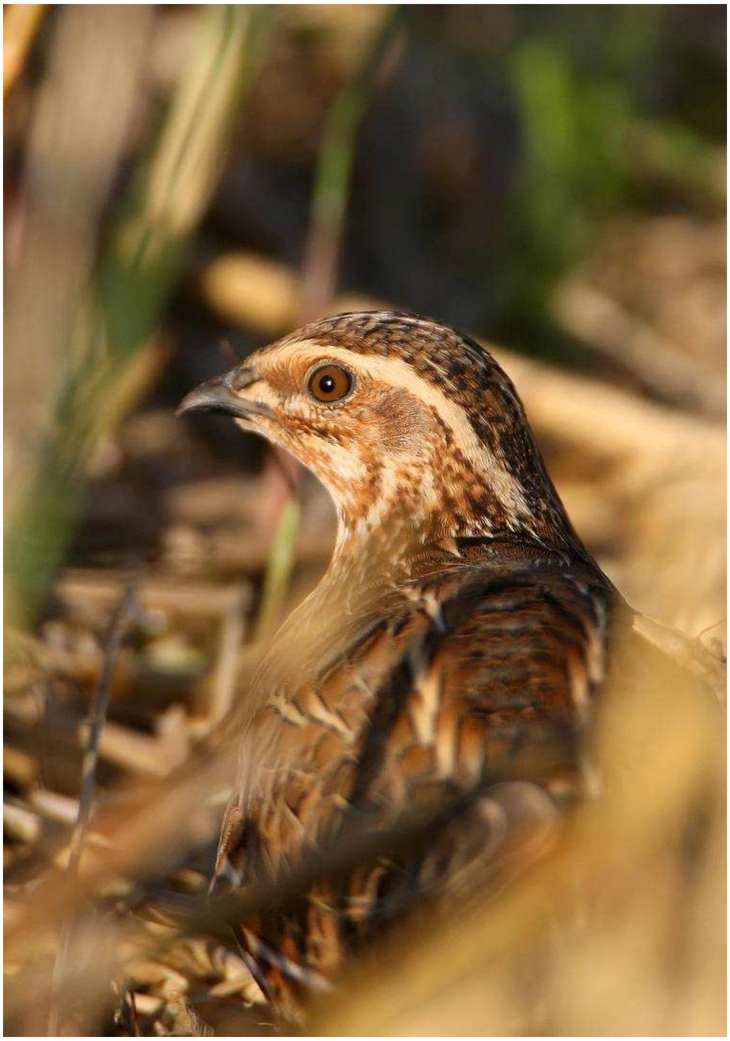
GENERAL INTRODUCTION



Common quail male, *Coturnix coturnix*
by Manel Puigcerver

GENERAL INTRODUCTION

Hybridization and introgression

Hybridization is a process by which individuals from two different species or lineages interbreed. It is a common event in nature, but only when the descendants of the intercross – called hybrids – are fertile and successfully reproduce, hybridization has an impact on the species' evolution.

When these descendants intercross with one of the parental species by repeated backcrossing, a flow of alleles from one parental species into the other occurs. This process is called introgression. Introgression differs from simple hybridization in the sense that alleles of one species move into the gene pool of the other and results in a complex mixture of parental alleles. On the other hand, hybridization can occur even if the genomes of the two species keep perfectly separated when hybrids do not produce offspring. Interbreeding of two species in the wild implies introgression of alleles from one species into the other only when admixed individuals survive and successfully backcross with the parental species. So, successful introgression is a long-term process and does not always occurs after hybridization. If they are not removed by random genetic drift or by purifying selection, introgressed alleles can indefinitely remain in the population, modifying the species' gene pool (Rhymer & Simberloff 1996). This change in the genetic composition of a population can be of great evolutionary importance since it can lead to the origin of novel species or adaptations (Arnold 1997). However, hybridization and introgression can also result in the loss of intra-specific mating opportunities and the disruption of local adaptation or coadapted gene complexes (Frankham et al. 2010).

Anthropogenic hybridization and introgression, though, are usually recent processes resulting from the introduction of species or from the invasion of alien species as a consequence of human activities. These, very often, lead to serious conservation problems (Levin et al. 1996, Rhymer & Simberloff 1996). Introgressed alleles may be detrimental in a new habitat and individual fitness could thus be reduced. If a reduction of population density occurs at a faster rate than species adaptation and evolution, introgression could even lead to the extinction of the population.

Box 1. Glossary

Hybridization: interbreeding between two different species or lineages.

Hybrid: offspring resulting from the interbreeding of two different species or lineages.

Parental species: species from which the hybrid descends.

Backcrossing: interbreeding between a hybrid and one of the parental species.

Backcross: descendant of a backcrossing event.

Gene flow: movement of alleles between groups of individuals.

Gene pool: set of all genes, or genetic information, in a population.

Introgression: flow of genes from one species into the gene pool of another by the repeated backcrossing of hybrids.

Genetic drift: change in the allele frecuencies of a population due to random sampling by reproduction.

The common quail

The common quail (*Coturnix coturnix*) (Linnaeus, 1758) is a migratory Galliform (Fam. Phasianidae) that may be under risk due to anthropogenic introgression (Fig. 1).

Figure 1. Common quail male.

This species is broadly distributed across Europe, North-Africa and West-Asia during the breeding season (February – August) (Moreau & Wayre 1968). At the end of this season, most individuals migrate thousands of kilometers to winter mainly south of the Sahel, in Africa, and in the Indian subcontinent (Guyomarc'h et al. 1998, Rodríguez-Teijeiro et al. 2012). In addition, some individuals winter in the South of the Iberian Peninsula (Delibes 1972) and in Northern Morocco. Similarly, small breeding populations exist in South Africa, which move north to winter in Angola, Zaire, Namibia and Zambia (BirdLife International 2012a, Del Hoyo et al. 1994) (Figure 2).

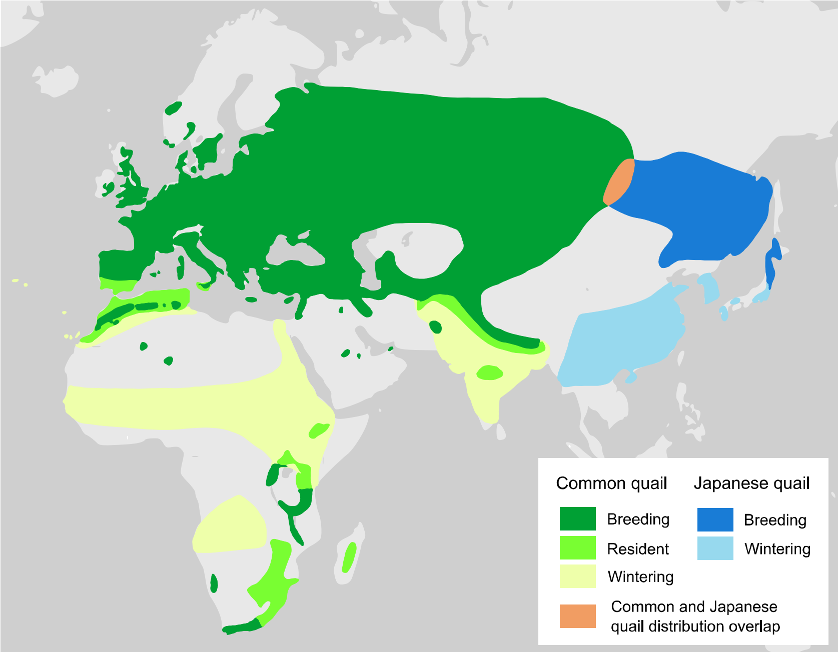


Figure 2. Distribution range of the common quail and the Japanese quail (adapted from BirdLife International 2012a and Del Hoyo et al. 1994).

This is a bird that inhabits grasslands and croplands -mainly winter cereal crops- during the breeding season (Guyomarc'h 2003). It shows some characteristic adaptations associated with this habitat: cryptic brownish plumage, it tends to walk stealthily inside the fields for short displacements, and it only takes flight when it feels really threatened.

Its life history is heavily determined by the limitations imposed by the habitat used. The structure of the habitat is subjected to strong fluctuations due to the harvesting of the cereal, which completely depletes vegetation cover. When that happens, starting in the

South and slowly advancing to the North, quails move in search of new available crops (Rodríguez-Teijeiro et al. 2009).

The species is highly mobile, showing three kinds of movements: (a) migratory movements of thousands of kilometers (Guyomarc'h et al. 1998), (b) nomadic movements during the breeding season in search of suitable but ephemeral habitats (Puigcerver et al. 1989, Rodríguez-Teijeiro et al. 2009) (c) and in search of mates within a given habitat patch (Rodríguez-Teijeiro et al. 2006). Previous studies have shown that in the same area from where the data for this thesis came from, 95% of the male population is replaced in less than 15 days (Rodríguez-Teijeiro et al. 1992). Consequently, this is an open population, with continuous and asynchronous inflow and outflow of individuals.

This species shows a complex mating system. Males aggregate in pseudo-leks (Sardà-Palomera et al. 2011) and display calls to make themselves detectable by females and other males. Females only make themselves detectable by replying to the calls of males. Male and female create a bond that lasts several days (Rodríguez-Teijeiro et al. 2003). During that time, opportunistic extra-pair copulations and mate-switching often occur (Rodrigo-Rueda et al. 1997). Satellite males have been observed seeking extra-pair copulations with paired females (Sardà-Palomera et al. 2011).

Females are polyandrous and usually mate with several males seriated in time during a single reproductive attempt and during all the breeding season (Rodríguez-Teijeiro et al. 2003). Males are also polygynous, mating with several females during the breeding season (Rodríguez-Teijeiro et al. 1992). After the male guards the female for some days, the pair bond breaks. Females stay in the area taking care of the clutch, while males move north in search of new potential partners (Puigcerver et al 1984, Rodríguez-Teijeiro et al. 1992). Because of this difference in behavior and mobility between the sexes, sex ratio is biased in favor of males following a latitudinal gradient (Schleidt 1983, Hemon et al. 1988, Puigcerver 1990, Rodríguez-Teijeiro et al. 1992). Nests are settled on the ground, the average number of eggs per clutch is 10.6 (Gallego et al. 1993), and chicks are precocial. Females can lay several clutches during one breeding season. The number varies from 1 to 3 clutches depending on habitat availability and latitude (Puigcerver et al. 1997). They can also produce replacement clutches in cases of nest predation. Quail average life-span is 0.8 years (Puigcerver et al. 1992).

A game species

Census size is estimated to be globally large, around 35 to 300 million individuals according to the International Union for Conservation of Nature (IUCN, BirdLife International 2012a), with important fluctuations from year to year (Puigcerver et al. 2012). Since visual detection is difficult, the more commonly used census method for quails involves the detection of calling males during the breeding season when stimulated with a female decoy (Gallego et al. 1993). However, this approach is far from perfect, and reliable population size estimates with narrow confidence intervals do not exist. For this reason, the assessment of population trends and conservation status is controversial. According to some authors (Burfield 2004), the species has suffered a decline during recent decades and has an unfavourable conservation status. However, other researchers consider that its conservation status is “least-concern” (BirdLife International 2014). In any case, this is a popular game species, with long tradition and an important socio-economic impact in Mediterranean countries and the Near East, where millions of individuals are hunted annually. Only in Spain, where the largest breeding population in Western Europe is located (Rodríguez-Teijeiro et al. 2006), more than 1,300,000 individuals were hunted annually during the period 1973–2010 (Yearbooks of Agro-alimentary Statistics of the Spanish Ministry of Agriculture, Fisheries and Food).

Because of the hunting interest in this species and with the aim of increasing hunting bags, the populations in many of those hunting areas (as in Italy, Greece, the Republic of Serbia, Montenegro, Romania and Spain) are annually restocked with thousands of quails bred in farms (Puigcerver et al. 2007, Rodríguez-Teijeiro et al. 1993, Tsiompanoudis et al. 2011). For example, in Catalonia (Northeast Spain), where data from this thesis was taken from, more than 68,000 farm-reared quails have been restocked annually during the period 1990–2006 (personal communication from the Catalan Department of Agriculture, Farming, Fisheries, Food and Environment) while the wild breeding population is estimated to be between about 5000 and 21,000 males (Rodríguez-Teijeiro et al. 2004). At least in this area, which could be representative of the situation in other areas, the number of restocked quails is several times larger than the size of the breeding population.

The Japanese quail

The Japanese quail (*Coturnix japonica*, Temminck & Schlegel, 1849) is a close relative of the common quail. It is also a migratory bird with very similar phenotype and habitat requirements to those of the common quail (Taka-Tsukasa, 1941).

The Japanese quail is distributed over East Asia, including Japan (Johnsgard 1988). The natural ranges of common and Japanese quail only overlap in the Lake Baikal area in Russia (Fefelov 1998), and in the Kentei region in Mongolia (Kozlova, 1932) (Fig. 2). Although this overlap occurs during the reproductive period, despite some anecdotal evidence (Barilani et al. 2005), no extensive natural hybridization has been reported (Del Hoyo et al. 1994, Guyomarc'h et al. 1998, Moreau & Wayre 1968).

For several centuries Japanese quails have been domesticated by breeding in captivity and selected for meat and egg production (Yamashina 1961) (Figure 3). Domestic Japanese quail first entered Europe (France and Italy) in the 1950s (Rizzoni & Lucchetti 1957, 1972). Nowadays it is commonly bred in captivity across Europe as poultry. Domestic Japanese quails have been artificially selected to show, among other traits, a reduced migratory drive (Derégnaucourt et al. 2005b). As a consequence of the selection for life in captivity, they also show difficulties to move and fly, as well as a lower anti-predatory instinct.

Figure 3. Domestic Japanese quail female

Concerns on the identity of restocked quails

Rearing common quails in captivity is difficult because of their restlessness (Caballero de la Calle & Peña Montañés 1997) and because capture of wild females is very unlikely (males are easily attracted by female calls, but females are difficult to attract). This has led to some concern about the genetic origin of quails reared in farms for restocking practices, where they are bred by the millions. A possible explanation for the great reproductive success in farms is that they could be of hybrid origin, resulting from crosses between wild common quails and domestic Japanese quails.

Since captive breeding represents a general relaxation of natural selective forces and adaptation to life under a very special set of conditions, domestic Japanese quails could show sets of alleles favourably selected in captivity but maladaptive in the wild. Studies in captivity have shown that migratory phenotypes are not common among hybrids (Derégnaucourt et al. 2005a, b), which have inherited the reduced restlessness of domestic Japanese quails (Derégnaucourt 2000, in Barilani et al. 2005). This is a key point for the conservation of common quails: if restocked individuals interbreed with common quails in the wild, the native population could gradually loose the mobility needed to track changes in the habitat. Sedentariness could drastically affect common quail viability, leading to a decrease in the fraction of migratory quails and to a decrease of the common quail population density (Guyomarc'h 2003). Sedentary individuals would spend the winter in a depleted habitat (harvested cereal crops), with reduced food resources, where predation risk would probably be higher, and would suffer the adverse climatic conditions, presumably leading to a higher mortality (Rodríguez-Teijeiro et al. 1992, Guyomarc'h 2003). Individuals would likely also change their nomadic behaviour in search of mates and suitable habitat, potentially affecting their fitness.

Even in the case that fitness of admixed individuals was not diminished, the massive releases of farm-reared quails could lead to a counter-intuitive reduction in the effective population size through Ryman-Laikre effects: a large portion of the breeding population could potentially derive from a small number of breeders from farms, resulting in a reduction of the genetic diversity and evolutionary potential (Ryman & Laikre 1991). And, also, even if the restocked individuals did not show a reduced migratory behaviour, a change in the genetic composition of the common quail gene pool should be expected, with unforeseeable consequences.

At least in theory, hybridization between common and farmed quails is likely to lead to a rapid increase in the proportion of hybrids and admixed individuals in wild populations.

Once admixture has begun it is difficult to stop if hybrids are fertile and mate both with other hybrids and with both parental species (Allendorf et al. 2001). As a result, in a few generations this process might result in a hybrid swarm in which almost all individuals are of hybrid origin (Allendorf et al. 2001, Huxel 1999).

Legal framework

As a result of these concerns, the European Union Management Plan for the common quail (period 2009–2011) has stressed the need to ban Japanese/hybrid quail releases throughout Europe and across the rest of the common quail distribution (Perennou 2009). Several European countries and regions have already banned or regulated restocking practices, making illegal the release of Japanese quails and hybrids (Portugal and France since 2002, Greece since 2007). In Italy, the Japanese quail is considered an introduced species (BirdLife International 2012b). In Spain, the country where the study has been carried out, the Japanese quail is also currently considered an introduced species (Spanish Royal Decree 630/2013). In this same country, the Natural Heritage and Biodiversity Law 42/2007 allows restocking with native species but not Japanese quail or hybrids; before the approval of this law, no special regulations were applied.

Although the current legal framework seems appropriate to prevent the introgression of maladaptive alleles, its enforcement is not sufficient. Many farm-reared individuals are still being released assuming that they are pure common quails. Farm managers sell them as common quails with a veterinarian certificated of purity. But the diagnosis of the individuals' origin is only based on their morphology, despite being very difficult to unequivocally differentiate between pure common quails and admixed individuals on the basis of their phenotype, as they look very similar, even indistinguishable in some cases (Derégnaucourt 2000, in Barilani et al. 2005). The correct assessment of the origin of restocked farm-reared individuals is central for the design of appropriate common quail conservation and management plans. Genetic analyses are useful for this purpose.

Restocking: a real threat

Several authors have already warned that restocking with domestic Japanese quails and hybrids can pose serious threats to the genetic integrity and survival of common quails (Guyomarc'h 2003, Barilani et al. 2005, Chazara et al. 2006, 2010, Puigcerver et al. 2007). This may not be just a theoretical threat. On one hand, hybrid individuals have been detected in the wild across Europe (Guyomarc'h 2003, Rodríguez-Teijeiro et al. 1993). However, previous studies (Puigcerver et al. 2007, 2013) have shown that the proportion of individuals identified as hybrids in areas close to the one studied in this thesis (in Catalonia, North-East Spain) has not clearly increased over a period of 20 years since the first detection of hybrids in 1990 (Figure 4). In these studies hybrids were identified by the mating call of males, which may lead to underestimations in the number of admixed individuals (Derégnaucourt et al. 2001), so it is not a reliable method for hybrid identification. On the other hand, some studies have shown that, in captivity, there are not pre- or post-zygotic barriers preventing the interbreeding between common and domestic Japanese quails or backcrosses between common quails and hybrids (Taka-Tsukasa 1941, Derégnaucourt et al. 2002, Derégnaucourt & Guyomarc'h 2003). However, very little is known about the fate of restocked quails and their impact on the native populations.

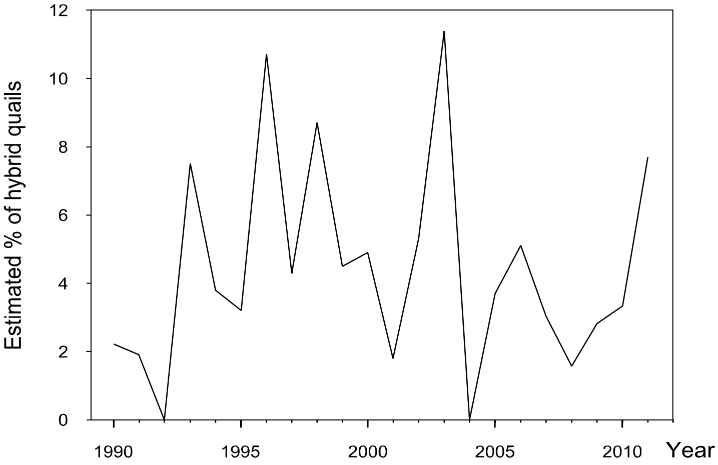


Figure 4. Estimated percentage of hybrid quails detected during the period 1990–2011 in three areas of Catalonia (N-E Spain) (adapted from Puigcerver et al. 2013).

Genetic markers

Since the phenotype of pure common quails is not clearly differentiable from that of hybrid and admixed individuals, genetic analyses seems to be the only way to reliably characterize the origin of the individuals. Population genetic approaches have probed very useful to better understand the ecology and behavior of wild species and to solve conservation problems. Genetic tools have been used for diverse objectives, like the characterization of population structure (Latch et al. 2011), the identification of migrants (Reddy et al. 2012), the study of mating systems by paternity analyses (Huck et al. 2014) or the detection of hybrids (Barbanera et al. 2011, Mirimin et al. 2014).

One of the most common methods currently used to characterize the genetic composition of populations is by genotyping individuals with a panel of microsatellites

(Allendorf et al. 2013). Microsatellites are molecular markers located in the nuclear DNA consisting on nucleotide sequences containing tandem repeats (Ellegren 2004). The number of repeats is usually very easy to characterize and tends to be highly polymorphic, so different alleles can exist in a population, providing a powerful tool for individual identification. Several microsatellites (usually between 8 and 25) are characterized in every single individual, providing its genotype: an individual identification code constituted by the combination of different alleles identified for each microsatellite. Most commonly used microsatellites do not code for any protein and have simple inheritance (in diploid organisms, one allele is inherited from the father and the other from the mother). All these characteristics make them very useful for population genetic studies.

Alleles present exclusively in one species are most useful for hybrid identification. The detection of only these alleles in an individual would be unequivocal evidence of membership to that species, whereas hybrids and introgressed individuals would carry alleles from several origins. However, when comparing closely related species -such as common and Japanese quail- it is more common to observe that alleles at one locus (a certain location in the genome) just differ in their relative frequencies. In this case the characterization of groups of individuals (populations or species) will be determined by the probability of finding a given genotype in each of the groups. Thus, the study of allele frequencies across a large number of individuals can allow the detection of population structure. Bayesian clustering procedures are used to detect this population structure (Vähä & Primmer 2006). Analyzing a pool of genotypes and the allele frequencies in that sample, some procedures, as the ones implemented in the program STRUCTURE (Pritchard et al. 2000), allow inferring the proportion of the individual genotype belonging to a genetic cluster. Based on the genetic variability of the analyzed sample, this software aggregates individuals in clusters based on their genotype similarity. Other Bayesian methods, like the ones implemented in NEWHYBRIDS (Anderson & Thompson 2002), calculate the individual probability of membership to a hybrid category (pure species A, pure species B, first generation hybrid ...).

However, the power of these analyses is limited by the number of microsatellites used and their variability (Vähä & Primmer 2006). In addition, due to the inherent variability in the individual genetic composition resulting from interbreeding of individuals of different genetic origin (Figure 5), usually genetic identity of individuals is far less reliable and more uncertain beyond second generation hybrids and first backcrosses. The reliability of the identification of the different hybrid classes can be increased by maximizing the number of unlinked genetic markers used and their variability.

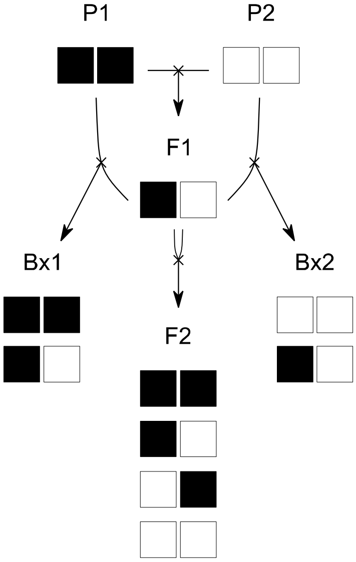


Figure 5. Genotypes expected at one locus resulting from the interbreeding of two species. P1: parental species 1; P2: parental species 2; F1: first generation hybrid; F2: second generation hybrid; Bx1: backcross with P1; Bx2: backcross with P2. Black and white boxes represent alleles originating in the two parental species. A genotype is given by the combination of two boxes. While F1 hybrids are expected to have one allele from each origin at all loci, backcrosses will have either two alleles from one of the parental species or one allele from each, but never two alleles from the other parental species. Crosses beyond this point (as in F2) could result in any combination of alleles at any single locus and the classification into one hybrid category or another is much more difficult and depends on the probability of each combination at each locus. The information from several microsatellites can increase the power to determine the category to which an individual belongs to.

Paternity assignment is also possible by microsatellite analyses (Xue et al. 2014, Li et al. 2014). Basically, the probability of each genotype being the father or sibling of another genotype is estimated by looking at the alleles that they share after correcting for the frequency of these alleles in the entire population (an allele could be shared between two individuals not because they are related but because it is very common in the population). Softwares implemented for paternity assignment, like CERVUS (Kalinowski et al. 2007), make a pair-wise likelihood comparison between offspring genotypes and putative parent genotypes, and establish its probability with a certain confidence level.

Another molecular marker commonly used in population genetic studies is mitochondrial DNA (mtDNA). This marker shows some characteristics that make it easy to amplify and study: it is maternally inherited, does not recombine and has many copies per cell (some times thousands of copies). These characteristics have made this the traditional marker of choice in phylogeny and phylogeography studies (Avise 2000). Since this marker is uniparental, it can be useful to identify the directionality in hybrid matings. If the mtDNA lineages are clearly separated for two species, its analysis in a hybrid will inform about the species of the mother of this individual (for example, see Muñoz-Fuentes et al. 2007).

Box 2. Some major events in the life cycle of a restocked common quail population

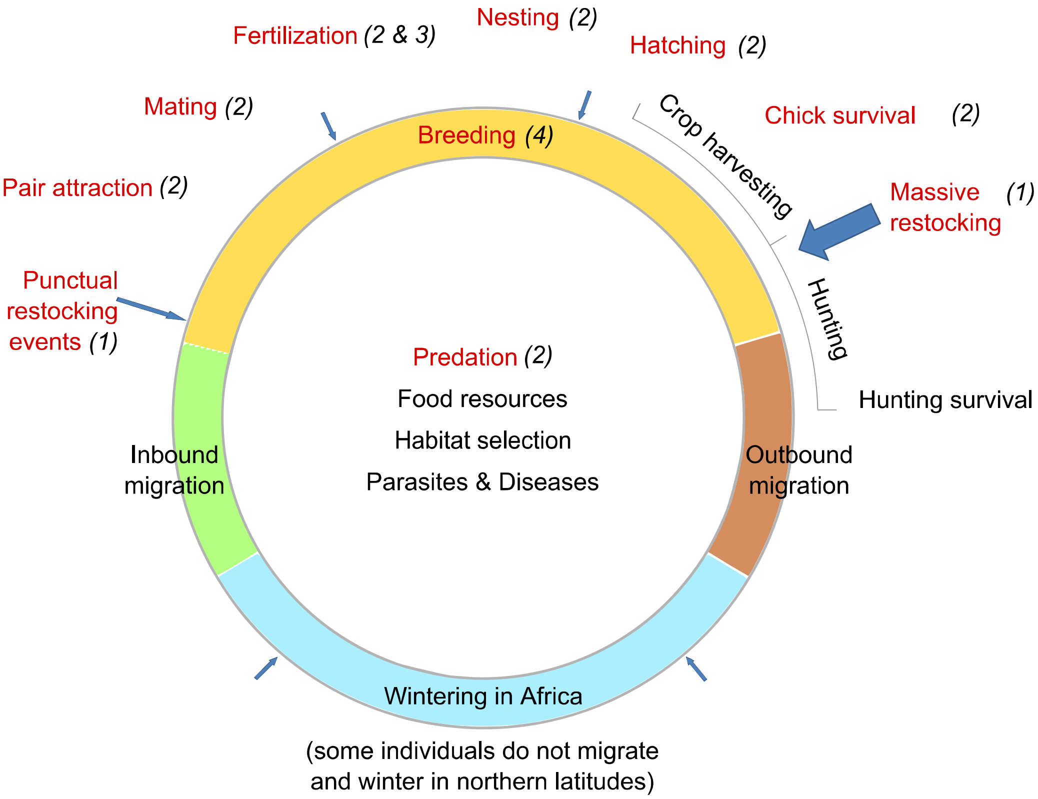
During their life cycle, and in areas where restocking with farm quails occurs, common quails overcome several events that modulate their fitness. In this thesis, I study how some of the events occurring during the breeding season are affecting the relative fitness of both kinds of quails. Although the precise date of these events depends on the local phenology of the habitat and the movements of the common quail, taking place later at higher latitudes and altitudes, they occur in the same order across populations.

After the wintering at lower latitudes, quails gradually arrive to Europe and start searching for mates. The complex mating system of the quail includes several key moments when the coexistence of common quails with quails of farm origin could impact their relative fitness: pair attraction, pair-bond establishment, mating and fertilization of the ova.

By the time females start incubating, the pair-bond had been broken and males look for new mates in the same or different areas or they move to other areas (Rodrigo-Rueda et al. 1997). Not all eggs are fertilized, neither all eggs develop in embryos that will hatch, but the hatching of eggs occurs almost synchronically (in a 24h period of time). Chicks abandon the nest after hatching but stay close to the female during at least three weeks. Since during that time chicks do not regulate their body temperature and they depend on the protection of their mother, chick survival can be low until they lose the down and get their first plumage. In a short time, juveniles develop enough to perfectly fly. When chicks do not depend on her, the female can engage in a second reproduction attempt. Sometimes this is precipitated earlier because of nest predation. In other cases, the habitat is harvested and the female has to move to other suitable areas in search of new mates.

In Spain (where the data of this thesis has been taken from), the hunting season starts some time after the harvest of cereals. Just before and during the hunting season, the restocking of thousands of farm-reared quails takes place in order to increase hunting bags. There are also intensive hunting grounds where hunting and restocking is allowed all year round. These areas are spread all over Spain, occupying just a small area. The genetic origin of individuals used for game restocking is one of the key points for the conservation and management of common quails that is addressed in this thesis.

During the breeding season, quails are also under the pressure of predation, food finding and habitat availability (Guyomarc'h 2003), as well as fighting parasites and diseases, which modulate their survival and fitness. After the breeding season, most quails, including the recruited ones, migrate back to winter in Africa.



Annual cycle and major events in the life history of the common quail that determine individual fitness. These events could also affect restocked quails, so they are of importance in the degree of introgression of domestic Japanese quail alleles into the native common quail population. In red, events that are subject of study in this thesis; in brackets, number of the paper in which they are studied.

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STUDY GOALS

The main goal of this PhD thesis is to understand the effects that game restocking has over common quail populations. In addition, to gain knowledge applicable to improve common quail conservation and management.

These general goals are addressed by investigating more **specific objectives**:

- Assess genetic origin of quails reared in Spanish game farms for restocking. Are they pure common quails? (Paper 1)
- Evaluate the power of genetic methods used in this thesis for the identification of quails of hybrid origin. (Paper 1 and Paper 4).
- Evaluate mating and breeding success of restocked quails compared to native common quails, by:
    - Testing if interbreeding in the wild is possible by the establishment of heterospecific matings. (Paper 2)
    - Studying effect of different breeding parameters over the fitness of restocked quails compared to common quails. (Paper 2)
    - Studying whether postcopulatory sexual selection between farm-reared hybrids and common quails could modulate the admixture between these two lineages in the wild and impact the rate of introgression. (Paper 3)
- Evaluate to what extent the releases of farm quails are affecting the genetic composition of the native population. Is there an introgression of domestic Japanese quail alleles into common quail populations? (Paper 4)
- Forecast changes in the genetic composition of the local population in the near future under different scenarios of genetic admixture. (Paper 4)

SUPERVISOR'S REPORT

Dr. José Domingo Rodríguez Teijeiro, Dr. Manel Puigcerver Oliván and Dr. Carles Vilà Arbonés co-supervisors of the PhD thesis entitled "Impact of game restocking on common quail populations" certify that the dissertation presented here has been carried out by Inés Sánchez Donoso in its totality and grants her the right to defend her thesis in front of a scientific committee. We also certify that none of the manuscripts included in this PhD thesis has been used as a part of another PhD thesis.

As supervisors, we have participated in designing, guiding and correcting earlier drafts of the chapters and manuscripts written by the PhD candidate. The contribution of the PhD candidate to each manuscript is detailed below:

PAPER 1. Sanchez-Donoso I, Vilà C, Puigcerver M, Butkauskas D, Caballero de la Calle JR, Morales-Rodríguez PA, Rodríguez-Teijeiro JD (2012) Are Farm-Reared Quails for Game Restocking Really Common Quails (*Coturnix coturnix*)?: A Genetic Approach. *PLoS ONE* 7 (6): e39031.
doi:10.1371/journal.pone.0039031.

Impact factor 2011: 4.092. 5-year impact factor: 4.244; First quartile in Multidisciplinary.

I.S.-D. contribution: Experimental design, sampling, laboratory work, genetic analyses, data analyses, paper writing.

PAPER 2. Puigcerver M, **Sanchez-Donoso I**, Vilà C, Sardà-Palomera F, García-Galea E, Rodríguez-Teijeiro JD (2014) Decreased fitness of restocked hybrid quails prevents fast admixture with wild common quails. *Biological Conservation* 171: 74-81.
doi: 10.1016/j.biocon.2014.01.010.

Impact factor 2012: 3.794. 5-year impact factor: 4.241; First quartile in Ecology, Evolution, Behavior and Systematics.

I.S.-D. contribution: Experimental design, experiment performance, sampling, laboratory work, genetic analyses, data analyses, paper writing.

PAPER 3. **Sanchez-Donoso I**, Morales-Rodriguez PA, Puigcerver M, Caballero de la Calle JR, Vilà C, Rodríguez-Teijeiro JD. Postcopulatory sexual selection favors fertilization success of restocking hybrid quails over native common quails. Submitted to *Ibis*.

Impact factor 2012: 2.361. 5-year impact factor: 2.488; First quartile in Animal Science and Zoology.

I.S.-D. contribution: Experimental design, sampling, lab laboratory work, genetic analyses, data analyses, paper writing.

PAPER 4. **Sanchez-Donoso I**, Huisman J, Echegaray J, Puigcerver M, Rodríguez-Teijeiro JD, Hailer F, Vilà C (2014) Detecting slow introgression of invasive alleles in an extensively restocked game bird. *Frontiers in Ecology and Evolution* 2: 00015.
doi: 10.3389/fevo.2014.00015

Impact factor: Recently founded journal, without impact factor yet.

I.S.-D. contribution: Experimental design, sampling, lab laboratory work, genetic analyses, data analyses, paper writing.

Another scientific paper coauthored by the PhD candidate and published in a peer-reviewed journal during the PhD, but not included in this thesis is:

Puigcerver M, **Sanchez-Donoso I**, Vilà C, Sardà-Palomera F, Morales-Rodríguez PA, Caballero de la Calle JR, Rodríguez-Teijeiro JD (2013) Hibridación entre la codorniz común (*Coturnix coturnix*) y la codorniz de granja: estado de un problema de conservación. *Ecosistemas* 22: 48-53. doi.: 10.7818/ECOS.2013.22-2.08

RESEARCH PAPERS

Marisa García-Andrés taking notes in a field crop (Mas Esplugues, Catalonia)
by Ines Sanchez-Donoso

PAPER 1

Are farm-reared quails for game restocking really common quails (*Coturnix coturnix*)?: A genetic approach

Ines Sanchez-Donoso, Carles Vilà, Manel Puigcerver, Dalius Butkauskas, José Ramón
Caballero de la Calle, Pablo Antonio Morales-Rodríguez,
José Domingo Rodríguez-Teijeiro

PLoS ONE 7 (6): e39031

Cereal crop (Figuerola, Catalonia), by Ines Sanchez-Donoso

"I tu què en penses?" ("What do you think about it?")
Carles Vilà

ABSTRACT

The common quail (*Coturnix coturnix*) is a popular game species for which restocking with farm-reared individuals is a common practice. In some areas, the number of restocked quails extensively surpasses the number of breeding quails. However, common quails are difficult to be raised in captivity and this arouses suspicion about a possible hybrid origin of the farmed individuals from crosses with domestic Japanese quails (*C. japonica*). In this study we used a panel of autosomal microsatellite markers to characterize the genetic origin of quails reared for hunting purposes in game farms in Spain and of quails from an experimental game farm which was founded with hybrids but has been systematically backcrossing with wild common quails. The genotypes of these quails were compared to those of wild common quails and domestic strains of Japanese quails. Our results show that more than 85% of the game farm quails were not common quails but admixed individuals and domestic Japanese quails. In the experimental farm a larger proportion of individuals could not be clearly separated from pure common quails. We conclude that the majority of quails sold for restocking purposes were not common quails. Genetic monitoring of individuals raised for restocking is indispensable as the massive release of farm-reared hybrids could represent a severe threat for the long term survival of the native species.

INTRODUCTION

Restocking has become one of the most commonly used practices for the conservation and management of endangered and game species. One important reason for restocking is to supplement fisheries and game bags in order to increase productivity or maintain recreational activities (Fischer & Lindenmayer 2000), in some cases even while threatening local gene pools (Mank et al. 2004). Restocking for hunting purposes has been carried out with diverse species and taxonomic groups such as deers (DeYoung et al. 2003), marmots (Kruckenhauser & Pinsker 2004), wild boards (Vernesi et al. 2003), waterfowl (Champagnon et al. 2009) and different Galliform species (Sokos et al. 2008, Lamont 2009), including partridges (Negro et al. 2001, Randi et al. 2003, Sevane et al. 2011), pheasants (Bagliacca et al. 2008) and quails (Rodríguez-Teijeiro et al. 1993, Barilani et al. 2005, Puigcerver et al. 2007).

The common quail (*Coturnix coturnix*) is a migratory Galliformes species distributed across Eurasia during its breeding period (Guyomarc'h 2003) and currently with an unfavourable conservation status in Europe due to a large historical decline Burfield 2004). Even so, the common quail is a popular game species with an important socio-economic impact. Only in Spain, more than 1,300,000 quails have been hunted annually during the period 1973-2010 (*Yearbook of Agro-alimentary Statistics* of the Spanish Ministry of Agriculture, Fishing and Food). Restocking with farm-reared quails to increase bag numbers is a very common practice in several European countries, such as Italy (Galli 2003), Greece (Barilani et al. 2005, Tsiompanoudis et al. 2011), Serbian Republic, Montenegro, Rumania and Spain (Rodríguez-Teijeiro et al. 1993, Puigcerver et al. 2007). According to Guyomarc'h (2003), 430,000-480,000 released quails were shot in France during the 1983-1984 hunting seasons, corresponding to 67%-75% of the 640,000 total individuals hunted. In Catalonia (Northeast Spain), an average of more than 68,000 farm-reared quails have been restocked annually during the period 1990-2006 (Hunting order plans of the Regional Government of Catalonia, unpublished data). Taking into account that the estimated wild male quail population in Catalonia ranges between 5,347 and 20,847 individuals (Rodríguez-Teijeiro et al. 2004), the number of quails restocked widely exceeds the breeding population.

However, rearing common quails in captivity for restocking is difficult because of their restlessness (Caballero de la Calle & Peña Montañés 1997). This has led to some concern about the genetic origin of farm-reared quails, and a possible explanation for this great reproductive success in farms is that they could be of hybrid origin, resulting from crosses between wild common quails and domestic Japanese quails (*Coturnix japonica*). The Japanese quail is a sister species of the common quail that is distributed over eastern Asia (Johnsgard 1988). For several centuries Japanese quails have been bred in captivity and selected for meat and egg productivity (Yamashina 1961). These domestic Japanese quail first entered Europe (France and Italy) in the 1950s (Rizzoni & Lucchetti 1957, 1972). As a result of the selection for captivity, these birds have lost their migratory restlessness (Derégnaucourt et al. 2005), show some difficulties to move and fly, and a lower anti-predatory instinct.

These differences in behaviour suggest that restocking with farm-reared domestic Japanese quails or hybrids (also called “admixed”) between the two species would result in a conservation problem. In fact, several authors have warned about the risk that restocking can represent for common quails when carried out with domestic Japanese quails and hybrids (Guyomarc’h 2003, Barilani et al. 2005, Puigcerver et al. 2007, Chazara et al. 2006, 2010). It entails a potential threat to the native common quail because it could lead to the introgression of domestic Japanese quail alleles and a subsequent loss of migratory behaviour and decline in fitness in the native common quail populations (Rodríguez-Teijeiro et al. 1992, Guyomarc’h 2003). Individuals that do not migrate or that show low mobility could be more exposed to adverse climatic conditions, loss of habitat after the harvesting of cereal crops (the usual habitat of the species), predation or a lack of food resources (Guyomarc’h 2003). Thus, the arrival of maladaptive genes from domestic Japanese quails could reduce the survival and adaptive potential of wild common quails.

This conservation concern seems to be a real one: hybrid individuals have already been detected in common quail breeding areas in different European countries such as Portugal, France, Italy and Spain (Rodríguez-Teijeiro et al. 1993, Guyomarc’h 2003, Barilani et al. 2005, Puigcerver et al. 2007). Moreover, recent field experiments with released radio-tagged farm-reared individuals of hybrid origin have shown that they can mate with wild common quails and produce fertile offspring (unpub. data) as it happens in captivity (Derégnaucourt et al. 2002). Closely related native species, as the red-legged partridge (*Alectoris rufa*) (Martínez-Fresno et al. 2007, Blanco-Aguiar et al. 2008) and the rock partridge (*A. graeca*) (Barilani et al. 2007), are threatened by hybridization resulting from game restocking with sister species or hybrids. As a result of the concern about hybridization, the release of Japanese quails and hybrids is illegal in Spain, Portugal, France and Greece. However, the diagnosis of the specific origin of farm quails before restocking is usually based on their morphology despite being very difficult to unequivocally differentiate between pure common quails and admixed individuals on the basis of their phenotype, as they look very similar (Derégnaucourt 2000 in Barilani et al. 2005). Thus, genetic analyses are absolutely necessary to assess the genetic identity of restocked farm-reared individuals.

The aim of this study was to identify the genetic origin of quails reared for restocking and hunting purposes in five different Spanish game farms by using a panel of nuclear microsatellite markers. We have also analysed quails from an experimental farm that is subject to a specific breeding management aimed at reducing the genetic contribution of the founders, which had domestic Japanese quail ancestry, by crossing farm-born individuals with wild common quails during several generations.

MATERIALS AND METHODS

Ethics Statement

All work related with animals in this study has been conducted according to recommendations of Federation of European Laboratory Animal Science Associations (FELASA) guidelines. It fulfills the ethic recommendations of the European Union and the Spanish legislation and has been approved by the Ethic Committee on Animal Experimentation from the University of Barcelona and from the University of Castilla-La Mancha.

Samples origin and collection

One hundred and fifty-two quails were analysed for this study. They were collected from four different origins (Table 1). 1) Males and females randomly sampled from five different Spanish game farms in 2009 and 2010, purchased for restocking and hunting purposes. 2) Quails from an experimental farm from the University of Castilla-La Mancha (Spain) managed for about 20 years to reduce the genetic contribution of founders. These individuals are descendants of an initial cross between hybrid females with wild common quail males whose offspring has been backcrossed with wild common quail males in successive generations. 3) Wild quail males captured from the field (see Puigcerver et al. 2007) during 1996-2009 in the regions of Seville (South Spain), Barcelona (Northeast Spain) and Drenthe (The Netherlands) which were identified as common quails on the basis of their song and phenotype. 4) Domestic Japanese quails from two laboratory lines from the Laboratory of Molecular Ecology of the Institute of Ecology of the Nature Research Centre, Vilnius University (Lithuania) (Kayang et al. 2002), and from four different Spanish meat farms.

Blood (100 µl) or muscle samples were individually stored at -20 ºC in 95% ethanol, until DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen) following manufacturer's protocols.

Group	N_G	Sampling origin	N
Game farm quails	52	Game farm 1	13
		Game farm 2	7
		Game farm 3	20
		Game farm 4	6
		Game farm 5	6
Experimental quails	19	Experimental farm	19
Wild common quails	42	Seville (S Spain)	25
		Barcelona (NE Spain)	5
		Drenthe (The Netherlands)	12
Domestic Japanese quails	39	Meat farm (4 farms, 4 samples from each)	16
		Laboratory lines (2 lines with 9 and 14 samples)	23
Total			152

Table 1. Quail samples studied. N_G: number of individuals per group. N: number of individuals per sampling origin.

Typing of microsatellite loci

Individuals were genotyped for eleven unlinked autosomal microsatellite loci originally developed for Japanese quail (Kayang et al. 2002, 2004): GUJ0001, GUJ0017, GUJ0028, GUJ0039, GUJ0044, GUJ0057, GUJ0065, GUJ0074, GUJ0085, GUJ0093 and GUJ0097. Loci were amplified by polymerase chain reaction (PCR). While some markers were PCR-amplified in a multiplex, others were amplified separately and subsequently pooled before electrophoresis. Detailed protocols are available upon request. All PCR products were electrophoresed on an ABI 3730 sequencer (Applied Biosystems), following manufacturer's protocols. Alleles were sized and scored using the software GeneMapper v3.5 (Applied Biosystems).

Analysis of microsatellite data

Average number of alleles per locus and allelic richness (mean number of alleles per locus corrected for sample size) (Leberg 2002) were calculated using FSTAT version 2.9.3.2 (Goudet 1995). Polymorphic Information Content (PIC) (see Botstein et al. 1980) and expected and observed heterozygosities (H_E and H_O) (Nei 1987) were calculated with EXCEL MICROSATELLITE TOOLKIT 3.1.1 (Park 2001).

Patterns of genetic differentiation were visualized by plotting the individuals in a two-dimensional space according to their microsatellite composition, independently of any *a priori* species designation, using a factorial correspondence analysis (FCA) in GENETIX (Belkhir et al. 1996-2004).

To identify genetically distinct clusters present in the data we used a Bayesian clustering procedure implemented in STRUCTURE 2.3.2 (Pritchard et al. 2000). STRUCTURE identifies the number of genetically distinct clusters that maximize the likelihood of the data (*K*) and estimates, for each individual, the fraction of the genome (q_i) that belongs to each one of the clusters. Analyses were performed using all individuals under the “admixture model” (as each individual may have ancestry in more than one parental population), with correlated allele frequencies and without prior population information (USEPOPINFO inactive), nor prior sampling location information (LOCPRIOR inactive). Simulations were run for 100,000 steps following a burn-in period of 30,000 steps, considering values of *K* ranging between 2 and 10, and were replicated five times (Vähä & Primmer 2006) after verifying that results did not vary significantly with longer cycles of burn-in iterations. Likelihood values were observed to converge during the runs. For *K*=2, we estimated the 90% probability interval for each individual proportion of membership to a cluster (q_i). The best value of *K* was chosen following the method proposed by Evanno et al. (2005), with STRUCTURE HARVESTER (Earl & vonHoldt 2011), which takes into account the rate of change in the log probability of data between successive *K* values. Each individual was assigned exclusively to one of the inferred clusters when its q_i to that cluster was equal or larger than a threshold corresponding to the minimum value observed among the non-admixed individuals used as reference (see below). Alternatively, individuals that showed lower q_i values could not be assigned exclusively to one cluster and were considered putatively admixed.

After confirming with STRUCTURE that none of the wild common quails and domestic Japanese quails had a genome that seemed admixed, we considered them purebred and used them as reference in analysis with NEWHYBRIDS 1.1 (Anderson & Thompson 2002) to assess if farm quails could be the result of interbreeding between the two species. We used NEWHYBRIDS to compute the posterior probability (P) for each individual to belong to each of the following genotypic classes: parental purebred 1 (P_1), parental purebred 2 (P_2), first generation hybrid (F_1), second generation hybrid (F_2, offspring from crosses between F_1 hybrids), backcross of F_1 with P_1 (Bx_1) and backcross of F_1 with P_2 (Bx_2). Posterior distributions were evaluated after running five independent analyses to confirm convergence, starting with different random seeds, for 10^5 Monte Carlo Markov Chain iterations after 10^4 burn-in steps, without using prior allele frequency information.

Analyses were run for four combinations of prior distributions (JEFFREYS or UNIFORM for Theta and Pi) to explore the robustness of the results, as recommended by the software authors (Anderson & Thompson 2002). The affinity of an individual to the genotype classes was assessed by its posterior probability values (P): those that showed P≥0.85 to one genotype class were assigned to that class; if no value reached 0.85, but the sum of all hybrid classes was above this threshold, individuals were identified as hybrids of unknown generation (Vähä & Primmer 2006); individuals whose origin could not be identified under these criteria were left unclassified.

RESULTS

Loci and population genetic characteristics

All 152 individuals studied were successfully genotyped at 7 or more of the 11 markers, and more than 95% of them were typed for 8 or more loci. All loci were polymorphic in the four groups of quails studied. A total of 224 alleles were found, 145 of which were exclusive of the reference wild common quails and 8 of the reference domestic Japanese quails (excluding individuals from game and experimental farms), implying great power for hybrid identification.

Domestic Japanese quails showed the lowest average number of alleles per locus, allelic richness and PIC while the highest values were found in wild common quails (Table 2). Values for game and experimental farm quails were intermediate. Observed heterozygosity was higher than expected in experimental farm quails (p-value = 0.0003). On the other hand, observed heterozygosity was lower than expected in domestic Japanese quails (p-value = 0.0012) as could be expected considering that the samples originated from separate breeding lines.

Group	N	Average number of alleles	Allelic richness	PIC	H_E	H_O
Game farm quails	52	14.00	7.65	0.80	0.83	0.80
Experimental farm quails	19	8.64	6.93	0.77	0.82	0.84**
Wild common quails	42	17.73	9.82	0.87	0.90	0.90
Domestic Japanese quails	39	5.27	4.11	0.59	0.66	0.60*

Table 2. Genetic diversity for each group of samples. N: number of individuals. PIC: Polymorphic Information Content (Botstein et al. 1980). H_E: expected heterozygosity; H_O: observed heterozygosity (Nei 1987). Significant differences between H_E and H_O are indicated by * (p-value ≤ 0.05) and ** (p-value ≤ 0.001).

Population differentiation

Wild common quails appeared completely separate from domestic Japanese quails along the first factorial component (FA-I) of the FCA (Figure 1). Game farm quails occupied an intermediate position between common quails and domestic Japanese quails along the same axis showing almost no overlap with either of them. Individuals from the experimental farm occupied the same range of values than wild common quails along the first axis, but showed a clear separation along the second axis (FA-II), with some individual values overlapping the range observed for wild common quails.

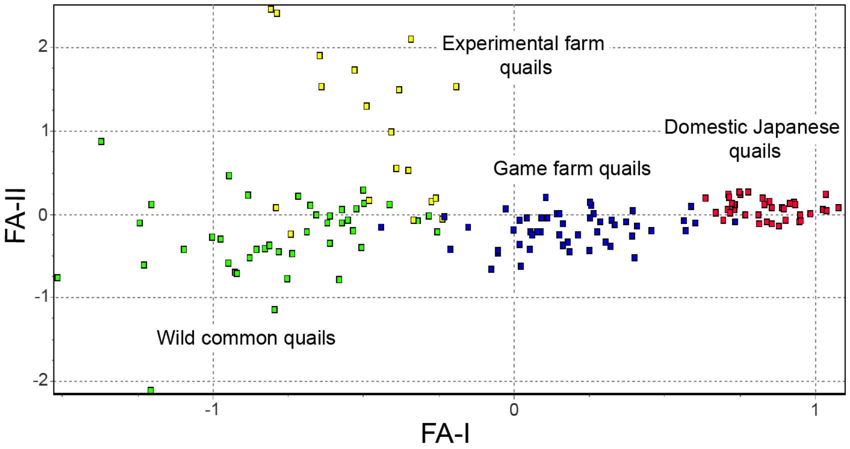

Figure 1. Factorial correspondence analysis. It shows the relationship among individual quail genotypes. Each colour represents a group of samples. Green: wild common quails; red: domestic Japanese quails; blue: game farm quails; yellow: experimental farm quails.

According to the Evanno et al. (2005) approach, the total sample could be subdivided in four clusters (Figure 2). Likelihood values converged during the runs and results did not change across replicates. Common quails and domestic Japanese quails were completely separated in two different clusters. All wild common quails (N=42) were unequivocally assigned to cluster 1 (C_1), with $q_i \geq 0.84$, while all domestic Japanese quails (N=39) were assigned to cluster 2 (C_2), with $q_i \geq 0.92$. Considering these results, we established that q_i values under a threshold value of 0.84 could be suggesting admixed ancestry. Of the game farm quails (N=52), 73% grouped in a separate cluster (C_3) while 27% appeared as admixed. Experimental farm quails (N=19) split up into their own cluster (C_4) (48%), and C_1 (26%) (with common quails), while 26% of them were identified as admixed. These results show that none of the game farm quails were assigned to the same cluster than common quails, and this was also the case for 74% of the experimental farm quails.

Hybrid identification

The partition of the sample in 4 groups was the result of the relative isolation between the different groups. However, since the sample included two evolutionarily distinct lineages (common and Japanese quails) and perhaps admixed individuals, we also investigated the partition of the sample into two groups (*K*=2). The likelihood values converged during the runs and results did not change between replicates. Common quails and domestic Japanese quails appeared completely differentiated in separate clusters, as in the previous analysis (Figure 2). All wild common quails (N=42) were unequivocally assigned to cluster 1 (C1), with $q_i \geq 0.87$, while all domestic Japanese quails (N=39) were assigned to cluster 2 (C_2) with $q_i \geq 0.83$ (Figure 2). In this case and considering these results, we established the value of 0.83 as the threshold below which individuals could be classified as admixed. Forty-three of 52 (83%) game farm quails had admixed genotypes, 8 (15%) clustered in C_1 with common quails and 1 (2%) with domestic Japanese quails in C_2. Sixteen (84%) of the experimental farm quails clustered in C_1, while the remaining 3 individuals (16%) had admixed genotypes (Figure 2).

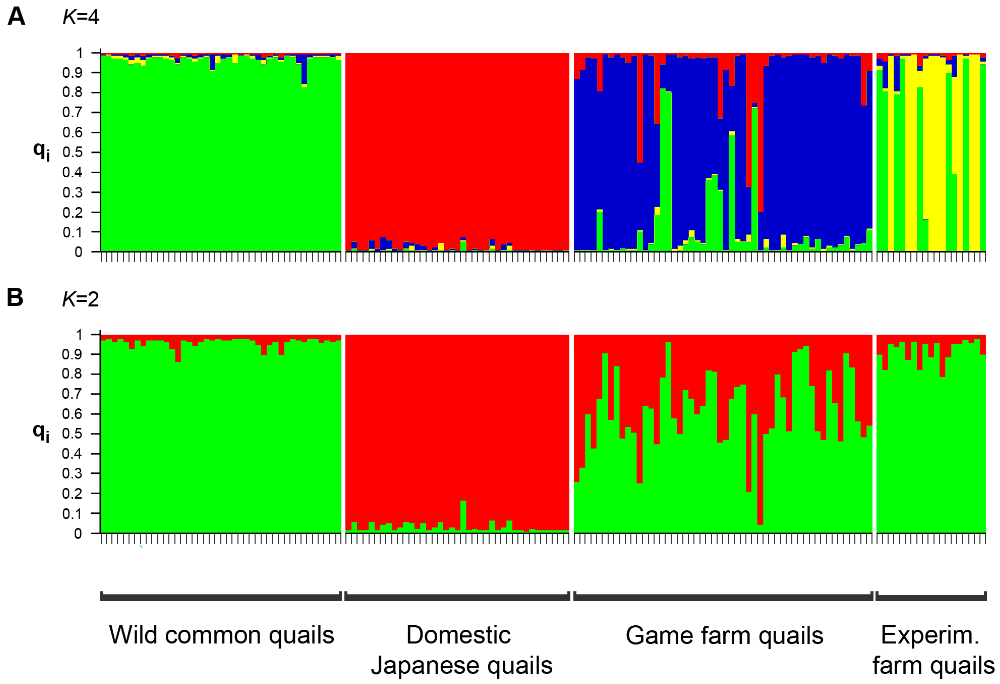

Figure 2. Clustering of individual genotypes into 4 (A) or 2 (B) clusters according to STRUCTURE. Each vertical bar represents one individual and clusters are represented by colours. The extent of the colours within each column represents the individual proportion of membership (q_i) to each one of the clusters. A) For *K*= 4: green: C_1; red: C_2; blue: C_3; yellow: C_4. B) For *K*= 2: green: C_1; red: C_2.

We also evaluated the width of the 90% probability interval for each q_i when *K*=2. While none of the wild common quails or domestic Japanese quails had probability intervals that exclude the possibility of being pure (q_i to C_1= 1 or 0, respectively; Figure 3), purebred ancestry could be excluded in 71% of the game farm quails. On the contrary, probability intervals did not exclude the possibility of being a pure common quail for any of the individuals from the experimental farm (Figure 3).

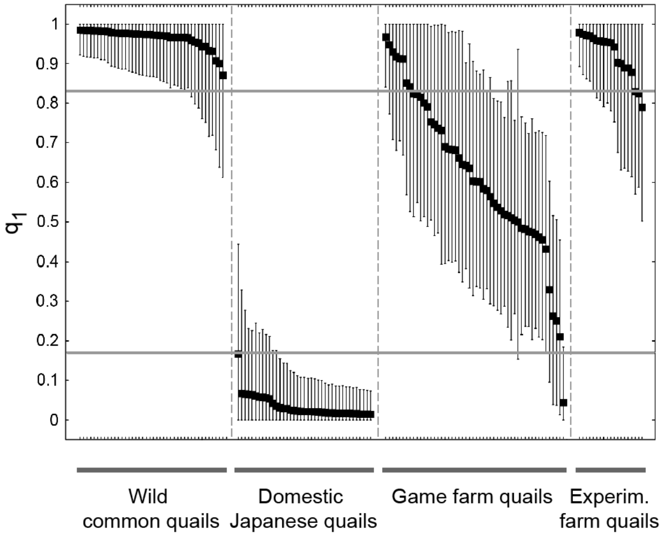

Figure 3. Individual proportion of membership (q_i) and 90% individual probability intervals according to STRUCTURE with *K*=2. Distribution of the q_i values as the individual proportion of membership to the same cluster as wild common quails (C_1), and 90% probability intervals for each individual. Individuals are sorted by group and by q_i value. Probability intervals excluding 0 and 1 are indicative of admixed ancestry. Horizontal lines indicate threshold values for q_i used for a first separation of pure and admixed individuals (see text).

After defining wild common quails and domestic Japanese quails as reference purebred groups, we used NEWHYBRIDS to identify the origin of all farm quails. More than 95% of wild common quails and domestic Japanese quails showed $P \geq 0.85$ to their genotypic class (P_1 and P_2, respectively), independently of the prior combinations used, what corroborated these samples could be used as reference groups. Game farm individuals that were classified as F_2 hybrids using a Jeffreys distributed prior for Theta were classified as domestic Japanese quails using a uniform prior. This last classification was considered unlikely due to the previously known information regarding the management of game farms (see discussion) and due to the results obtained with STRUCTURE when *K*=2. For this reason, we only took into account results obtained using Jeffreys prior for Theta (individual classification did not show significant differences depending on the prior used for Pi when Jeffreys prior was selected for Theta). The analyses indicated that 46 out of 52 (88%) game farm quails were admixed (Figure 4). Thirty of these 46 could be identified as F_2 hybrids, while 16 of them were hybrids of unknown generation (probably indicating older admixture). Three of the game farm quails (6%) could not be classified into a unique class, 2 were assigned as common quails and 1 as domestic Japanese quail. On the other hand, 10 out of 19 of the experimental farm quails (53%) were identified as common quails, 7 (37%) could not be classified and 2 (10%) were hybrids of an unknown generation.

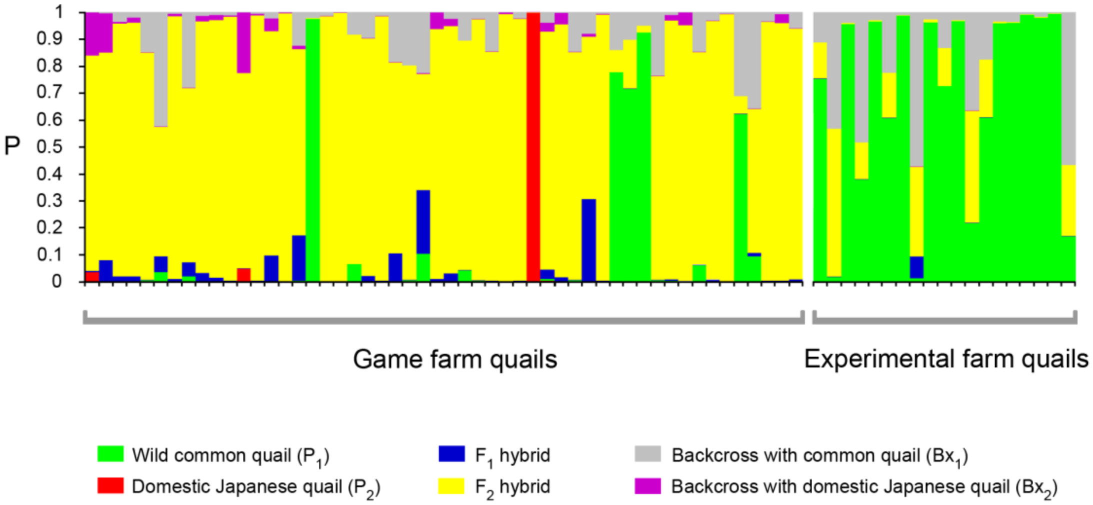

Figure 4. **Individual genotype classification according to NEWHYBRIDS**. Each vertical bar represents one individual. Each colour represents the posterior probability (P) of each individual to belong to the six different genotypic classes. Game farm and experimental farm individuals are sorted as in Figure 2.

Comparing the results regarding game farm and experimental farm quails obtained with STRUCTURE when *K*=2 and with NEWHYBRIDS, we observe assignment inconsistencies in only three individuals. For the three cases (three game farm quails) individuals were classified as common quails by STRUCTURE but as hybrids of unknown generation by NEWHYBRIDS. This is likely to result of ancient admixture with multiple generations of backcrosses with pure common quails. While all individuals were classified as either pure or admixed by the criteria that we defined in STRUCTURE, the classification with NEWHYBRIDS was more conservative and some of the individuals were left unclassified.

DISCUSSION

Our results show that each one of the groups of quails studied has a unique identity that allows its separation in genetic analyses. Thus, wild common quails, domestic Japanese quails, game farm quails and experimental farm quails form four well separated groups, as suggested by STRUCTURE (Figure 2). Among these, wild common quails have the largest genetic diversity, as should be expected since the other groups represent smaller captive lineages that are more or less reproductively isolated from each other. Probably, the arrival of domestic Japanese quails from Asia into Europe did not involve many different lines (Rizzoni & Lucchetti 1957, 1972). This bottleneck could have produced the low genetic diversity present in the domestic Japanese quail lines analysed. Similarly, the number of breeders in game farms is expected to be relatively small. In addition, domestic Japanese quails do not constitute uniform populations but are fragmented (with separate meat and laboratory lines, for example), as shown by the difference between observed and expected heterozygosity resulting from a Wahlund effect (Hamilton 2009). The opposite pattern is observed in the experimental farm, where outbred matings are regularly imposed.

The differentiation is largest between wild common quails and domestic Japanese quails since both groups appeared in the two extremes of the first axis of the FCA without any overlap (Figure 1). All STRUCTURE analyses clustered them in separate groups, including those with K=2, which allows separating the two evolutionary lineages. Also, a large number of alleles are not shared between the two groups, although this can be greatly affected by the sample size and by the fact that domestic Japanese quails are represented by inbred lines. This clear separation for the studied markers facilitates the identification of admixed individuals, overcoming the difficulties derived from their similar phenotypes.

Our analyses suggest that, contrary to the claims commonly expressed by farm managers, at least between 85% and 90% (depending on the approach used) of the analysed game farm quails commonly used for restocking purposes in Spain are not pure common quails and show obvious signs of admixture with domestic Japanese quails. This estimate is based on the assumption that the reference individuals indeed represent purebred common and domestic Japanese quails. However, it is possible that some of them could have slightly admixed ancestry. Nevertheless, if this was the case, our numbers would be underestimating the proportion of admixed individuals in game farms, and the real proportion could be even larger.

This large proportion of admixed individuals does not seem exclusive to Spanish game farms. Previous genetic studies included a few game farm-reared quails and suggested hybrid origin in Portugal (Amaral et al. 2007), France, Italy and Spain (Barilani et al. 2005). Most of the time, farm managers avoid revealing their procedures but indirect reports have suggested that they may often interbreed individuals of hybrid origin for many generations. We investigated the probability of farm individuals being pure or offspring of two generations of intercrossing or less (F_1, F_2 or a backcross of F_1 to one of the two parental classes). It is likely that many of the individuals studied are the result of a larger number of generations of intercrossing, but in order to estimate the ancestry of each individual with higher precision we would require a larger number of markers with high PIC, which would allow a better separation between hybrid classes (Vähä & Primmer 2006). Nevertheless, our results clearly show the admixed ancestry of the studied game farm birds.

The analyses with NEWHYBRIDS indicated that, depending on the priors used, most game farm individuals were classified as domestic Japanese or F_2 hybrids. We assumed that identification as F_2 was more likely because of the results obtained with STRUCTURE with K=2 and because of the mating strategies that may have been taking place in game farms to obtain individuals for hunting purposes (Guyomarc'h 2003). Domestic Japanese quails and common quails (Pérez 1974, Derégnaucourt et al. 2005) could have been crossed, most likely domestic Japanese females with wild common quail males trapped in the field (Amaral et al. 2007), and the offspring could has been subsequently intercrossed. With this procedure, fertile hybrids (Derégnaucourt et al. 2002) may easily be obtained showing a “wilder” instinct, flying better and being smaller than the domestic Japanese quails (Guyomarc'h 2003), and thus being more attractive for sportive hunting. However, these hybrids inherit the reduced restlessness of domestic Japanese quails (Derégnaucourt 2000 in Barilani et al. 2005). Among the game farm quails analysed, between 4% and 15% were identified as common quails, depending on the approach used. This could reflect the occasional introduction of wild quails into the captive populations to act as breeders in order to decrease inbreeding and genetic load.

In the case of the experimental farm, common quail males have been introduced to the farm breeding population with a regular and much higher frequency than in conventional game farms during the last 20 years. What is more, in the last ten years (about 10 generations) only females that are descendants of a common quail father have been used as breeders. In this case, we expect a higher frequency of common quail alleles in experimental farm quail genomes. As a result, between 53% and 84% (depending on the methodological approach used) of the analysed experimental farm quails were classified as common quails. This implies that, after 20 generations of experimental crossing, between 10% and 16% of them are still identified as hybrids and 37% cannot be classified into any of the genotypic classes. In addition, we expect all individuals from this farm to carry Japanese quail mitochondrial DNA (see also Barilani et al. 2005). The management strategy implemented in the experimental farm, trying to obtain individuals genetically more similar to the common quail than individuals from ordinary game farms, achieves its goal. However, we have no information neither about the possible cytonuclear conflicts deriving from the different origin of their mitochondrial and nuclear genomes, nor about the relative fitness, behaviour and survival of these individuals in the wild compared to pure common quails. We ignore to which extent this management could minimise the impact of releasing admixed individuals.

Due to the concern about the risk of restocking hybrid farm-reared quails, several European countries and regions have banned or regulated restocking practices (Portugal and France since 2002, Greece since 2007). In Spain, although different regions have different policies, the national law allows restocking with common quail, but not with Japanese quail or hybrids (Spanish Law, 42/2007, Natural heritage and Biodiversity). Even so, many farm-reared individuals are still being released assuming that, in fact, they are pure common quails. Authorities allow quail releases trusting on the diagnosis of veterinarians that identify them as common quails on the only basis of their phenotype despite the fact that this identification method is usually ambiguous (Derégnaucourt 2000 in Barilani et al. 2005). Genetic analyses should be required to certify the origin of individuals used for restocking.

Our results show that restocking with farm-reared quails entails a potential severe risk for native populations of common quail because of their admixed origin. There is an urgent need for controlling the genetic origin of restocked quails and preventing the release of hybrid quails to preserve the genetic integrity of the common quail. Similarly, there is a need for an extensive survey of the diversity in wild quails to assess the impact that these releases may have had in the populations across its distribution range.

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PAPER 2

Decreased fitness of restocked hybrid quails prevents fast admixture with wild European quails

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Biological Conservation 171: 74-81

Hybrid chick hatched out in the lab, by Ines Sanchez-Donoso

"Your time and effort are very valuous, even more than the money you are going to spend"
Jennifer A. Leonard

ABSTRACT

Restocking with non-native species for hunting purposes is a widespread practice in some Galliformes species that may result in the introgression of maladaptive alleles into native populations. Quails farmed for restocking are produced by interbreeding domestic Japanese quails (*Coturnix japonica*) with European quails (*Coturnix coturnix*). Massive releases of these animals could represent a threat to native European quails. In this study we radio-track 16 female native European quails and 51 female farm-reared quails over four breeding seasons in a single locality. Our results show that farmed female quails attracted more wild common quail males than European quail females, probably because they produce more rally calls. Here for the first time we show empirical evidence that European quails and restocked farmed quails interbreed in the wild. Further, hybrid farmed females and European females had similar probabilities of mating, nesting success, clutch size, fertility, hatching probability and chicks' survival probability. However, female farmed quail had higher mortality than European quail females, and their nests had a higher rate of predation. These last observations could explain why the two lineages do not appear completely admixed after more than 20 years of massive restocking practices. However, our results clearly show a lack of reproductive barriers in the wild and that introgression may not be completely prevented. Thus, banning restocking with Japanese quail or hybrids is necessary to prevent the progressive introduction of maladaptive alleles into the European quail populations.

INTRODUCTION

Anthropogenic hybridization, either resulting from the introduction of species or from the invasion of alien species due to the modification of habitats, may create serious conservation problems (Levin et al., 1996; Rhymer and Simberloff, 1996). Introductions may be accidental (Kidd et al., 2009; McDowell, 2002) or part of a management plan, for example, to bolster endangered populations (Armstrong and Seddon, 2008; Massemin-Challet et al., 2006; Santos et al., 2009) or to increase hunting bags in the case of game species (Barbanera et al., 2010; Blanco-Aguiar et al., 2008). Restocking with non-native species for hunting purposes is a widespread management action for Galliformes (Barbanera et al., 2005, 2007, 2008; Blanco-Aguiar et al., 2008; Casas et al., 2012; Derégnaucourt et al., 2002; Derégnaucourt et al., 2005a, b; Porkert et al., 1997; Puigcerver et al., 2007; Tejedor et al., 2007). These restocking practices are carried out mainly with farm-reared birds, in most cases of doubtful genetic origin (Perennou, 2009), and thus constitute a real danger for native populations (Chazara et al., 2010; Laikre et al., 2010; Olden et al., 2004).

The European quail (Coturnix coturnix), also called common quail in Europe, is a migratory Galliform with a distribution from the British Islands to Lake Baïkal and from the Arctic Circle to the tropics (Del Hoyo et al., 1994; Guyomarc'h et al., 1998; Moreau and Wayre, 1968). It is of conservation concern (SPEC 3) due to a large historical decline (Burfield, 2004). Even so, it is a very popular game bird with a high socio-economic value, especially in some Mediterranean countries. In Spain alone, which hosts the largest breeding population in Western Europe (Gallego et al., 1997; Rodríguez-Teijeiro et al., 2006), more than 1,300,000 individuals were hunted annually during the period 1973–2010 (Yearbooks of Agro-alimentary Statistics of the Spanish Ministry of Agriculture, Fisheries and Food). Because of hunting interest in this species, restocking with farm reared quails is a very common practice in several European countries such as Italy, Greece, the Republic of Serbia, Montenegro, Romania and Spain (Puigcerver et al., 2007; Rodríguez-Teijeiro et al., 1993; Tsiompanoudis et al., 2011). For example, in Catalonia (Northeast Spain), more than 68,000 farm-reared quails have been restocked annually during the period 1990–2006 (personal communication from the Catalan Department of Agriculture, Farming, Fisheries, Food and Environment) in an area with a wild population of between about 5000 and 21,000 males (Rodríguez Teijeiro et al., 2004).

Recent genetic studies show that these farm-reared quails are hybrids of European and domesticated Japanese (Coturnix japonica) quails (Amaral et al., 2007; Barilani et al., 2005; Chazara et al., 2010; Sanchez-Donoso et al., 2012) (in this manuscript we use the term ''hybrid'' to refer to all individuals whose genomes show extensive admixture of the two lineages, without limiting the definition to first generation hybrids). The Japanese quail is distributed across East Asia, including Japan. The natural ranges of the two species only overlap in the surroundings of Lake Baïkal in Russia (Fefelov, 1998) and in the Kentei region in Mongolia (Kozlova, 1932). No natural hybridization has been reported (Del Hoyo et al., 1994; Guyomarc'h et al.,1998; Moreau and Wayre, 1968). However, hybrids of the two species generated in captivity are or have been used for releases indifferent European countries, and, as a result, the Japanese quail is considered an introduced species in Italy by the IUCN (http://maps.iucnredlist.org/map.html?id=100600195; accessed on November 5 2013) and in Spain by the Spanish Government (Royal Decree 630/2013). Thus, there is a real possibility that restocking could lead to the extensive introgression of domestic Japanese quail alleles into the European quail gene pool.

The European quail and the Japanese quail have almost identical habitat requirements (Taka-Tsukasa, 1941), and experiments in captivity show that they present only partial mate discrimination (Derégnaucourt and Guyomarc'h, 2003). Derégnaucourt et al. (2002) have also shown that hybridization between European and domestic Japanese quail, and backcrosses between hybrids and the parental species readily occurs in captivity and no differences have been found in average fertility, hatch and chick survival rates between hybrid pairs and European quails.

However, the arrival of Japanese quail alleles may represent a threat for European quails if they represent different adaptation to environmental conditions, co-adapted gene complexes or even sets of maladaptive alleles favorably selected in captivity. The European quail is a partially migratory species, whereas the domestic Japanese quail lost its migratory impulse during the domestication process (Derégnaucourt et al., 2005a,b; Guyomarc'h, 2003). As released hybrid quails have a lower frequency of migratory phenotypes (Derégnaucourt et al., 2005a, b), the introgression of their genes into wild populations could cause a decrease in the migratory tendency of European quail populations. This could lead to a decrease of the European quail population density, as non migratory quails are more likely to suffer seasonal depletion of food resources in winter and a longer hunting season.

At least in theory, hybridization between European and farmed quails is likely to lead to a rapid increase in the proportion of hybrids and admixed individuals in wild populations. Once admixture has begun it is difficult to stop, especially if hybrids are fertile and mate both with other hybrids and with both parental species (Allendorf et al., 2001). As a result, in a few generations this process might result in a hybrid swarm in which almost all individuals are of hybrid origin (Allendorf et al., 2001; Huxel, 1999), leading to the collapse of the European quail population. But this may not be just a theoretical threat. Hybrid individuals have been detected across Europe (Guyomarc'h, 2003; Rodríguez-Teijeiro et al., 1993), and genetic evidence for introgressive hybridization has been reported in Portugal (Amaral et al., 2007), Spain (Barilani et al., 2005; Sanchez-Donoso et al., 2012) and France (Chazara et al., 2010). However, these studies do not provide evidence of hybridization occurring in the wild. Consequently, Guyomarc'h (2003) called for changes in European Union law to stop the introgression of Japanese quail genes by banning the production and commercialization of Japanese quails as well as the breeding of hybrid quails for restocking purposes. Finally, the European Union Management Plan for the European quail (period 2009–2011) has stressed the need to ban Japanese/hybrid quail releases throughout Europe and across the rest of the European quail distribution (Perennou, 2009).

However, in spite of this persistent threat, Puigcerver et al. (2007, 2012) monitored the phenotype and song in wild quail populations in Catalonia over a period of 28 years (from 1984 to 2011) and failed to detect an increase in the proportion of hybrids. At the same time, genetic analyses over a 15 year period (1996–2010) showed that an increase over time in the proportion of admixed individuals cannot be detected (Sanchez-Donoso et al., 2014). These data strongly suggest that despite the annual release of tens of thousands of hybrids, the introgression of Japanese quail alleles into the wild population is lower than initially expected.

The lack of an obvious increase in the proportion of admixed individuals can be explained by different factors:

(a) A very high mortality rate of restocked hybrids, which could be poorly adapted to life in the wild, because they lack the ability to protect themselves against cold weather, antipredator behavior and the ability to find appropriate food (Guyomarc'h, 2003). High mortality is probably also due to hunting, since restocked individuals are usually released just before the opening of the hunting season, which starts before the birds are habituated to the new environment.

(b) In spite of the lack of reproductive barriers in captivity, ecological or behavioral barriers could prevent admixture in natural conditions. There is no direct evidence of mating between European quails and farmed birds in natural conditions.

(c) Mixed pairs (or pairs of farmed animals mating in the wild) could suffer high chick mortality (this could be due to causes such as lack of parental care resulting from adaptation to captivity, or susceptibility to parasites), as has been observed for other Galliformes. For example, Duarte and Vargas (2004) found a chick mortality rate of 91% in restocked farm-reared females of red-legged partridge (*Alectoris rufa*).

The aims of this study were to investigate whether massive restocking with farm quails of hybrid origin quails in one of the European countries where restocking with farm-reared quails is legal (Spain) may lead to extensive admixture with European quails in the wild, and to study mating and productivity differences between female wild European quails and females restocked from game farms. Finally, we investigate how these differences could contribute to explain why the proportion of admixed individuals is not clearly increasing through time in the wild population. Our results showed decreased survival and fitness for the released birds. The results also emphasized how the putative consequences of restocking practices cannot be directly inferred from just the number of individuals released.

MATERIAL AND METHODS

In Spain, the country where the study has been carried out, the Law 42/2007 of Natural Heritage and Biodiversity allows restocking with native species but not with non-native ones or hybrids. In this case, restocking with European quail is allowed, but not with Japanese quail or hybrids. In our study, we have used farm reared quails intended for restocking that were certified as European quails by the regional Administration based on a veterinarian analysis. However, these farm-reared individuals turned out to be hybrids (Sanchez-Donoso et al., 2012). As indicated below (see ''Field Experiment Design'') we obtained all necessary permits required to carry out this study, and the released farmed birds that survived to the end of the study as well as their chicks were recaptured.

Study area

The study was carried out in a 1 km2 area in Northeast Spain (41º25’N, 1º23’E, 628 m above sea level), in a short and narrow valley with winter cereals (mainly barley and wheat) which constitutes a suitable habitat for European quail reproduction. The study area is surrounded by pine forest patches unsuitable for quails, but other cereal fields with quails are located less than 2 km away.

Field experiment design

The study was conducted during the breeding seasons of the period 2007–2010. The field work started around April 23rd, coinciding with the arrival of the first migrants, and finished around August 4th with the end of the breeding season due to the harvest of cereals, which implies the loss of the breeding habitat. On average, the field work lasted 102.5 days (SD=7.2) per breeding season.

During the period 2007–2009, 20 walk-in funnel traps (144 cm x 67 cm x 87 cm) were randomly distributed along the edge of the cereal plots facing the cereal. Inside these traps we put a smaller cage containing a sexual decoy (Rodríguez-Teijeiro et al., 2003; Sardà-Palomera et al., 2011). In ten of these traps, the sexual decoy was a hybrid female from a game farm close to the study area, in Catalonia (Spain). These farm-reared hybrids showed small phenotypic differences when compared to their wild counterpart (as in Guyomarc'h, 2003): they had shorter wing and longer tarsus.

Groups of male European quail have been shown to attract both males and females (Sardà-Palomera et al., 2011). For this reason, the remaining ten traps were set in five pairs to replicate the aggregations of males observed in the field (normally involving 2 to 4 males, pers. obs.). Each pair was set in parallel separated by about 5 m. One trap of the pair contained one cage and the other one contained two cages, each cage containing one male decoy. Thus, each pair of traps represented an aggregation of 3 males (hereafter, male aggregation trap). Two of the male aggregation traps contained male European quail captured in neighboring areas and the remaining three contained male hybrid quail (Table 1).

Sexual decoy in the trap	**2007**	**2008**	**2009**	**2010**	**TOTAL**
One hybrid female	10	10	10	6	36
One European quail female	0	0	0	3	3
Hybrid male aggregation (3 males)[a]	3	3	3	3	12
European quail male aggregation (3 males) [a]	2	2	2	2	8
Total	15	15	15	14	59

[a] Each one of these is composed by two traps and were intended to imitate groups of males found in European quail populations.

Table 1. Number and types of traps used each breeding season.

In 2010, 19 walk-in funnel traps were placed in the study area. Six traps contained hybrid females as sexual decoy, while for comparative purposes three contained European quail females (captured in neighboring areas and kept in a pen for several months). No changes were made with regard to male aggregation traps (Table 1).

Traps were checked every 2–3 days to keep disturbance caused by researchers to a minimum. Water was supplied from an aseptic 8-l tank; food trays (with vitamin-supplemented wheat) had a capacity of 3 kg which allowed individuals to eat ''ad libitum''. Cages had double wire to protect the quails against predators, and trap, which was protected against sunlight and rainfall, was sprayed at every visit with a mammal repellent. At every visit, food and water supplies were checked.

When a free-ranging male entered a funnel trap containing a female (either European quail or hybrid), we attached backpack radio transmitters (PIP-3 Biotrack button cell tags) to the females and we released them. The released female was replaced in the cage by another one of the same origin. Three days per week, the released individuals were monitored with a telemetry receiver (R1000, 148–174 MHz; Communications Specialists, Inc., Orange, CA) and a three element Yagi antenna to locate them and to check if they had mated and nested, following Rodríguez-Teijeiro et al. (2003). We consider that a female mated successfully when she succeeded in establishing a pair bond with a male, no matter whether this female would succeed in nesting or not (she could be predated before that). For nesting success we understand the success in nesting by a female, no matter whether this nest was finally predated or not. At the end of the study, hybrid radio tagged individuals were captured and returned to captivity to avoid the risk of genetic pollution. A total of 67 individuals were radiotagged (51 hybrid females and 16 European quail females). Table 2 shows the number of radio-tagged females and the number of nests found in each year of study.

Type of female	2007	2008	2009	2010	TOTAL
European quail females	1 (1)	2 (2)	2 (1)	11 (7)	16 (11)
Hybrid females	11 (8)	15 (4)	17 (8)	8 (1)	51 (21)

Table 2. Number of females radio-tagged in each year of study. In brackets: number of nests found.

Once a nest was located, we determined the clutch size. After 10–12 days of incubation (more than 60% of the incubation period), we took the eggs from the nest to put them in a Masalles G-180 THP incubator (Sant Cugat del Vallès, Barcelona, Spain) and raised the chicks in captivity. This procedure allowed us to know the number of fertilized eggs, the number of hatched eggs and the number of chicks surviving in captivity during the critical period of the first three weeks of life (while flying abilities are not yet developed) for European quail and hybrid females. Also, it allowed us t o know the number of nests preyed upon during the 10 days of the incubation period that were monitored. After the study was completed, these chicks remained in captivity in a large pen.

In addition, telemetry also allowed finding dead individuals. Since females tend to stay in the same area after their release, we defined the known survival time for each female as the time from the release until its death or until the study period reached its end due to harvest (at that time we proceeded to the recapture of the radio-tagged individuals).

Blood samples (100 µl) from the jugular vein were taken and used to genetically identify the origin of all individuals used in this study as European quails or hybrids following Sanchez-Donoso et al. (2012). All individuals were genotyped for 11 unlinked autosomal microsatellite loci (from Kayang et al., 2002, 2004) and genotypes were analyzed with STRUCTURE 2.3.2 (Pritchard et al., 2000) and NEWHYBRIDS 1.1 (Anderson and Thompson, 2002).

The study strictly followed the requirements of the Spanish Law 5/1995 of protection of animals used in experimentation and for other research goals, and the Decree 214/1997, which regulates the use of animals for experimentation and for other research goals. The animal experimentation procedure for this study (number B9900035) was approved by the Ethics Committee on Animal Experimentation of the University of Barcelona and by the Autonomic Government of Catalonia (‘‘Direcció General del Medi Natural’’, ‘‘Departament de Medi Ambient i Habitatge’’), which has all the competences in this subject. Moreover, the University of Barcelona institutional code of good research practices was followed (Vicerectorat de Recerca, 2010).

Data analysis

Attraction ability

The attraction of wild quails to traps was analyzed with regard to the sex and origin (European quail or hybrid) of the individuals used as decoy. For each kind of trap (with a female decoy or a male aggregation decoy), the number of captures was modeled with Generalised Linear Models (GLM) as a function of the origin of the decoy in the trap. The number of captured wild quails was the response variable and the origin of the individuals acting as decoy was the independent variable of interest in both models. GLM models were constructed assuming a Poisson error distribution and log link function. They were tested for overdispersion (residual deviance/residual df > 1) and the heterogeneity of residuals was assessed by visual examination of the figures. The number of traps with each kind of decoy was also added as an offset term because they were not equally represented not only between years, but also within years (Table 1). As female decoys do not attract wild females, an additional independent variable was the sex of the captured quails only for traps simulating aggregations of males. In this model, ‘‘male aggregation trap’’ was also added as a random factor because males and females captured in the same male aggregation trap are not independent observations. Thus, this model was a Generalised Linear Mixed Model (GLMM). Since only in 2010 hybrid females and European quail females were simultaneously used as decoys, the comparison of their mate attraction was carried out only in this year. The interaction between explanatory variables was initially added to the model and removed if not significant. To establish the significance of the full model we used a likelihood ratio test, comparing its deviance with that of the null model including only the intercept. To test the significance of the interaction between explanatory variables we compared the deviance of the full model with that of a corresponding reduced model without interactions. These models allowed us to test whether there were differences in the number of quails captured per trap and breeding season considering the two different decoys. We could also investigate differences between sexes in the degree they are attracted by male aggregations.

Female mating and breeding success

A GLM was fitted for each variable related to female breeding success: (1) mating success (binary); (2) nesting success (binary); (3) clutch size (count); (4) fertilized eggs per laid egg (proportion); (5) nest predation (binary); (6) hatched eggs per fertilized egg (proportion); and (7) surviving chicks per hatched egg (proportion). Binary and proportion variables were fitted assuming a binomial error distribution and logit link function and clutch size was fitted assuming a Poisson error distribution and log link function. If a model presented overdispersion (residual deviance/ df > 1), the corresponding variable was refitted using, depending of the case, a quasi-Poisson or a quasi-binomial error distribution.

A factor representing years and its interaction with origin (hybrid or European) were also initially added to the models to take into account changing conditions across years. These additional factors were removed from the final models if no significant effect was found.

Since female mating and nesting success are not only influenced by female ability to mate and nest but also by their survival probability, when modeling those variables, we added as covariate the female known survival time. Thus, this covariate allowed the models to test for differences in female mating and nesting success due to their origin independently of possible differences due to different mortality. These models also allowed us to estimate how long it takes for a female to achieve 95% probability of mating or nesting.

Survival

We used a Cox proportional hazards regression model (Cox, 1972) to assess the effect of female origin on its survival during the breeding season. This model assumed that the relative risk of death for both types of females (hybrid or European quail) remained constant over time. This risk was estimated by the model by considering the known survival periods. The model took into account if the end of the known survival period of each female was due to death or to the beginning of harvest. We also took into account the possible differences across years, and the interaction origin - year. If the interaction was not significant, it was removed from the models.

All analyses were conducted in R ver. 2.15.2 (R Core Team, 2012). GLM's models were fitted using the `glm()` function. Cox proportional hazards model was fitted using the function `coxph()` present in the package `survival` (Therneau, 2012). The significance of the factors added into a model were evaluated performing an analysis of deviance with the function `Anova()` available in the package `car` (Fox and Weisberg, 2011). In not overdispersed, the test for the analysis of deviance for GLM and Cox models was a likelihood-ratio (χ^2). In overdispersed GLM, an F-test was used. The differences between the levels of a significant factor were tested with the function `esticon()` from the package `doBy` (Højsgaard et al., 2013). The p values obtained from `esticon()` function were corrected using the `p.adjust()` function which controlled type I errors performing a false discovery rate correction (Benjamini and Hochberg, 1995). Confidence intervals (CI) of the estimated parameters were calculated for a 95% confidence level.

RESULTS

Genetic origin of all individuals was unambiguously assigned and according to the expectations for wild (European quails) and farm (hybrid) quails (results not shown). Analyses with STRUCTURE AND NEWHYBRIDS produced consistent results.

Attraction ability

In 2010, hybrid females acting as sexual decoys in walk-in funnel traps attracted 2.58 times more wild European quail males than did female European quails (confidence interval, CI: 1.16–6.87, χ^2_1=5.52, p=0.019, Fig. 1a).

Male aggregation traps attracted 5.75 times more wild males than females (CI: 2.87-13.16, χ^2_1=20.85, p<0.001, Fig. 1b). European quail male aggregation traps were 2.55 times more visited, by both wild males and females (interaction not significant), than hybrid male aggregation traps (CI: 1.48-4.58, χ^2_1=11.03, p<0.001). The number of individuals entering the traps varied between years (χ^2_3=12.68, p=0.005); thus, in 2008 only 0.5 individuals were captured per trap, whereas in the rest of years 3.5 individuals were captured. These results strongly suggest that: (a) female hybrids are able to attract wild European quail males more effectively than female European quail; (b) male aggregations attract more males than females (although we do not have precise information about the sex ratio in the study area; males are known to be several times more numerous than females in the area); (c) hybrid male aggregations attract less wild quails (males and females) than European quail aggregations.

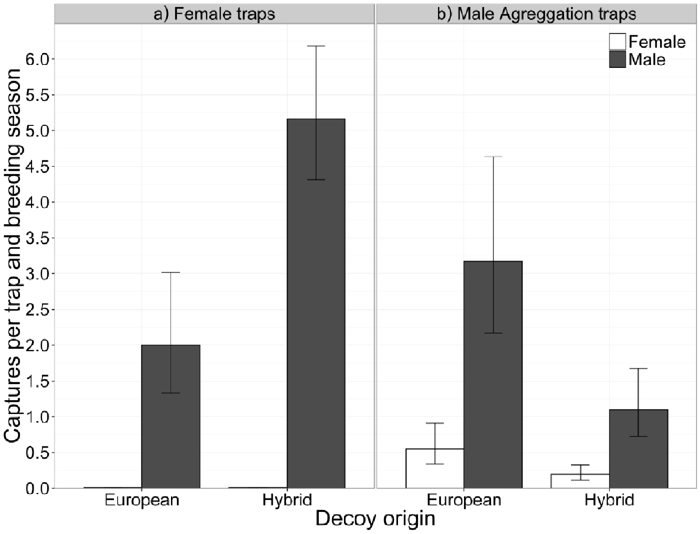

Figure 1. Female and male wild quails captured per trap and breeding season (mean ± se) as a function of decoy origin (European quail or hybrid originating from game farms) for two types of trap: a) one female used as decoy (only 2010 data) and b) a group of males used as decoy (from 2007 to 2010 data).

Female mating and breeding success

During the four years of the study, a total of 51 hybrid females and 16 European quail females were radio-tagged and monitored. The majority of the matings (94%) took place with a common quail male. For hybrid females, 61% (31) succeeded in mating, whereas 81% of European quail females (13) mated. Two hybrid females (4%) paired with freely roaming hybrid males. Mating probability was first modeled including ‘‘female origin’’ and ‘‘year’’ as factors, together with the ‘‘known survival period’’ of each female as covariate. Due to the low frequency of matings with free-roaming male hybrids, male origin was not included as a factor. This model showed a significant effect of year in female mating probability (χ^2_3=8.24, p=0.041). Mating probability in 2009 was higher than in 2007 (χ^2_1=4.16, p=0.041) and 2008 (χ^2_3=4.55, p=0.033). This mating probability also depended on the female known survival period (χ^2_1=37.68, p<0.001, Fig. 2a). But the model failed to show differences in the probability of mating between hybrid and European quail females (χ^2_1=0.93, p=0.335). On average, the model shows that mating probability reached 95% 40.8 days (CI: 25.3-72.8) after the release of the female (Fig. 2a).

Radio-tracking of the 51 hybrid females monitored showed that 21 of them (41%) nested, whereas of the 16 European quail female monitored, 11 of them (69%) succeeded in nesting. In the model for nesting success, neither female origin nor year had a significant effect. However, covariate ‘‘known survival period’’ had a significant effect. None of the interactions were significant. For these reasons, the final model was constructed using ‘‘female origin’’ as independent variable and ‘‘known survival period’’ as a covariate. According to this model, female origin did not have a significant effect on nesting success (χ^2_1=0.43, p=0.510), whereas known survival period showed a strong influence (χ^2_1=25.94, p<0.001, Fig. 2b). Females reached a 95% probability of nesting 49.6 days (CI: 27.7-99.2) after their release.

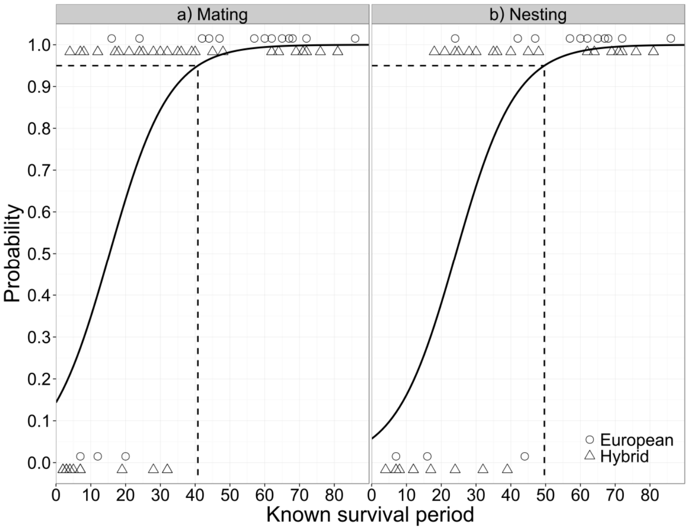

Figure 2. Female mating (a) and nesting (b) probabilities as a function of the number of days in which females remained alive in the study area (known survival period). Adjusted functions: (a) y=inv.logit(-1.79+0.12·x); b) y=inv.logit(-2.81+0.12·x). No differences were observed in these variables depending on female origin (European quail or hybrid). Dashed lines in both graphs show the time at which each Generalised Lineal Model predicts a mating or nesting probability of 95%. Observations of both European and hybrid female quails, from which the models were built, are represented by circles and triangles respectively.

Factor ''year'' showed a significant effect only on fertility (χ^2_3=10.11, p=0.0182). Fertility in 2009 was lower than in 2007 (χ^2_1=6.61, p=0.010). Since factor ''year'' did not have a significant effect on clutch size, hatching success or chick survival, it was excluded from these models. The final models did not show differences between hybrid and European quail females regarding clutch size (χ^2_1=2.74, p=0.098, Table 3), fertility (χ^2_1=0.01, p=0.915, Table 3), hatching success ($F_{(1,20)}$=2.67, p=0.102, Table 3), or chicks survival ($F_{(1,19)}$=0.46, p=0.496, Table 3).

Finally, the probability of having the nest preyed upon was higher for female hybrids (χ^2_1 =4.31, p=0.038, Table 3), and did not change from year to year (initial model: χ^2_3=7.45, p=0.059).

Variables studied	Hybrid female	European quail female
Clutch size	9.14 (7.30-11.50)	11.09 (9.24-13.18)
Fertility	0.97 (0.88-0.99)	0.97 (0.88-0.99)
Hatching success	0.93 (0.80-0.98)	0.83 (0.72-0.92)
Chick Survival	0.85 (0.73-0.93)	0.79 (0.69-0.87)
Nest predation[a]	0.22 (0.08-0.41)	0[b]

[a] Significant differences depending on female origin.

[b] Confidence intervals cannot be calculated due to the lack of variability.

Table 3. Nests variables. Means and 95% confidence intervals (in parentheses) for clutch size, fertility, hatching success, chick survival during the first three weeks of life, and nest predation probability for both hybrid and European quail female nests.

Survival

The mortality of the released hybrid females was 73% by the end of the annual study season; 23 of them were killed by predators (62% of the dead hybrid females) and 14 were found dead from unknown causes. This mortality rate is much higher than for European quail females (31%, 3 of them killed by predators and 2 found dead from unknown causes), clearly indicating that hybrid females proportional hazards regression model revealed that there is a significant difference in the relative risk of death for females of different origin (χ^2_1=17.55, p<0.001) and from different year (χ^2_3=12.47, p=0.006). More specifically, 2010 was the worse year. In 2010, the risk of death for a female was 5.47 (CI: 1.89–15.84) times higher than in 2007 ($\chi^2_1=9.84$, p=0.010) and 3.54 (CI: 1.43–8.77) times higher than in 2009 ($\chi^2_1=7.5$, p=0.018). The risk of death of a hybrid female, once released, was 7.22 (CI: 2.52-20.65) times higher than that of a European quail female. Fig. 3 shows the survival curves for European and hybrid quail females once released into the field. These curves show that 40.8 days after the release (time at which 95% of females have already mated, see Section 3.2) survival probability was higher for European quails (0.7, CI: 0.51-0.96) than for hybrids (0.29, CI: 0.19-0.44). Similarly, by the time 95% of females had nested (49.7 days after release, see Section 3.2), survival probability for female European quails was 0.68 (CI: 0.49-0.95), while for hybrids it was 0.27 (CI: 0.17-0.42).

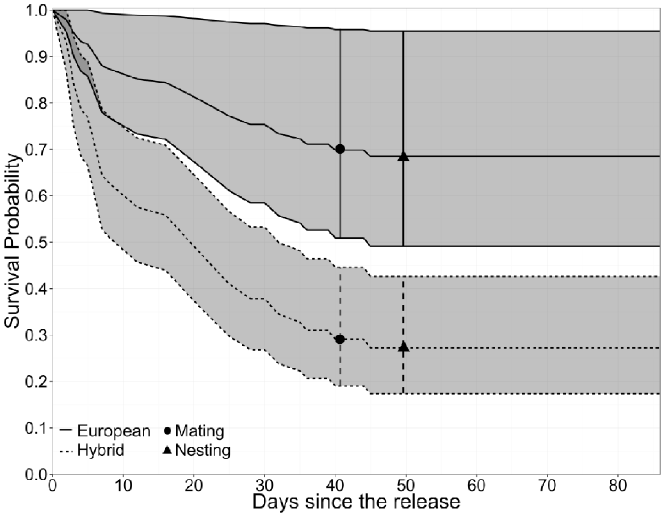

Fig. 3. Estimated survival probabilities (mean and 95% confidence intervals, based on a Cox proportional hazards regression model) for European (solid lines) and hybrid (dashed) female quails. Survival probabilities when mating (circles) and nesting (triangles) probabilities reach 95% (see Fig. 2) are represented to show that the probability of surviving long enough to mate and nest is higher for European quail females than for hybrid females.

DISCUSSION

Previous studies have shown that, in captivity, there are not pre- or post zygotic barriers preventing the interbreeding between European and Japanese quails or between European quails and hybrids (Derégnaucourt et al., 2002; Derégnaucourt and Guyomarc'h, 2003; Taka-Tsukasa, 1941). However, other studies suggest that the massive releases of farm bred quails have not resulted in a hybrid swarm (Puigcerver et al., 2007, 2012), indicating that perhaps ecological or behavioral barriers acting in the wild maintain separation (Pierotti and Annet, 1993). Nevertheless, this does not seem to be the case. Our results show, for the first time, that European quails and hybrids interbreed in the wild. Thus, the presence of admixed individuals in European quail populations in different countries during the breeding season may be explained not just as a result of restocking practices (as suggested by Puigcerver et al. (2007)), but also as the result of released hybrids reproducing in wild European quail populations.

Our results showed that female hybrids attract more wild common quail males than common quail females (Fig. 1). This could be due to the observation that they produced more rally calls from the trap, attracting more males (pers. obs.). This contrasts with the results by Derégnaucourt and Guyomarc'h (2003) who have shown that, in captivity, European quail females are very selective, emitting the greatest number of rally calls in response to mating calls from conspecific males, a lower number in response to mating calls produced by Japanese quail and an intermediate number responding to mating calls from hybrid males. A possible explanation for this difference could be that hybrid females produced more vocalizations because they were less stressed in a cage. Although we tried to minimize this effect by keeping wild caught females in captivity for some months before starting the experiments, we cannot assess if the differences in behavior are associated to the hybrid origin or are result of adaptation to life in captivity. In addition, female hybrids and European quails (Fig. 2a) had a similar probability of mating. These matings occurred mostly with European quail males (94%), which were much more abundant than hybrids in the area (Puigcerver et al., 2007). Therefore, mixed pairs formed in the wild are most likely formed by a hybrid female and a European quail male.

We did not know in advance whether our artificial hybrid male aggregations could attract wild European quails of both sexes, but we expected so because Sardà-Palomera et al. (2011) showed that European male aggregations attracted both males and females as a consequence of the mating system of the species. Our results show that wild European quail males, which have a complex polygamous mating system (Rodríguez-Teijeiro et al., 2003), are more attracted by artificial European quail male aggregations than to those of hybrid males, suggesting that natural European quail male aggregations found in the wild (Guyomarc'h et al., 1998) may be reasonably preserved despite the release of hybrids.

With regard to the breeding success of the females released from the traps together with a male, restocked hybrid females had similar mating and nesting success rates to European quail females. As in captivity (Derégnaucourt et al., 2002), fertility, hatching rate and chick survival rate (within the first three weeks of life) are also very similar between hybrid and European females (Table 3), although we evaluated chick survival after hatching the eggs in captivity, and this may not reflect survival rates in the wild. As farm-raised hybrid quails have been selected for life in captivity, their chicks should be expected to survive better in captivity than chicks coming from wild common quails. Therefore, the lack of differences in survival in captivity of chicks from hybrid and common quail does not imply similar survival in the wild. Since mating preferences do not show the existence of strong pre-zygotic barriers and the breeding success does not seem obviously lower, other mechanisms must explain why the proportion of hybrids has not been increasing in European quail populations (Puigcerver et al., 2007, 2012).

The reason for this may be found, at least in part, in the lower probability for female hybrids to survive long enough to mate and nest (Fig. 3). In addition, the nests of hybrid females are preyed upon more often (Table 3). These differences could contribute to explain why the massive releases of farmed quail have not resulted in a hybrid swarm. Mortality rates of restocked farm-reared birds are usually higher than for native populations because they are poorly adapted to the natural environment and lack the ability to defend themselves against cold and to forage and select food in those conditions (Guyomarc'h, 2003). After hunting, predation is probably one of the main causes of mortality for farm-reared hybrid quails, as happens for other farmed game bird species (Casas et al., 2012; Duarte and Vargas, 2004; Gortazar et al., 2000; Putaala and Hissa, 1998) due to the lack of an antipredatory behavior (Guyomarc'h, 2003; McPhee, 2003). The same poor knowledge about the local conditions might explain the higher nest predation rate for hybrid females.

It is unsurprising that hunting is the main cause of mortality for restocked farm quails since these tend to be released immediately before the hunting season or before special hunting events. We do not have data about the mortality during the hunting season of hybrid quails that were present during the breeding season compare to that of European quails. However, it seems reasonable to consider that the same lack of anti-predatory behavior would favor a higher hunting pressure on hybrids, thus increasing their mortality rates, as suggested by Guyomarc'h (2003), who reported 75% of restocked individuals from a total of 4959 hunted quails in a large hunting estate (64,000 ha) in France.

Previous field surveys spanning a long time period (Puigcerver et al., 2007, 2012) did not show an increase in the proportion of hybrids detected in nature. This could apparently suggest that restocking European quail populations with hybrid quails does not represent a conservation problem. However, our results show that the two groups are not genetically isolated and that interbreeding occurs in the wild. These observations seem incompatible, but can be explained by the lower chances of survival and nesting of hybrid females in the wild, as well as their higher rate of nest predation. This translates into a lower fitness for the quails of hybrid origin. This lower fitness could be due either to carrying maladaptive alleles of Japanese quail origin or to the adaptation to life in captivity. This result is in agreement with the results of a 15-year-long genetic study showing that the proportion of hybrid individuals in the population was not increasing over time, but also emphasizing that the results were compatible with a slow rate of introgression if hybrids had reduced fitness (Sanchez-Donoso et al., 2014). In this case, a slow introgression of farm alleles could be expected, which would translate into extensive admixture in some generations. Our results confirm reproduction and also lower fitness for the hybrids, and so we expect a progressive build-up of alleles of farm origin into the wild population. This is likely to affect the genetic composition of European quail populations, which could result in a decrease in the adaptive and evolutionary potential of the species.

Our results show that in the case of small populations of European quails, the interbreeding with restocked hybrids could represent a loss of homospecific matings and a reduction of the potential for population growth. It is paradoxical that restocking practices for this species, instead of increasing the long-term chances for survival, could result in a reduction of the genetic effective population size and evolutionary potential (Ryman and Laikre, 1991) and could limit population growth. In addition, captive breeding of European quails for restocking could make sense if the species was under conservation concern; however, it is currently classified as ‘‘least concern’’ by the IUCN. In this case, restocking practices make no sense from the conservation point of view, especially considering that the introduction of farm-reared individuals in wild populations may lead to other threats, such as transmission of pathogens, that could cause substantial mortality in the native populations (Peeler et al., 2006).

The results obtained also emphasize how the putative consequences of restocking practices cannot be directly inferred from just the number of individuals released.

As reported in Casas et al. (2012) for the red-legged partridge (*A. rufa*), public Administrations should ensure that releases for hunting purposes do not represent a threat for the conservation of native species and populations. These authors suggest some management strategies to control genetic introgression and help the original population structure to recover: (1) stop releases of hybrid birds; (2) implement effective inspection procedures for farms providing individuals for restocking; (3) promote management plans avoiding releases in areas where restocking programmes have not yet been performed, and where there is reliable evidence that populations consist of non admixed individuals.

It is important to highlight that the results of this study suggest that some restocked individuals could have been interbreeding with native European quails during the last decades. Until we better understand the long-term consequences of the release of quails with – potentially – maladaptive genes, we believe all restocking programs involving Japanese quails or their hybrids within the natural distribution range of the European quail should be banned.

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PAPER 3

Postcopulatory sexual selection favors fertilization success of restocking hybrid quails over native common quails

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Submitted to *Ibis*

Common quail male (*Coturnix coturnix*), by Manel Puigcerver

> *"Aunque tengas que desviarte a veces, hay que mantener el rumbo"*
> *Armando Mújica*

ABSTRACT

Postcopulatory sexual selection plays an important role in the reproductive success of males in many species. Thus, differences in fertilization success could affect rates of admixture and genetic introgression between divergent lineages. We investigated sperm precedence in matings in captivity involving common quails (*Coturnix coturnix*) and farm quails of hybrid origin (*C. coturnix* x domestic Japanese quail, *C. japonica*), the last used in restocking practices to increase hunting bags. These matings in natural conditions are claimed to represent an important threat for the conservation of native common quail populations. Results showed that fertilization success of each male depended on 1) the time it spent with the female, 2) the presence of sperm from a previous male in the female oviduct, 3) the time that the previous partner had been copulating with the female, and, most importantly, 4) the genetic origin of the male (wild or farm). Farm hybrid males showed higher fertilization success than wild common males, they needed less time with the female to fertilize the same proportion of eggs. The presence of alien sperm in the female oviduct reduced the percentage of fertilized eggs by a male. However, this reduction was higher for wild males when the precedent mate was a farm male. In summary, the sperm of farm hybrid males may outcompete the sperm of native males and this could be favoring the introgression of domestic Japanese alleles into the common quail population, constituting thus a severe conservation threat for wild common quail populations.

INTRODUCTION

Female promiscuity is widespread in nature (Birkhead & Møller 1998), creating a background for sexual selection both before and after copulation (Shuster *et al*. 2013). Postcopulatory sexual selection plays an important role in evolutionary diversification (e.g. Birkhead & Møller 1998, Birkhead & Pizzari 2002, Birkhead *et al.* 2009), occurring through different mechanisms. One of them is sperm competition, where gametes of different males compete to fertilize the same set of ova (Parker 1970, Pizzari & Parker 2009). Another mechanism is cryptic female choice, by which females are able to bias paternity in favor of a particular male after having copulated with several (Eberhard 1996, Pitnick & Brown 2000, Birkhead & Pizzari 2002, Dean *et al.* 2011). Both mechanisms are usually related and are difficult to disentangle (Birkhead 1998), resulting in sperm precedence patterns by which some gametes take precedence over others when fertilizing ova.

Sperm competition and cryptic female choice are facilitated by the capability of females to store viable sperm in their reproductive tract. Sperm storage permits that sperm from several males compete to fertilize the ovum and allows females to impose some kind of postcopulatory selection, playing an important role in mating strategies (Smith 1984, Birkhead & Parker 1997). Sperm-Storage Tubules (SST) are the structures for sperm storage found in the oviduct of birds and some other taxa (Birkhead & Møller 1993, Pearse & Avise 2001). The persistence of viable sperm within the female varies among bird species, from days to weeks (Birkhead & Møller 1992a), allowing the fertilization of ova long after copulation (Cohen 1977, Shugart 1988, Birkhead & Møller 1993).

The Common quail (*Coturnix coturnix*) is a migratory Galliform species that inhabits grasslands and croplands in Europe, North-Africa and West-Asia during the breeding season and African grasslands during winter (Guyomarc'h *et al*. 1998, Rodríguez-Teijeiro *et al.* 2012). Although the census size is estimated to be globally large, it is a game species, with economic importance in several European countries, where millions of birds are hunted every year. In many of those hunting areas, their populations are annually reinforced with the release of thousands of quails bred in farms with the aim of increasing the hunting bags. The number of individuals released can be up to 5 to 20 times bigger than the breeding population in same areas (Sanchez-Donoso *et al*. 2012). A recent genetic analysis has shown that farm individuals are not common quails but descendants of crosses with domestic Japanese quail (*C. japonica,* Sanchez-Donoso *et al*. 2012). Released farm quails are competing with native quails for mating opportunities and their admixture in the wild has been identified as the main conservation problem for common quail populations (Guyomarc'h 2003, Perennou 2009). Domestic Japanese quails have been artificially selected to show, among other traits, a reduced migratory drive. It has been suggested that this character could be inherited to some degree by farm quails intended for restocking (Derégnaucourt et al. 2005a,b) and could impact natural populations if they interbreed. This admixture could entail a loss of migratory impulse and a sedentariness of native populations, which would thus winter in unfavourable latitudes. Moreover, growing in captivity makes farm quails poorly fitted to the wild conditions when released. These individuals suffer a higher predation (Puigcerver *et al*. 2014) and could also be subject to farm diseases or parasites due to lower immune system response as a result of relaxed selection in captivity (as in other related species, Villanúa *et al.* 2008). Recent studies have confirmed that farm and wild quails interbreed and produced offspring in the wild (Puigcerver *et al*. 2014, Sanchez-Donoso *et al*. 2014).

One of the factors that could determine the success of the admixture between wild and farm quails is postcopulatory sexual selection. Since domestic Japanese quails may have been artificially selected to be highly fertile (Pérez y Pérez 1966), their male farm descendants could be outcompeting wild males, which would be promoting the introgression of invasive alleles. Several characteristics of both common and domestic Japanese quail lead to think that they can be exposed to postcopulatory sexual selection (either by sperm competition or by cryptic female choice), which could be influencing the success of hybridization in the wild.

(i) Females are polyandrous in both species. Domestic Japanese quail females are receptive to multiple males in laboratory settings and males can force copulations if females are not receptive (Adkins-Regan 1995). Common quail females usually mate with several males in a single reproductive attempt (Rodríguez-Teijeiro *et al.* 2003). Although male and female create a bond that lasts several days while the female is sexually active, opportunistic extra-pair copulations or mate-switching often occur during that time (Rodrigo-Rueda *et al.* 1997).

(ii) Both species present SST which would allow ejaculates from different males to accumulate, even if matings occur on different days. Domestic Japanese quail females can store viable sperm for up to 11 days (Sittmann & Abplanalp 1965, Birkhead & Fletcher 1994).

(iii) Males from both species have large testes relative to their body mass (Puigcerver 1990, Rodríguez-Teijeiro *et al*. 1992) which is a common feature in bird species with high frequency of extrapair paternity (Møller 1991, Møller & Briskie 1995) and in species with intense sperm competition (Møller 1988, Jennions & Passmore 1993, Gage 1994), because the investment in sperm production is increased (Clulow & Jones 1982, Møller 1989, Cook 1997).

(iv) Common quail males have penis (Puigcerver *et al*. 1994). Penis could facilitate forced copulations (as in waterfowl, Coker *et al*. 2002) and some degree of postcopulatory female selection could have developed to manage the output of these copulations (Briskie & Montgomerie 1997).

In this paper we investigated whether postcopulatory sexual selection exists between the sperm of common quail males and hybrid males intended for restocking. We assessed if fertilization success for both kinds of males was the same and also how fertilization success was modified when other sperm was found in the female's oviduct. To study these questions, we carried out an experiment in captivity with females mating with wild common males or with hybrid farm males. Some of those females were secondly exposed to another male of the same or different genetic origin. We studied the sperm precedence pattern from these crosses by genetically analyzing the resulting embryos and, thus, we assessed the fertilization success for each kind of male. All in all, we investigated whether postcopulatory sexual selection between farm-reared hybrids and common quails could contribute to the admixture between these two lineages in the wild, increasing the threat imposed by the massive restocking campaigns.

METHODS

Experimental design

Experimental crosses were initially attempted using wild common females captured from the field. However, they did not breed in captive conditions. It is very difficult for pure common quail females to lay eggs in captivity (Caballero de la Calle & Peña Montañés 1997). Therefore, experiments were conducted with 16 females from an experimental farm of the University of Castilla-La Mancha (Spain). These females were descendants of admixed females backcrossed with wild common quail males for about 20 generations, so they had domestic Japanese mitochondrial DNA (Barilani *et al*. 2005) but nuclear DNA indistinguishable from common quail (Sanchez-Donoso *et al.* 2012).

Twenty-seven males were used in the experimental crosses. Ten of them were captured from the wild in three cereal crop areas in Spain (four in Barcelona, four in Ciudad Real and two in Seville provinces; see Puigcerver *et al.* 2007 for capture methods); the remaining 17 males were born and reared in three different Spanish game farms intended for restocking. Wild males were genetically identified as pure common quails (without any evidence of admixed ancestry) while farm-reared males were hybrid individuals with variable degree of admixture. Species identification was done following Sanchez-Donoso *et al*. 2012 (data not shown). All individuals were about one year old to avoid any possible effect of age on their fertility (Santos *et al*. 2013).

Quails were kept in aviaries during at least eight months before the start of the experiments, in order to get them habituated to captive conditions. They were moved to individual 20x20x20 cm cages fourteen days before the beginning of the experiments to ensure that no viable sperm was stored in the female's oviducts (Birkhead & Fletcher 1994, Miranda *et al.* 2013) and to avoid sperm fatigue in males (Preston *et al.* 2001). All experimental crosses were done during the natural breeding season of the common quail (from the beginning of June to the end of July).

Females remained in their cage during the experiments to avoid disturbances and males were the ones moved in and out the cages as needed (see below). All individuals were sexually active during the experiments and used only once. Female maturity was confirmed by their capability of laying eggs while for male its sexual activity status was assessed by their swollen cloaca (measured in length; Siopes & Wilson 1975) and/or the presence of foam in the cloacal gland (Adkins-Regan 1995). Physical condition and health were assessed by the weight and physical appearance and only individuals in good condition were used in the experiments.

Statistical analyses were conducted in R 2.15.2 (R CoreTeam 2012). Generalized Linear Models (GLMs) were fitted using the `glm()` function and constructed assuming a Binomial error distribution and a Logit link function. GLMs were tested for the presence of outliers, homoscedasticity, normality and overdispersion. Overdispersed models were recalculated assuming a Quasibinomial error distribution. The significance of the variables studied was evaluated by an analysis of deviance with the function `Anova()` available in the package `car` (Fox & Weisberg 2011). In models that were not overdispersed, the analysis of deviance was evaluated by a likelihood-ratio test (Chi-square test); an F-test was used in overdispersed models.

Experiment 1: Simple crosses - Male fertilization potential

A set of simple crosses (one male and one female) was designed to study the fertility of farm and wild common quail males without other sperm in the female's oviducts. Six wild common males and 10 farm males were caged with a female each. After the female laid 3 to 8 eggs, the male was removed from the cage and Experiment 1 finished. The number of days that female and male were caged together was registered, as well as daily laying events (absence/presence of egg laid). Eggs were collected every day, individually labeled and incubated in the laboratory during a week. After that time, they were opened to determine whether they were fertilized or not by the presence or absence of an embryo.

We analyzed with a GLM whether genetic origin of males could affect their egg fertilization capability. The proportion of fertilized eggs during the time male and female were caged together was the response variable and male origin was the explanatory factor tested. The number of days the male spent with the female was added as a covariate, since we suspected that it could have some effect on the proportion of fertilized eggs.

Experiment 2: Double crosses - Sperm competition

To study the possible sperm competition between males of the same or different origin, we carried out a second experiment with 11 females after concluding Experiment 1 (four females that had been with wild males and seven with farm males). At the same time that the first male (M1) was taken out of the cage, it was replaced by a second male (M2). M2 was caged with the female until she laid 3 to 11 more eggs. After that, M2 was removed from the cage, and Experiment 2 finished. Three different types of crosses were done regarding the origin of the males and the order in which they encountered the female: 1) wild M1 - farm M2 (w-f); 2) farm M1 - wild M2 (f-w); 3) farm M1 - farm M2 (f-f). Four replicates were performed for the first and second type of cross, and three for the last one.

Eggs were handled as in Experiment 1. Number of eggs laid and eggs fertilized were registered. We genotyped the embryos and assessed their paternity. Since sperm stored in the female oviduct can fertilize ova with the same probability during six days after mating (Figure 1 in Birkhead & Fletcher 1994), we registered the number of eggs fertilized by each male and the number of non-fertilized eggs laid during the first six days after the male exchange. During that period, fertilization by M1 or M2 would only depend on the possible postcopulatory selection over the sperm of the two males.

Firstly, we analyzed whether the presence of sperm from a previous male, stored in the female's oviduct, had an effect on the proportion of eggs fertilized by a second male. The proportion of eggs sired by a male over the total number of eggs laid was the response variable of the GLM. Presence or absence of alien sperm was the factor of interest. Wild and farm males were analyzed independently to observe the alien sperm effect over each kind of male. Secondly, we tested whether the proportion of non fertilized eggs changed before and after male exchange, to assess if it was dependent on the presence of sperm of one or two males in the oviduct. Finally, we studied whether fertilization capability of M2 was determined by its own origin and the origin of the previous male. We tested differences in the proportion of embryos sired by M2 among the three male arrangements: w-f, f-w and f-f. The number of days M1 had been with the female was included as a covariate.

Experiment 3: Time alone – Sperm durability

After removing M2, the female was left in the cage during 14 days. Eggs laid were collected, incubated and checked for embryos as before, and paternity analyses were done for all embryos. We registered the number of eggs and length of time during which these were fertilized in the absence of males.

Paternity analyses

Paternity of embryos was assessed by genetic analyses using a panel of autosomal microsatellite loci. A blood sample (100 µl) was obtained for each adult and a tissue sample for each embryo. Samples were stored at -20 ºC in 95% ethanol until DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen), following manufacturer's protocols. Individuals were genotyped for 11 unlinked autosomal microsatellite loci originally developed for Japanese quail by Kayang *et al*. (2002, 2004). These markers were highly polymorphic and had been previously used for hybrid identification (Sanchez-Donoso *et al*. 2012, Sanchez-Donoso *et al*. 2014). Some loci were amplified by polymerase chain reaction (PCR) in a multiplex (some of them with primers modified to facilitate multiplexing, as in Sanchez-Donoso *et al*. 2014), while others were amplified separately and subsequently pooled before electrophoresis (detailed protocols are available upon request). All PCR products were electrophoresed on an ABI 3730 sequencer (Applied Biosystems), following manufacturer's protocols. Alleles were sized and scored using the software GeneMapper v3.5 (Applied Biosystems).

Paternity of embryos was assigned by using the maximum likelihood method implemented in CERVUS 3.0.3 (Kalinowski *et al*. 2007). This software assigns parentage based on a pair-wise likelihood comparison. Simulations, based on allele frequency analyses, were run in advance to estimate the significance of these differences between likelihoods for a defined confidence level (see Marshall *et al.* 1998, Kalinowski *et al*. 2007, for details). Each mating experiment was analyzed separately. Simulations of parentage were run for 100,000 offspring. For all embryos analyzed, paternity was unambiguously assigned to one of the putative parents with a probability over 99%. In all cases, genetic analyses confirmed as mother the female in the cage, showing the reliability of the markers used in the study.

RESULTS

The egg laying pattern was very variable among females. Females did not lay eggs every day, neither all eggs were fertilized. In most cases, no more than one egg was laid per day. Only 3 of the 16 females laid 2 eggs in one day. In these three cases, only one of the eggs was fertilized. Fertile eggs did not necessarily occur on successive days, but could be separated by days with non-fertilized eggs or without egg. The number of eggs laid by each female was not related to the proportion fertilized (Spearman's r_s=0.15, N=18, p>0.05).

Experiment 1: Simple crosses – Male fertilization potential

The duration of the experiment was variable among cases, lasting on average 18.6 days (standard deviation, SD=12.1). During this time, females laid 6.0 eggs on average (SD=1.2).

Both male origin and number of days the male spent with the female had a significant effect on the proportion of eggs fertilized. The longer the male spent caged with the female, the larger the proportion of fertilized eggs (χ^2_1=5.93, p=0.015, Fig. 1). Although this pattern was observed for both types of males, there were clear differences between them (χ^2_1=7.19, p=0.007): for the same amount of time, farm males fertilized more eggs than wild males (Fig. 1).

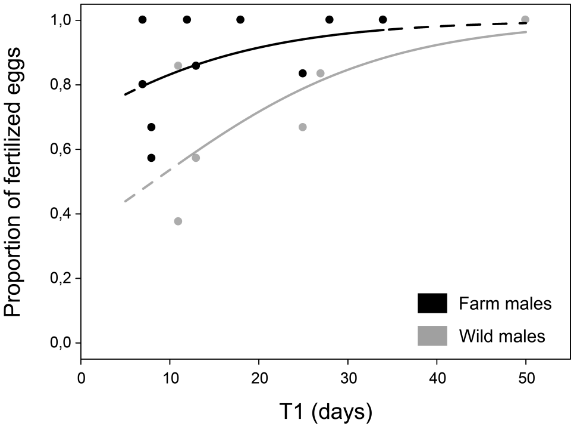

Figure 1. Proportion of eggs fertilized by hybrid farm males (black dots) and wild males (grey dots) depending on the days spent with the female (T1). Curves show the generalized linear model that best fitted the data. Dashed curves show the inferred fitting of the model beyond observations. Probability of egg fertilization increases over time for both male types, but the probability of fertilization was higher for farm males.

Experiment 2: Double crosses - Sperm competition

Experiment 2 lasted 10.4 days on average (SD=2.5) during which each of the 11 females laid 7.0 eggs on average (SD=2.8).

The first egg sired by M2 was one of the first three eggs laid during this experiment and during the four first days after changing males. After that, a change in paternity in the egg series occurred and none of the following eggs was sired by M1. In eight cases, eggs were fertilized by sperm from M1 stored in the female's oviduct after the change in males had taken place. In two of these eight cases, M1 sired only the first egg after the change of males, in four M1 sired the two first eggs, and in two M1 was the father of the three first eggs.

The presence of sperm from a previous male stored in the female's oviduct led to a reduction in the proportion of eggs fertilized by a male (Fig. 2). For wild males, the reduction was 74% (proportion of eggs fertilized by a wild male as M1 vs. proportion of eggs fertilized as M2 -with a farm male as M1-: $F_{(1,8)}$=12.88, p=0.007, Fig. 2). For farm males, the reduction was 31%, less accentuated but still significant (farm male as M1 vs. farm male as M2: $F_{(1,15)}$=5.01, p=0.041, Fig. 2). In this case, the origin (wild or farm) of the previous male (M1) did not have a significant effect ($F_{(1,5)}$=0.01, p=0.923).

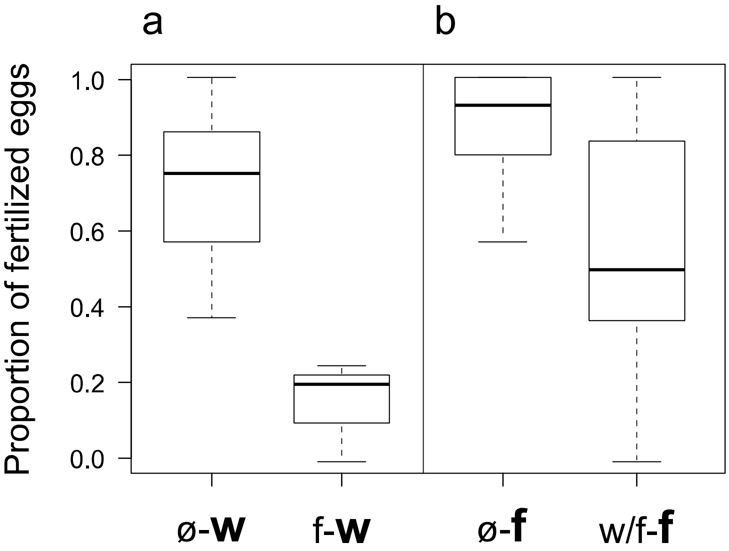

Figure 2. Proportion of eggs fertilized by the second male quail (marked in bold) mating with a female in different kinds of crosses. The bottom and top of each box are the first and third quartiles, the band inside the box is the median and the range of the data is marked by the whiskers. "Ø": no male before; "w": wild male; "f": farm male. The proportion of eggs fertilized by a wild **(a)** or a farm **(b)** male when the female had mated before with another male was lower than when the female had not. In the case of farm males, there were no differences regarding the origin of the first male.

The proportion of non-fertilized eggs did not differ before and after the male exchange ($F_{(1,25)}$=0.26, p=0.617). This indicated that the reduction in the proportion of fertilized eggs by a male when another male (M1) had been with the female before was due to the presence of the eggs fertilized by M1, and not due to an excess of non fertilized eggs after the male exchange.

The proportion of embryos sired by M2 (thus, excluding non-fertilized eggs) was dependent on the combination of males with regard to their origin while controlling for the time M1 had been before with the female (χ^2_2= 10.66, p=0.005). The longer the stay of M1 with the female, the lower proportion of eggs fertilized by M2 (Fig. 3). Farm males that copulated after another male (independently of its origin) fertilized a higher proportion of eggs than wild males that copulated after a farm male (χ^2_1=10.54, p=0.001). Even though the proportion of eggs fertilized by M2 males was reduced, the proportion of eggs fertilized by farm males as M2 was not different from M1 wild males ($F_{(1,11)}$=0.25, p=0.625).

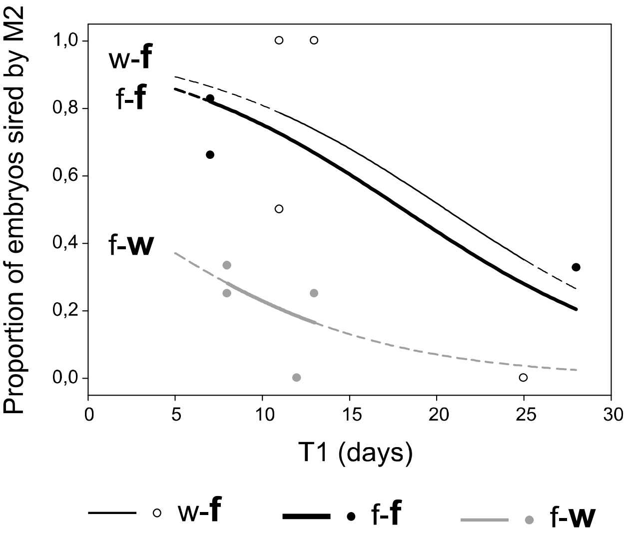

Figure 3. Proportion of embryos sired by the second male (M2, in bold) depending on the time the first male (M1) had been with the female (T1, in days), for each male's origin combination ("w": wild male, "f": farm male). The longer the previous male had been with the female, the lower proportion of embryos was sired by the following male. Wild males acting as second partner sired significantly less embryos than farm males did. Although plotted separately to facilitate understanding, levels "w-f" and "f-f" were not significantly different.

Experiment 3: Time alone – Sperm durability

Out of the 11 females in Experiment 2, ten were used for Experiment 3. From these, one did not lay eggs during the time she was left alone, three laid only non fertilized eggs, and six laid fertilized eggs. These females retained viable sperm during a maximum of 10 days (average: 6.2; SD=2.5). During this time, a maximum of 7 eggs could be fertilized by the sperm stored in the SST (average: 3.5; SD=2.2). Eggs were sired in all cases by the last male that stayed with the female.

DISCUSSION

Several factors can modulate a male's ejaculate competitiveness (Cook *et al*. 1997, Pitnick *et al*. 2009, Pizzari & Parker 2009), such as ejaculate size (volume and absolute number of spermatozoa; Martin *et al.* 1974, Gage & Morrow 2003), spermatozoan traits (length, swimming speed or longevity), seminal plasma biochemistry, and the interactions between the ejaculates of competing males and also with the female's oviduct (Cook *et al*. 1997, Pitnick *et al*. 2009, Pizzari & Parker 2009). In the case of the quail, also the production of foam by the proctodeal gland has been found to be of importance in male fertility (Cheng *et al*. 1989, Adkins-Regan 1999, Finseth *et al*. 2013). All these traits, together with cryptic female choice, can contribute to determine the relative fertilization success of the different males mating with the same female. However, in this paper we have not focused on the mechanisms underlying the sperm precedence pattern, but on the output of this process.

Results showed that the time a male spent with a female had a positive effect on his fertilization probability. The longer the male stayed with the female, the higher the proportion of eggs from the clutch that was fertilized by his sperm. This period had also an effect on the fertilization success for any male subsequently copulating with that same female. This time dependency of the fertilization success would explain the natural mating behavior of the common quail. Male and female establish a pair-bond for a period of time during which the male guards his partner against possible extra-pair copulations (EPC). Since mate-switching has also been observed, the longer a male guards its mate, the higher the chances will be to fertilize the clutch even if the female copulates later with another male. In addition, our results also show that quail sperm could be viably maintained in the female's oviduct during an average of 6 days, with an average of 3.5 eggs fertilized during those days. Taking into account that common quail clutches usually total about 10 eggs (Gallego *et al.* 1993), males would be unlikely to fertilize entire clutches by mating just once or a few times over a short time period with the same female, so the guarding behavior is likely to increase their siring potential.

Previous studies have already reported that males that copulate for longer time have higher fertilization success (Dickinson 1986, Parker & Simmons 1991). A higher number of copulation events increases the volume of sperm introduced into the female, what increases the siring probability of that male. A male's fertilization success will generally increase with the quantity of his sperm relative to that of other males (Parker *et al.* 1990). In our study, after the first egg sired by M2 was laid, a change in the paternity in the egg series occurred, and none of the following eggs was sired by M1, neither while being with M2 nor when she was alone. Our results did not support the last-male precedence pattern observed in other bird species (Warren & Kilpatrick 1929, Cheng *et al.* 1983, Briskie 1996, Birkhead & Pizzari 2002) - by which the last male that copulates with the female is the one siring the majority of the clutch –, since in our case not only the order of the males would be affecting the sperm precedence pattern, but this pattern would be under the effect of the time male and female had been copulating. On the contrary, our results did not completely reject the "last in, first out" pattern (Birkhead & Møller 1992b, Stai & Searcy 2010), by which some kind of sperm stratification occurs in the SST: the sperm of the first male is stored at the bottom of the tubules and the sperm of subsequent mates is deposited in top of it and it is the one that would fertilize eggs first. When this has been used up, the sperm beneath it gets exposed, so the last eggs are fertilized by the first male sperm. We did observe a complete replacement of the male siring the embryos after a given point. However, since in our case embryo paternity was determined by the order of males and the length of time spent with the female, we do not know if a shorter stay of M2 would allow the reappearance of eggs sired by M1. In any case, some eggs were fertilized by M1 after M2 had been introduced in the cage, what implies that it is not just the last sperm inseminated the one that will fertilize eggs. When a male stops copulating with a female, the amount of sperm in the female's oviduct gets reduced through time (Birkhead & Fletcher 1994) because it is used up fertilizing ova and because there is a passive sperm loss that makes its viability decline (Birkhead *et al.*1995, Birkhead & Pizzari 2002). The reduction of viable sperm stored also reduces the probability of fertilization. Our results supported this observation, since fertilization of eggs stopped after some time being without a male. When a second male introduced sperm into the female's oviduct, the first male fertilization success would reduce faster, by passive sperm loss plus by competition with an increasing quantity of sperm from the second male.

Results from this study show that farm males had higher fertilization success than wild males. Farm males needed less time than common males to sire the same proportion of embryos in a clutch. This implies that farm males during the breeding season are potentially able to fertilize a larger number of eggs, even in just a few days, thus gaining a competitive advantage.

Previous matings by the female reduced the male's fertilization success. This reduction in the proportion of eggs fertilized by the second male was because some of them were sired by the previous mate, since the overall fertilization success was not affected by the presence of sperm from more than one male. This reduction depended again on the amount of time the previous male was with the female, it was smaller when the time the first male had been with the female was shorter. However, this fertilization reduction was also affected by the origin of the second male: farm males suffered a smaller reduction in the fertilization success that wild males. In fact, the proportion of eggs that farm males could fertilize in presence of alien sperm in the female's oviduct was not different to the proportion fertilized by common quail males when no other sperm was present. This could also give farm males a great advantage to fertilize eggs over common quail males.

Differences in sperm competitiveness have been observed between poultry breeds (Prieto *et al.* 2011, Santiago-Moreno *et al*. 2014) what implies that may have a heritable component. The domestication process and selection for increased productivity in captivity could have increased fertility in many domestic species (Darwin 1868). This could also be the case for domestic Japanese quails that contributed to the origin of the farm quails analyzed here. The results of this study suggest that restocked farm quails may have higher fertility than the wild common quails and may dominate sperm competition. Similar results could have been obtained if the wild birds used in the mating experiments performed worse due to the lack of habituation to life in captivity. However, we tried to prevent this possibility by keeping in captivity all the birds used in these experiments for at least eight months before the beginning of the study.

Our results indicate that restocking practices have the potential for a higher impact on the native population than initially suspected by just considering the number of crosses between wild and farm quails. Puigcerver *et al*. (2014) have shown that, despite a higher mortality for restocked quails and the preference for intra-specific matings, interspecific matings in natural conditions can occur. In fact, Sanchez-Donoso *et al.* (2014) have detected introgression of alleles of farm origin into the wild common quail populations. In the present study, we have detected an advantageous postcopulatory selection in favor of males of farm origin. These results indicate that a relatively small number of successful interspecific matings could have an important contribution to the introgression of farm alleles into common quail populations. Given the superior reproductive potential of the quails of farm origin, we advice against restocking practices.

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PAPER 4

Detecting slow introgression of invasive alleles in an extensively restocked game bird

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Frontiers in Ecology and Evolution 2: 00015

Cereal crops inhabited by quails (Punta Blava, Catalonia), by Ines Sanchez-Donoso

"Hacer un doctorado no es cuestión de inteligencia, es cuestión de cabezonería"
Alejandro González-Voyer

ABSTRACT

Interbreeding of two species in the wild implies introgression of alleles from one species into the other only when admixed individuals survive and successfully backcross with the parental species. Consequently, estimating the proportion of first generation hybrids in a population may not inform about the evolutionary impact of hybridization. Samples obtained over a long time span may offer a more accurate view of the spreading of introgressed alleles in a species' gene pool. Common quail (*Coturnix coturnix*) populations in Europe have been restocked extensively with farm quails of hybrid origin (crosses with Japanese quails, *C. japonica*). We genetically monitored a common quail population over 15 years to investigate whether genetic introgression is occurring and used simulations to investigate our power to detect it. Our results revealed that some introgression has occurred, but we did not observe a significant increase over time in the proportion of admixed individuals. However, simulations showed that the degree of admixture may be larger than anticipated due to the limited power of analyses over a short time span, and that observed data was compatible with a low rate of introgression, probably resulting from reduced fitness of admixed individuals. Simulations predicted this could result in extensive admixture in the near future.

INTRODUCTION

Interspecific hybridization and its evolutionary consequences are often studied by analysis of samples obtained over a relatively short time period, without aiming to monitor genetic changes over time. This approach may allow identification of admixed individuals, but it may not be informative about the evolutionary impact of introgressive hybridization. For instance, the presence of first generation hybrids does not necessarily imply the spread of invasive alleles in the native population (introgression). When hybrids have reduced fitness or are infertile, hybridization might not have a large impact on the genetic composition of the parental species. Hybridization in such cases will still imply a loss of intraspecific mating opportunities, reducing the effective breeding population (accelerating genetic drift). Alternatively, if alien alleles do introgress into the gene pool of the native population, hybridization can have long-term fitness consequences (Rhymer & Simberloff 1996; Haygood et al. 2003).

To estimate the impact and rate of admixture, the proportion of first generation hybrids and subsequent backcrosses can be assessed from analysis with a large panel of molecular markers (Vähä & Primmer 2006). If the analyzed population sample includes individuals with varying degrees of admixture, this implies that first generation hybrids are interbreeding with the parental species, leading to alleles from one species introgressing into the gene pool of the other (Rhymer & Simberloff 1996). However, such data can only offer a snapshot of the evolutionary process, without informing about changes over time. Furthermore, inference about long-term effects are susceptible to limitations due to small sample sizes (is the sample representative of the entire population?) and low frequency of interbreeding (is it possible to estimate the frequency of rare interbreeding events?). In addition, an important and often overlooked source of error is associated with varying levels of admixture from year to year (is the level of admixture changing over time?).

Samples obtained over several generations can help to assess temporal changes in the level of admixture and can offer a long-term view on the introgression of invasive alleles, more meaningful from a conservation and evolutionary point of view. The robust characterization of slow introgression requires the use of long time series to allow tracking the arrival of new alleles into the population.

Common quail (*Coturnix coturnix*) populations offer the opportunity to obtain long temporal series of samples to investigate the consequences of admixture of divergent lineages. Its European breeding population is estimated to consist of several millions of individuals (8,400,000-14,100, 000, BirdLife International 2004) that move nomadically across the breeding range looking for suitable habitat (Puigcerver et al. 1989; Rodríguez-Teijeiro et al. 2009) and mates (Rodríguez-Teijeiro et al. 2006). This game species is of high economic and recreational interest in southern Europe; millions of quails are killed every hunting season (Guyomarc'h 2003). Due to this interest, every year wild populations are extensively restocked prior to the game season with farm-reared quails. For example, in Catalonia (Northeast Spain), the wild common quail population has been estimated to range between 5000 and 20 000 males (Rodríguez-Teijeiro et al. 2004), and about 57 000 farm-reared individuals have been released yearly from 1990 to 2011 (Hunting order plans of the Regional Government of Catalonia). The number, size, timing and location of the releases change from year to year and very limited information is available for most of them. While quails for game restocking are sold as be common quail, recent genetic analyses have shown that they are, actually, hybrids between common quails and domestic Japanese quails (*C. japonica*) that have been interbreeding for a number of generations in game farms (Sanchez-Donoso et al. 2012).

Captive breeding represents a general relaxation of selective forces and promotes traits that are maladaptive in the wild (Lynch & O'Hely 2001; Frankham 2008; Baskett & Waples 2013). Thus, supplementation programs based on the release of captive-bred individuals have the potential to deeply alter the genetic composition of native populations (Ford 2002). These large-scale releases can lead to loss of genetic diversity, breakdown of adaptations and change in the population genetic structure (Eldridge & Naish 2007; Laikre et al. 2010; Marie et al. 2010). Specifically, several authors have pointed out that restocking with domestic Japanese quails and hybrids can pose serious threats to the genetic integrity and survival of common quails (Guyomarc'h 2003; Barilani et al. 2005; Chazara et al. 2006, 2010; Puigcerver et al. 2007). Their interbreeding can lead to introgression of maladaptive domestic Japanese quail alleles into the common quail population, potentially leading to alterations or loss of migratory behavior, and a decline in fitness in native quails (Guyomarc'h 2003). A decrease in average fitness means a lower population growth rate, which could even become negative. However, even in the case that fitness of admixed individuals was not diminished, the massive releases of farm-reared quails could lead to a counter-intuitive reduction in the effective population size through Ryman-Laikre effects: a large portion of the breeding population could potentially derive from a small number of breeders from farms, resulting in a reduction of the genetic diversity and evolutionary potential (Ryman & Laikre 1991). As a result of these concerns, the EU Quail Action plan has emphasized the need to preserve common quail genetic integrity (Perennou 2009).

Several lines of evidence support the existence of introgressive hybridization from farm quails into wild common quails. Japanese and admixed quails have vocalizations that differ from those of common quails, with first generation hybrid male crows being intermediate between domestic Japanese quail and common quail (Derégnaucourt et al. 2001), and this has often been used to characterize the identity of free-ranging quail males

(Guyomarc'h & Guyomarc'h 1996; Collins & Goldsmith 1998; Barilani et al. 2005; Puigcerver et al. 2007). Using this approach, hybrid quails were first detected in Catalonia by members of our research group in 1990 in an area close to the study site (Rodríguez-Teijeiro et al. 1993). Since then, and following the popularization of farm quails for restocking, hybrid individuals have been detected, using genetic methods, in common quail breeding areas in multiple European countries including Portugal, France, Italy and Spain (Barilani et al. 2005; Guyomarc'h 2003; Rodríguez-Teijeiro et al. 1993). Experiments in captivity have shown that common and Japanese quails present only partial mate discrimination (Derégnaucourt & Guyomarc'h 2003), and that the two species interbreed easily and hybrids do not have reduced survival (Derégnaucourt et al. 2002). These observations suggest that Japanese quail alleles may have been introgressing into the European common quail population since the early 1990s.

However, the occurrence and frequency of interbreeding under natural conditions between native and released quails is not known. Furthermore, while hybrids do not show reduced survival in captivity, selection against hybrids in the wild could potentially lead to strongly reduced rates of introgression. For this reason, it has been suggested that individuals identified in nature as hybrids could correspond to farm released animals, without requiring any hybridization in the wild at all (Puigcerver et al. 2007, 2012). This is in part supported by the radio-monitoring of female farm quails, which showed an increased mortality and predation risk for their clutches (Puigcerver et al. 2014). However, this same study also revealed their success at attracting wild males and reproducing in the wild. Consequently, it remains unclear to what degree these massive releases are affecting European common quail populations.

Here we studied the genetic composition of wild quails sampled in a single locality in Northeast Spain across a 15-year period (about 15 generations), to evaluate to what extent the releases of farm quails are affecting the genetic composition of the local population. We combined genetic analyses with simulations to test the power of our markers to detect introgression of farm alleles. We also carried out simulations assuming different levels of fitness for the admixed individuals to investigate which values are compatible with the observed data. We then used these values in further simulations to forecast changes in the genetic composition of the local population in the near future under different scenarios.

MATERIALS AND METHODS

Samples and datasets

The field survey was carried out in an area of about 1 km^2 in Northeast Spain (Mas Esplugues, 41º 25’ N, 1º 23’ E, 628 m above sea level), a short, narrow valley with winter cereal crops (mainly barley and wheat) which constitute suitable breeding habitat for quails. The breeding population has been continuously monitored for over 25 years in the framework of a long-term project to study the biology and mating behavior of common quails (J.D.R.-T. and M.P.). It is an open population and individuals continuously arrive during the breeding season, spend some time and leave in search of suitable habitat patches and mates (Puigcerver et al. 1989; Rodríguez-Teijeiro et al. 2006; Rodríguez-Teijeiro et al. 2009). This is a game area, and the hunting season starts in mid-August, after the end of the breeding season (from about mid-April to early July). The great majority of quails breeding in the area were estimated to be one year old, with very few breeders being aged two or more (Puigcerver et al. 1992). For this reason, we considered generation time to be about 1 year.

A feather or a blood sample was obtained from 468 wild quails, captured during several breeding seasons (i.e. prior to the majority of farm releases), following protocols approved by an authorized Ethics Committee (for sampling methods see Puigcerver et al. 2007). Our sample represents a very large proportion of the quails breeding in the study area every season, but is very difficult to have precise count of the number of breeders. These wild quails were genotyped at 9-13 autosomal microsatellite loci. Genotypes were generated as three different datasets (Table 1) produced by different researchers, in different labs and using different instruments. Dataset I included quail samples collected during the breeding seasons of 1996 and 1997, genotyped by I.S.-D. A subset of these genotypes was included in a previous study (Sanchez-Donoso et al. 2012). Dataset II included quail samples collected during the breeding seasons of 1999, 2002 and 2005, genotyped by J.H. as part of her MSc thesis at the University of Uppsala, Sweden (Huisman 2006). Dataset III included quail samples obtained during the breeding seasons from the period 2007-2010, genotyped by I.S.-D. for this study.

Quail samples from Spanish game farms were analyzed as reference for the three wild quail datasets: 52 individuals from 5 farms (farm set A, sampled in 2009 and 2010, included in Sanchez-Donoso et al. 2012) were used as a reference in analysis with datasets I and III, while 29 individuals from 3 game farms (farm set B, sampled in 2006) were used with Dataset II (see Table 1). All farm individuals were intended for restocking. In a previous study, no genetic differentiation was observed among individuals from the different farms (Sanchez-Donoso et al. 2012, including some of the samples studied here), suggesting that all of them derive from the same stock and/or that the exchange between them is extensive.

For this reason, and considering the large size of the overall population in farms and the lack of precise information about the origin and number of breeders, in the simulations we assumed that the allele frequencies in the farm population remained constant throughout the study period (see below).

Dataset	Year	N	N loci	Alleles per locus (average)	A	PIC	H_e	H_O
I	1996	42	9	17.2 ± 6.2	15.3 ± 4.9	0.87 ± 0.07	0.90 ± 0.02	0.88 ± 0.02
	1997	51	9	18.0 ± 5.3	15.5 ± 4.1	0.88 ± 0.06	0.90 ± 0.02	0.87 ± 0.02
II	1999	31	13	13.5 ± 4.5	12.4 ± 4.1	0.81 ± 0.13	0.84 ± 0.03	0.74 ± 0.02
	2002	30	13	14.0 ± 5.5	12.7 ± 4.6	0.81 ± 0.15	0.84 ± 0.04	0.75 ± 0.02
	2005	31	13	13.6 ± 4.2	12.4 ± 3.7	0.83 ± 0.10	0.86 ± 0.03	0.75 ± 0.02
III	2007	50	11	19.6 ± 6.6	15.9 ± 4.6	0.89 ± 0.05	0.90 ± 0.01	0.84 ± 0.02
	2008	33	11	16.6 ± 4.8	15.3 ± 4.0	0.87 ± 0.07	0.89 ± 0.02	0.84 ± 0.02
	2009	97	11	21.7 ± 6.6	15.3 ± 4.1	0.88 ± 0.05	0.90 ± 0.01	0.84 ± 0.01
	2010	103	11	23.6 ± 8.2	15.9 ± 4.4	0.89 ± 0.06	0.90 ± 0.01	0.85 ± 0.01
Farm set A		52	9	14.6 ± 4.6	12.0 ± 3.6	0.80 ± 0.07	0.83 ± 0.02	0.80 ± 0.02
Farm set A		52	11	14.0 ± 4.3	11.4 ± 3.3	0.77 ± 0.11	0.82 ± 0.02	0.80 ± 0.02
Farm set B		29	13	11.4 ± 4.3	10.4 ± 3.6	0.80 ± 0.08	0.81 ± 0.03	0.73 ± 0.02

Table 1. Genetic diversity (averaged across loci, ± standard deviation) of wild and farm quails at autosomal microsatellites. The entire sample is composed of three datasets genotyped independently and using partially different sets of markers (see text). Reference farm samples are the same for datasets I and III (farm set A), but with different number of loci. Farm set B is constituted by reference farm samples used with Dataset II. N: number of samples genotyped, N loci: number of loci genotyped, A: allelic richness, PIC: Polymorphic Information Content, H_e: expected heterozygosity, H_o: observed heterozygosity.

Joining the three datasets offered the opportunity to compile a time series spanning 15 years. They could not be fully integrated, as the sets of genetic markers used were not identical (see below) and genotype scoring was done by different researchers. Since many DNA samples derived from small feathers, each providing only a small amount of DNA, it was not possible to re-analyze them under a unique set of conditions. Datasets I and III could be merged when restricting analyses to the markers used in both cases after ensuring the consistency of the allele call by genotyping a panel of reference samples.

Laboratory procedures

For some individuals, DNA was extracted from blood samples (100 µl, stored at -20ºC in 95% ethanol until analysis) using DNeasy Blood & Tissue Kit (Qiagen) following manufacturer's protocols, or following standard phenol-chloroform extraction procedures. For the rest of individuals, DNA was extracted from feathers (stored at -20ºC in 95% ethanol) using a Chelex resin protocol (Walsh et al. 1991).

To characterize the maternal ancestry of quails and to determine directionality of hybrid matings, a 453 base pair (bp) fragment of the 5' of the mitochondrial DNA (mtDNA) control region was sequenced for 26 wild quails from Dataset II (7 from 1999, 7 from 2002 and 12 from 2005) and the 29 farm quails from farm set B. The wild quails were selected to include diverse phenotypes and, potentially, admixture. Having admixed individuals of farm and wild origin could allow inferring if both sets of admixed birds had the same maternal origin. DNA samples were amplified via polymerase chain reaction (PCR) using primers PHDL and PH-H521 (Barilani et al. 2005). Sequencing reactions were carried out using the same forward and reverse primers, and were run on a MegaBACE 1000^{TM} instrument (Amersham Biosciences) following manufacturer's protocols. From the two sequences obtained, a consensus was built using SEQUENCHER v. 5.1 (Gene Codes Corporation, Ann Arbor, MI, USA).

Individuals were genotyped for autosomal microsatellite loci originally developed for Japanese quail (Kayang et al. 2000, 2002) (Dryad reference number for genotype data sets: *pending*). Some loci (marked below with an asterisk) were analyzed for datasets I and III using primers modified to facilitate multiplexing (see Supplementary Material Table S.1). The number and combination of loci typed varied between datasets. Dataset I was genotyped for 9 unlinked markers (each marker was located on a different chromosome): GUJ01, GUJ17*, GUJ39, GUJ44, GUJ57*, GUJ65*, GUJ85, GUJ93 and GUJ97*. Dataset II was typed for these same markers, excluding GUJ85, plus five additional markers: GUJ14, GUJ28, GUJ33, GUJ62 and GUJ68 (13 loci in total). Although some loci used for Dataset II are on the same chromosome (Kayang et al. 2004), previous analyses on this dataset showed they are not in linkage disequilibrium (Huisman 2006). Dataset III was genotyped for the same 9 loci as Dataset I, plus GUJ28 and GUJ74 (11 loci in total, located on different chromosomes). Since datasets I and III were scored by the same researcher, and using a panel of reference samples to ensure that all allele calls were consistent, they could be analyzed together for the 9 shared markers.

Some markers were PCR-amplified in a multiplex, while others were amplified separately. Detailed protocols are available upon request. PCR products from dataset I and III, and from their reference farm set, were electrophoresed on an ABI 3730 sequencer

(Applied Biosystems) following manufacturer's protocols, and alleles were sized and scored using the software GeneMapper v3.5 (Applied Biosystems). Electrophoresis of Dataset II PCR products, and of their reference farm set, was performed on a MegaBace 1000 capillary instrument (GE Healthcare) following manufacturer's protocols. The associated software, Genetic Profiler 2.2, was used for the allele calling.

Data analysis

Mitochondrial DNA sequences were aligned with Muscle in MEGA v5.1 (Tamura et al. 2011). The same program was used to compare sequences and for phylogenetic reconstructions using maximum likelihood (ML) with the Tamura-Nei model of sequence evolution and a gamma distribution rate of variation across loci, and neighbor-joining (NJ) based on the maximum composite likelihood model. Support for nodes was assessed with 500 bootstrap replicates.

Average number of alleles per locus and allelic richness (mean number of alleles per locus corrected for the minimum sample size; Leberg 2002) were calculated using FSTAT version 2.9.3.2 (Goudet 1995). In order to measure marker informativeness we calculated the Polymorphic Information Content (PIC; Botstein et al. 1980) which takes into account the number of alleles per locus and the frequency of these alleles, using EXCEL MICROSATELLITE TOOLKIT 3.1.1 (Park 2001). We used the same software to calculate observed (H_o) and expected (gene diversity, H_e) heterozygosities (Nei 1987). Overall differentiation between wild and farm samples (for each sample set) was estimated using Weir & Cockerham's (1984) estimate of the fixation index F_{st} in GENETIX (Belkhir et al. 1996-2004), Hedrick's (2005) standardized measure of genetic differentiation (G'_{ST}) and Jost'D estimator of actual differentiation (D_{est}, Jost 2008) with SMOGD (Crawford 2010).

Genetic composition of wild and farm quails was analyzed using the Bayesian clustering procedure implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000). Other likelihood approaches could have been used to infer population structure, but we chose the approach implemented in STRUCTURE because its use is widespread for hybridization studies, and because it is easy to scale up to analyze thousands of simulated datasets across multiple computer processors (see below). Since our goal was to track the introgression of farm alleles into the wild population we chose to always use the same analytical approach and we avoided using approaches aimed at identifying individual hybrids and backcrosses (for example NEWHYBRIDS, Anderson & Thompson 2002).

For a given number (K) of genetically distinct clusters, STRUCTURE provides an estimate of the fraction of the genome (q_i) that belongs to each one of the clusters. Analyses were performed under the admixture model, as each individual may have ancestry in more than one parental population, and were run with correlated allele frequencies and without population or sampling location information (USEPOPINFO and LOCPRIOR set to 0). Analyses were done for K=2 to separate farm and wild clusters, since previous analyses showed that this is the highest level of population structure (Sanchez-Donoso et al. 2012). Analyses were run for 100 000 steps following a burn-in period of 100 000 steps, and were replicated five times. Results did not vary significantly with longer runs and likelihood values were observed to converge during the runs. STRUCTURE proportion of membership to each cluster (q_i) and 90% posterior probability intervals were estimated for each individual. For consistency across real and simulated datasets, STRUCTURE was always run using these parameters.

All STRUCTURE analyses included wild-caught quails together with quails from farms used as reference to allow the identification of two clusters that would facilitate tracking farm and wild origins. The value of q_i=0.90 was used as an arbitrary threshold above which individuals were assigned to cluster *i* as pure (see Barilani et al. 2005). Individuals that showed lower values ($q_i < 0.9$ for both clusters) were considered as potentially admixed.

To study if there was an increase through time in the proportion of farm alleles in the wild population, we focused on the estimated yearly proportion of wild-caught individuals classified as admixed ($0.1 < q_{farm} < 0.9$) in the population, rather than the degree of admixture for each individual. The classification as being admixed or not for each individual was the response variable (binary) and was analyzed using Generalized Linear Models (GLM). Sampling year was the continuous explanatory variable, since the interest was to test whether there was a trend in the ratio of admixed individuals over time. A binomial error structure and logit link function was used, and the models were fitted in R (version 2.15.2; R Core Team 2012) using the `glm()` function. The factor “dataset” was included to account for differences due to investigator and marker set when analyzing the three different data sets. All models were tested for overdispersion to avoid false positives and for collinearity between “year” and “dataset”. With these models we investigated if the probability of being classified as admixed was different between years and, if so, if it was increasing over time.

Simulation of farm allele introgression

Initial analyses of the observed data showed low levels of admixture between wild and farm quails. To test if these analyses had enough statistical power to detect an increase of admixture over time, we performed STRUCTURE analyses on data simulated with a series of scripts (available at http://www.consevol.org). These simulations were intended to represent the population at the breeding season (when field surveys took place). The scenarios that we investigated involved varying the proportion of farm individuals in the breeding population every generation, and different fitness values for admixed individuals born in the wild.

We first simulated genotypes for a wild population of quails where every generation a certain proportion of the potential breeders originated from farm releases. The simulations thus portrayed the composition of our study population during the breeding season. Because wild quails in the area show high mobility (Rodríguez-Teijeiro et al. 1992) and are part of a much bigger population (millions of individuals), we simulated a large population with a constant size of 10 000 breeding individuals to minimize the effects of random genetic drift. Genotypes at generation 0 were generated by randomly drawing two alleles for each locus according to their observed frequency. Starting allele frequencies were based on Dataset III, after excluding individuals whose genetic composition was clearly dominated by farm alleles ($q_{farm} \geq 0.40$ according to STRUCTURE analyses of that dataset alone; this threshold was selected to exclude individuals born in farms - q_{farm} close to 1 - and first generation admixed individuals - q_{farm} close to 0.5). Similarly, 10 000 genotypes were generated for farm quails by randomly drawing pairs of alleles for each locus based on the observed allele frequencies from farm set A. Since we had selected unlinked markers (see above), alleles at one locus did not influence allele selection at other loci (markers are assumed to recombine freely). Also, since our simulations only cover a limited time span (a few decades at the most), we did not consider the effect of mutations. Although Datasets III and farm set A may have an allelic composition different to the allelic composition of the populations before any admixture had taken place in the wild, we consider that these datasets may be an appropriate overall representation of the degree of differentiation between wild and farm quails and can offer useful insight on how introgression can be expected to proceed.

Successive generations (up to 20 generations to parallel the time period since the first hybrids were detected in the study area) of wild quails involved 10 000 new individuals per generation. To simulate the continuous restocking of quails, each of these new individuals had a certain probability of originating from farm releases (between 1% and 5%, according to the genetic analyses of our field samples; see below) and, in this case, was directly drawn from the file of farm genotypes. The genotypes for the rest of the individuals were generated by drawing alleles from two parental genotypes randomly selected from the previous generation. The simulation was run for 20 generations. We assumed non-overlapping generations and a generation time of one year. We did not assign sex to the genotypes and any pair of genotypes could act as parents for a genotype in the following generation. A schematic representation of these simulations is portrayed in Figure 1.

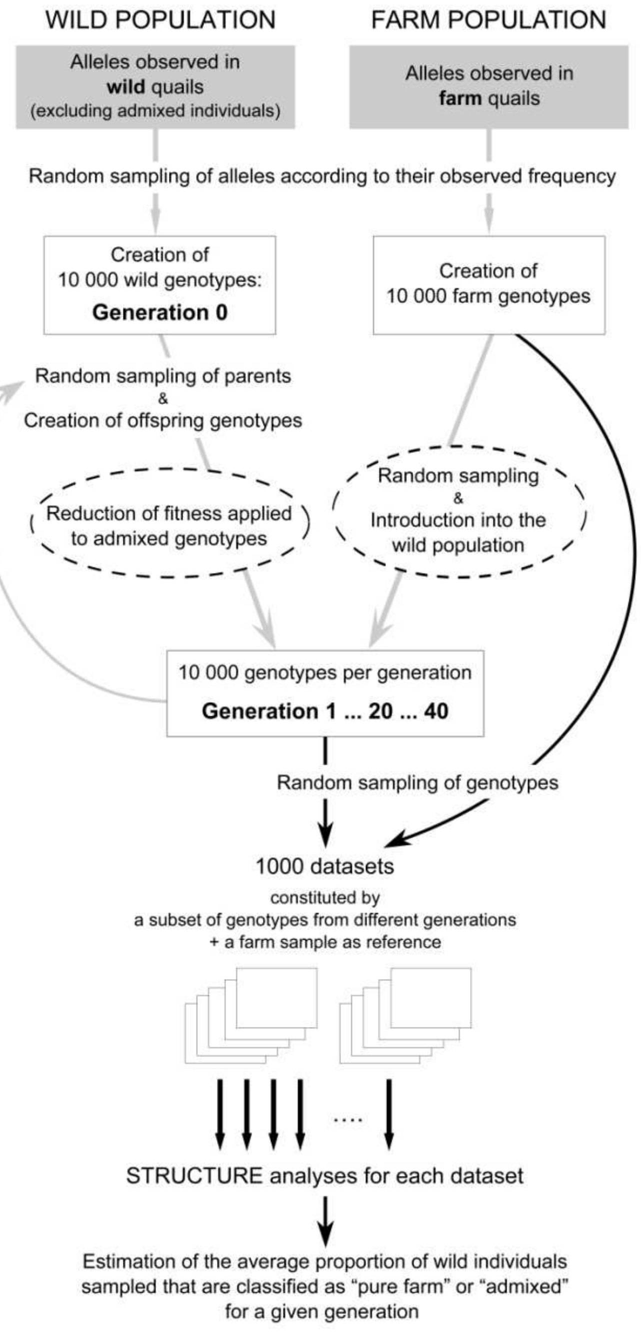

Figure 1. Schematic representation of the population simulations (gray arrows) and analysis of simulated data (black arrows). Boxes represent simulated populations or datasets. Simulations were used to assess the effect over time (across different generations) of the proportion of potential breeders of farm origin and varying fitness of admixed individuals (variables affecting the population simulations, marked with discontinuous circles). The first set of simulations was carried out assuming that all individuals, independently of having farm ancestry or not, had the same probability of contributing to the next generation (fitness= 1). For details, see text.

Initial simulations assumed that all individuals had the same chances of producing offspring in the following generation (fitness=1) independently of their origin (farm, wild or admixed). However, it is likely that farm individuals contribute maladaptive traits (Lynch & O'Hely 2001; Frankham 2008; Baskett & Waples 2013), and that they and their offspring have a lower fitness. To introduce this factor into a second set of simulations, we tracked for each individual the proportion of the genome expected to come from the farm population (1 for farm individuals, 0 for pure common quails, 0.5 for first generation admixed individuals). Each time that a new genotype was generated and one or both of the parents had a proportion of farm ancestry of 1 or 0.5, its probability of survival to the breeding season was determined by a predefined fitness value varying between 1 and 0 (1 when the probability of contributing to the next generation for admixed individuals was the same as for wild common quails, 0 when they never contributed; in the simulations we used the following fitness values: 1, 0.8, 0.6, 0.4 or 0.2). To simplify, we assumed that the fitness value was the same independently of the individual's proportion of farm ancestry. If the genotype did not survive, it was not included among the genotypes for a given generation (this would only include those that could participate as potential breeders), and a completely new genotype was generated to replace it and to keep the total population size constant. We assumed that lower fitness affected the chances of survival for the first two generations of admixed individuals, which are the ones that are most likely to suffer loss of extrinsic or intrinsic adaptation (i.e., presence of maladaptive alleles or breakup of co-adapted gene complexes, Laikre et al. 2010). After two generations genetic incompatibilities may be less likely and those individuals were considered as if they were pure wild quails for subsequent generations (proportion of farm ancestry: 0).

Analysis of simulated data and power tests

For a visual examination of the level of introgression in the simulated population, we randomly sampled individuals from multiple generations (50 individuals per generation for a few predefined generations) and analyzed them with STRUCTURE (K=2) together with a simulated farm dataset (50 farm individuals). The same analysis was also carried out breaking the temporal series into three sets of samples, covering a shorter time span each, mirroring the three datasets resulting from the field work (Table 1). In addition, in order to compare the results from the simulated data with the results obtained for the field datasets and to assess the power to detect introgression with the markers used, we prepared a program to replicate our field sampling pattern. Since the first hybrid individual in a neighboring area was detected by its vocalizations in 1990 (Rodríguez-Teijeiro et al. 1993), several years before the first of the wild-captured quails analyzed in this study, individuals from 1996 were assumed similar to the 5th simulated generation, 1997 to the 6th, and so on.

The program produced 1000 sets of simulated data extracting genotypes from the simulated population of 10 000 individuals per generation and with the same number of individuals per year as in real data (see Table 1). Each one of these simulated datasets was analyzed with STRUCTURE as described above (Figure 1).

For each STRUCTURE run we calculated the proportion of simulated genotypes that were not classified as pure wild ($q_{farm}>0.10$; admixed or pure farm). The 95% confidence interval for this proportion, for a given combination of potential breeders coming from farms and fitness value, was based on the 2.5th and 97.5th percentiles over each set of 1000 STRUCTURE runs. The proportion observed for the real data was then compared with these confidence intervals to assess which simulated scenarios were compatible with the real data (analysis of field samples).

RESULTS

To clarify analyses and results, a flow chart of the analyses and conclusions reached with them is provided as Supplementary Material (Figure S.1).

Mitochondrial DNA

Since mtDNA is maternally inherited and common quail and Japanese quail have well differentiated sequences (Barilani et al. 2005), this marker is useful for establishing directionality in hybrid matings. ML and NJ phylogenetic trees showed the existence of two clearly differentiated clades (Fig. 2). The control region sequences of 29 farm quails revealed 8 haplotypes and all of them were located within the same clade, with pairwise differences ranging between 1 and 10 substitutions (prefix F in Fig. 2). One of the haplotypes (F1W1 in Fig. 2) was observed in 22 individuals, while the remaining 7 were observed in one individual each. In contrast, the diversity observed in wild quails was much higher, with 13 haplotypes in 26 quails, mostly located within a second clade (prefix W plus F1W1 in Fig. 2). Five of the wild haplotypes were observed in more than one individual. One of these (F1W1), present in five wild individuals, was the only one shared with farm quails and corresponded to the most frequent farm haplotype. A GenBank BLAST search showed that F1W1 was identical to sequences previously identified in Japanese quails, supporting the hypothesis that farm quails derive from captive domestic Japanese quail females used as breeders, as suggested by Sanchez-Donoso et al. (2012). These sequences constitute the Japanese quail clade in Figure 2. Importantly, all analyzed farm individuals carried mtDNA sequences from this clade.

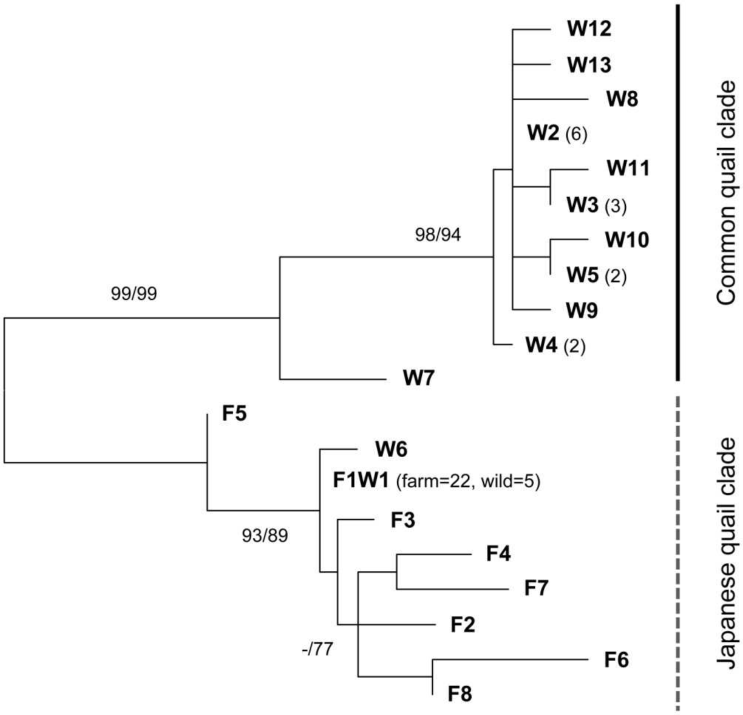

Figure 2. Unrooted maximum likelihood (ML) phylogenetic tree of mitochondrial control region haplotypes identified in wild (indicated with the prefix W) and farm (F) quails. Only one haplotype was shared between wild and farm quails (F1W1) (it was found in 22 farm quails and 5 wild quails). Number of quails sequenced with each haplotype is indicated in parenthesis when larger than 1. Bootstrap support for ML and NJ (in this order) is indicated at the nodes when higher than 70%. Two clades are found, separating common quail (*Coturnix coturnix*) from Japanese quail (*C. japonica*) sequences with high support.

Two of the haplotypes found in wild quails were either identical to (F1W1, in five individuals) or clustered with (W6, in one individual) farm haplotypes within the Japanese quail clade. The remaining sequences made up the second clade that did not include any farm sample (common quail clade), with differences between haplotypes also ranging between 1 and 10 substitutions. Despite the short size of the fragment studied here, sequences from the two clades were clearly differentiated by 17 to 24 substitutions. The existence of mitochondrial sequences from the Japanese clade in some wild quails confirms the presence of genetic material from quails of farm origin among the wild-sampled quails (all of these individuals were confirmed as being of farm origin or admixed in the microsatellite analyses, see below).

Identification of admixed individuals

All studied microsatellite loci were highly variable in both farm and wild quails. Despite the high polymorphism, previous paternity studies (not shown) revealed that the markers and protocols used did not result in false alleles. All individuals were successfully genotyped for at least 70% of the markers. The number of alleles per locus was high for all datasets and markers, with an average number of alleles per locus and allelic richness higher than 10 for all years and datasets (Table 1). As expected, the average number of alleles per locus was closely associated with sample size, but allelic richness remained practically constant from year to year within datasets. PIC values were high in all cases (with average PIC over 0.81 in wild quails and over 0.77 in farm quails), showing that the allelic variation was potentially very informative of population structure. Expected heterozygosity (H_e) was above 0.8 in all cases. This high heterozygosity results from the high number of alleles and similar values have been observed in other Galliform species with high allele diversity (He et al. 2008; Hassen et al. 2009; Bei et al. 2012). Observed heterozygosity (H_o) was slightly lower than H_e in all cases, which could be due to the presence of sets of alleles of different origin (farm and wild) that had not reached Hardy-Weinberg equilibrium. The values for H_e and H_o were almost constant within datasets, even when comparing datasets I and III (typed by the same person and equipment), which supports our initial assumption of negligible genetic drift.

Game farm quails showed lower average number of alleles per locus and allelic richness than wild quails analyzed with the same markers (Table 1). There was significant genetic differentiation between the wild (including possibly admixed individuals) and farm populations, and F_{st} values between them were very similar across datasets: F_{st} for Dataset I =0.05, for Dataset II =0.06, for Dataset III =0.05 ($p<0.05$ in all cases). Due to the high heterozygosity of our loci and the upper limit this imposes on F_{st} (1–[average within population H_e]; Hedrick 2005), these relatively low values actually represent a pronounced differentiation: Hedrick's standardized G'_{ST} values were between 0.35 and 0.41 (ca. 40% of the maximum differentiation possible), and D_{est} between 0.25 and 0.31.

We used the software STRUCTURE to divide each dataset (together with the corresponding farm set) in two clusters (K=2) in order to identify the contribution of the two quail lineages (wild or farm) to the genome of each individual. In each analysis (all five replicates produced practically identical output), a farm cluster and a wild cluster appeared clearly separated, and several individuals were identified as admixed (Fig. 3). Using $q_{farm} \geq 0.90$ as a threshold for the proportion of farm ancestry, between 79 and 90% of farm animals in each analysis were classified as pure farm, and none appeared as pure wild (Fig. 4A and Supplementary Material Table S.2a). Admixed individuals in the farm (between 10% and 21% of the farm animals) may represent descendants of crosses between farm quails and common quails (males) that are regularly introduced into farms as breeders (Sanchez-

Donoso et al. 2012). Of the quails sampled in the wild, between 81% and 94% were identified as pure wild ($q_{farm} \leq 0.10$). Some wild individuals (up to 10%) had very high q_{farm}, compatible with pure farm origin, and likely represent quails from restocking events. Overall, between 3% and 16% of wild-caught birds were classified as admixed ($0.10 < q_{farm} < 0.90$).

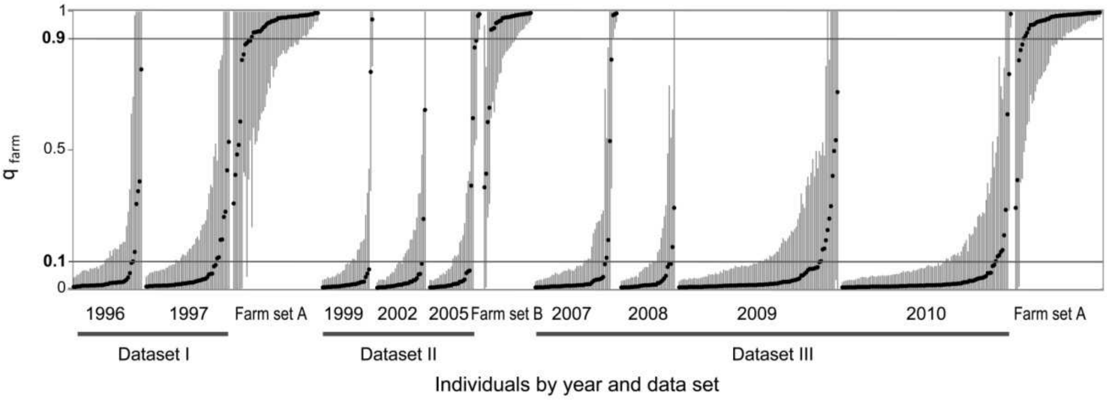

Figure 3. STRUCTURE analyses for K=2 for wild and farm quails for three datasets from different time periods. Dots represent individual proportion of membership to farm cluster (q_{farm}) and vertical lines are 90% highest posterior density intervals. Individuals are sorted by dataset, year and q_{farm} value. Horizontal lines at q_{farm}=0.10 and 0.90 indicate threshold values used to separate pure ($q_{farm} \leq 0.10$ for pure common quails, $q_{farm} \geq 0.90$ for pure farm quails) and admixed individuals ($0.10 < q_{farm} < 0.90$; see text).

Since we identified some individuals with admixed nuclear genotypes among farm quails, the presence of admixed individuals in the wild is not, *per se,* indicative of matings between restocked farm quails and native wild quails; these individuals could represent admixed animals released from farms. However, there are two lines of evidence that point to interbreeding in the wild. First, the ratio between admixed and pure farm, as classified by STRUCTURE, was much higher in the wild sample than in the farm sample (Fig. 4A, Supplementary Material Table S.2a; Yates Chi-square=67.21, df=1, p<0.0001). We would expect the same ratio in farms and in the wild if no interbreeding occurred in natural conditions; the observed admixed birds in the wild would just have resulted from their release from farms. Therefore, the inflated proportion of admixed quails in the wild compared to the proportion of freely roaming pure farm quails points to possible crosses in the wild between restocked and wild quails. However, the observed pattern would also emerge without crosses in the wild if restocked pure farm quails had lower survival in the wild than admixed quails, thus leading to an over-representation of the admixed individuals in the field sample.

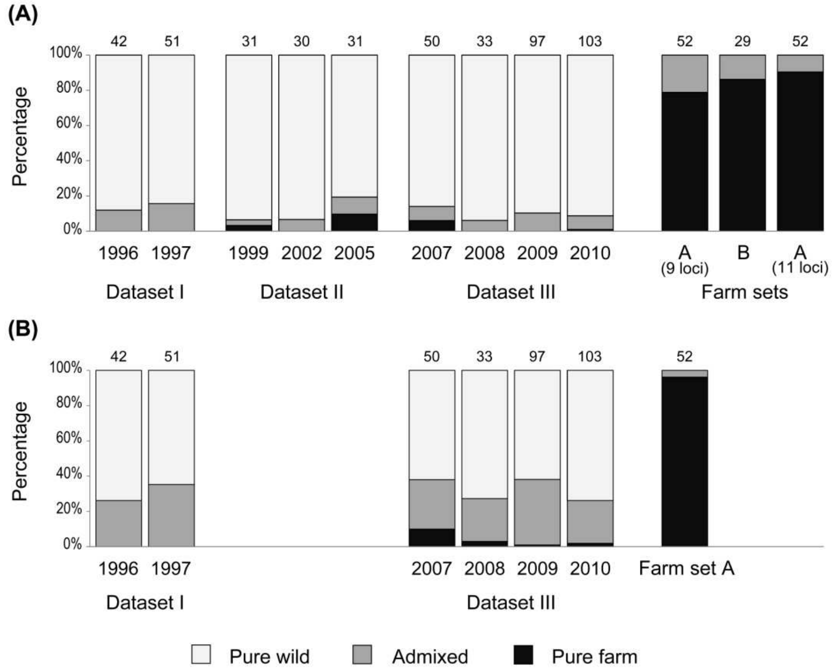

Figure 4. Percentage of individuals classified as pure wild (pale grey), pure farm (black) and admixed (dark grey) according to q_{farm} values obtained from STRUCTURE (K=2). Number of samples per year is indicated above each bar. **(A)** Analysis carried out separately for each dataset (as in Fig. 3 and Supplementary Material Table S.2a). **(B)** Datasets I and III analyzed together (as in Fig. 6 and Supplementary Material Table S.2b).

The second line of evidence derives from the mtDNA analysis. For the 26 wild samples from Dataset II used for mtDNA analyses, 6 were identified as admixed by their nuclear DNA and out of these, 4 had common quail mtDNA (3 had haplotype W2, and 1 had W3; Table 2, Fig. 2). The other 2 had Japanese quail mtDNA, as had the 4 wild-caught individuals assigned as farm quails by their autosomal genotypes. These last individuals probably represent animals released from farms. Since none of the farm individuals had common quail mtDNA (0 out of 29) and female common quails are known to reproduce very poorly in captivity (Caballero de la Calle & Peña Montañés 1997), finding four admixed individuals in the wild population with common quail mtDNA by chance is highly unlikely (Fisher exact test: p=0.008) if they just represented animals released from farms. Our findings therefore strongly indicate successful mating in the wild between restocked quails (males) and native wild quails (females).

	mtDNA haplotypes	
Autosomal genotype	Common quail	Japanese quail
Pure wild	16	0
Admixed	4	2
Pure farm	0	4

Table 2. Classification of 26 wild quails from Dataset II, based on autosomal microsatellites (as suggested by STRUCTURE: pure wild: $q_{farm} \leq 0.10$; admixed: $0.10 < q_{farm} < 0.90$; pure farm: $q_{farm} \geq 0.90$), and mtDNA (common quail or Japanese quail mtDNA, Fig. 2).

Changes through time in the level of introgression

During the 15 years encompassed by our study, there was no clear increase in the proportion of admixed individuals in the wild (Fig. 3 and Fig. 4A). GLM analyses using the year of sampling as independent variable and controlling for dataset failed to show a significant trend in the proportion of admixed individuals through time (β=0.122, SE=0.131, p=0.352). Results did not change when we did not control for dataset (β=-0.028, SE=0.030, p=0.347).

To assess our power to detect an increase of the proportion of admixture in the wild, we simulated 15 generations of a population where about 1.5% of the potential breeders every generation were quails restocked from farms. This proportion is below the estimated average from our field data (2.2%; see Fig. 4A and Supplementary Material Table S.2a) and our simulations could thus exemplify the changes that are expected to occur in the population with a low-moderate contribution of farm alleles every generation. We randomly selected 50 simulated genotypes from different generations and 50 simulated farm genotypes. The proportion of admixed genotypes significantly increased over time (β=0.133, SE=0.035, p <0.001; Fig. 5A) when the simulated dataset spanning 15 generations was analyzed as a whole. Ten additional sets of simulated genotypes were generated and all of them showed a significant increase in the proportion of admixed genotypes (p <0.001 in all cases). However, when the same data was analyzed subdivided into three datasets, mirroring the analysis of real data (Fig. 3), the degree of introgression was underestimated. A smaller proportion of the simulated wild population appeared as admixed in later generations, and this proportion did not show changes over time (β=-0.031, SE=0.039, p=0.432; Fig. 5B). The comparison of the q_{farm} values for the same simulated genotypes from the last generations (12 to 15) showed that fragmenting the data into shorter time periods led to an underestimation of q_{farm} in wild quails in later generations, and of the proportion of admixed individuals in the population (Fig. 5D). The same comparison for the first simulated generations (1 and 2) did not show the same differences in q_{farm} values (Fig. 5C). Thus, limiting the time span analyzed greatly reduced our ability to detect the progressive introgression of alleles from the farm population.

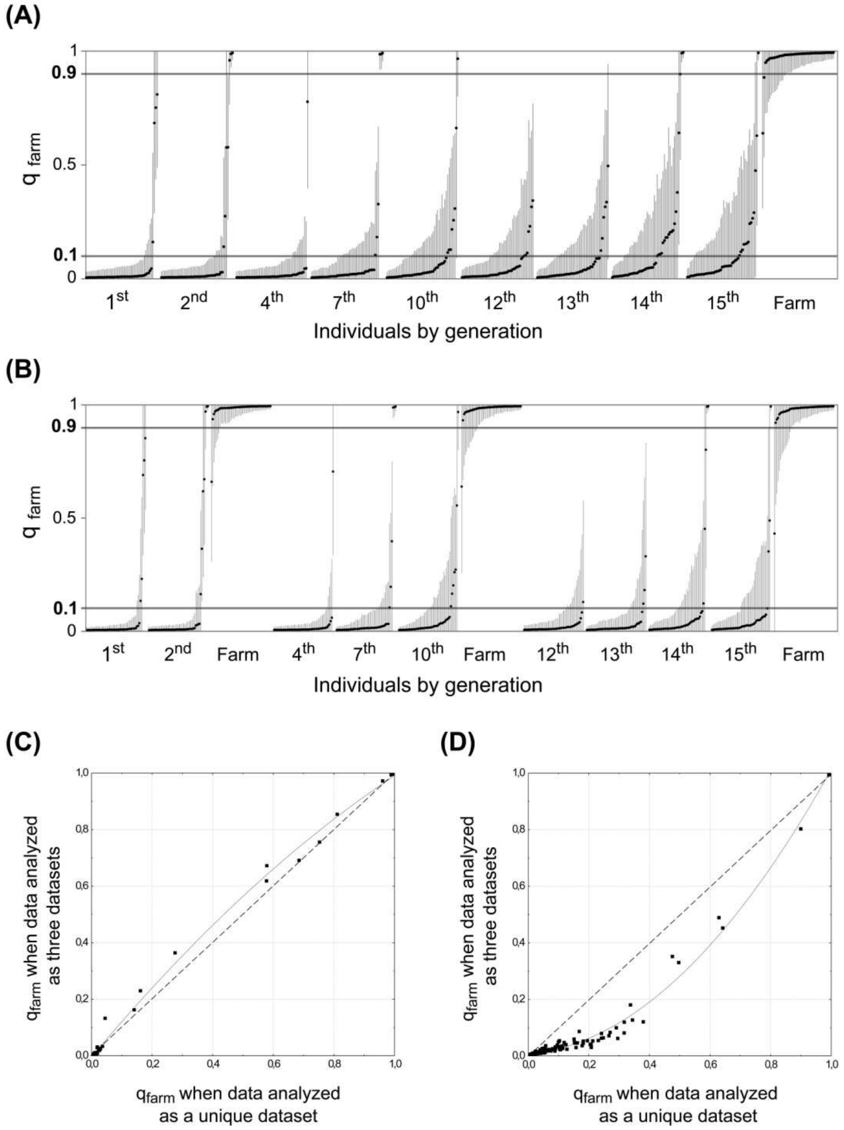

Figure 5. STRUCTURE analyses (K=2) for simulated data assuming that about 1.5% of the potential breeders are of farm origin. Fifty genotypes were sampled from each generation and 50 simulated farm genotypes were included in the analyses. **(A)** All data included in the analysis as a unique dataset. **(B)** Same simulated data as above, but analyzed in three datasets separately, analogous to those in Fig. 3 (same 50 simulated farm genotypes included in all analyses). Generations 12 to 15 do not show the degree of introgression that was inferred above. **(C)** Scatterplot comparing q_{farm} values for individuals from generations 1 and 2 obtained in (a) (X axis) B and (b) (Y axis). The curve represents the polynomial curve that best fits the data: q_{farm} values were almost identical for the two axes. **(D)** Similar scatterplot for individuals from generations 12, 13, 14 and 15. In this case, intermediate values of q_{farm} are underestimated when analyzed using short time series and more individuals appear as pure wild.

Similarly, the field dataset that spanned the longest time period (Dataset II, 6 years, Table 1) did not show a change in the proportion of admixed individuals over time (β=0.184, SE=0.183, p=0.314); the same result was obtained for a different wild population located over 100 km north of our study site monitored during the same 6 years (data not shown, see Supplementary Material Text S.1). We used simulations based on Dataset II to investigate the power to detect an increase of admixed individuals over a period of six years using the same number of markers and samples. We found that increased introgression of farm alleles would not be consistently detectable (in at least 90% of the cases) unless the proportion of potential breeders coming from farms was above 3% (see Supplementary Material Text S.2), higher than the proportion observed in our study area.

Since the simulations showed that the ability to detect introgression of farm alleles into the wild population (with the sample size available) depends on the length of the time series considered, we joined datasets I and III for a STRUCTURE analysis covering a longer time span (although reducing the loci used to the nine shared by both datasets). This resulted in wider confidence intervals for the estimates of q_{farm} (Fig. 6A). In agreement with the simulations (Fig 5A compared to Fig. 5B), joining datasets I and III indicated larger admixture (Fig. 6B). The proportion of individuals classified as pure wild decreased compared to previous analyses (Fig. 4B and Supplementary Material Table S.2b). Again, no noticeable change was observed in the proportion of admixed individuals through time (β=-0.007 SE=0.021, p=0.737): about 31% for the first two years of the series (years 1996 and 1997) and 28% for the last four years (years 2007 to 2010).

In conclusion, the genetic analysis of the field data did not show an increase in the proportion of admixed individuals in the population, even after joining datasets I and III to overcome limitations associated with a short time series. However, these analyses suggested that perhaps the level of admixture had been underestimated by considering only short time periods in each analysis.

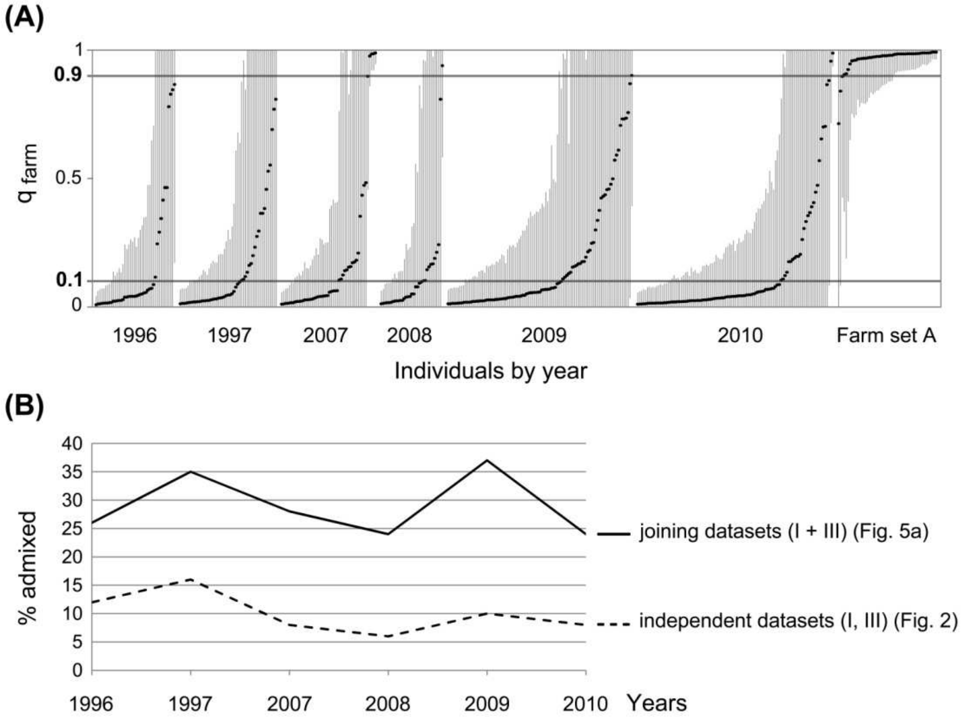

Figure 6. (A) Joint STRUCTURE analysis (K=2) for wild quails from datasets I and III. **(B)** Proportion of admixed individuals ($0.10 < q_{farm} < 0.90$) per year. Continuous line: datasets I and III analyzed together (as above). Dashed line, datasets I and III analyzed independently (as in Fig. 3). No increase in the proportion of admixed individuals is observed.

Introgression as a function of the proportion of farm breeders and fitness of admixed individuals

One limitation of our simulations was that we considered a fixed proportion of the potential breeders to originate from farms (1.5%) while in real data this proportion was variable between years. It was not clear if a lower proportion would also produce a detectable signal of introgression, nor what level of introgression could be expected if the fitness of admixed individuals was lower than that of the native common quail population. To assess the likely fraction of restocked farm individuals among the potential breeders that could produce the observed patterns of low admixture, we simulated 20 generations with fractions of 1, 1.5, 2, 2.5, 3, 4 or 5% per generation. For each case we generated 1000 datasets of simulated genotypes analogous to the field sampling (same number of samples per year as in datasets I + III) and we analyzed them with STRUCTURE. Since the proportion of individuals with

q_{farm}>0.10 in Dataset III was 32.4% (when combining datasets I and III; Fig. 4B and Supplementary Material Table S.2b), we investigated in which cases this value was inside the 95% confidence interval obtained from the simulations. The observed value was compatible with the simulations only when the proportion of potential breeders coming from farms was 1% (Fig. 7, not shown for values higher than 3%). However, this value was almost a third of the observed average in the field sample (2.7%; Fig. 4B and Supplementary Material Table S.2b).

We suggest two possible explanations for this mismatch: first, the percentage of farm individuals potentially contributing to the breeding population may have been overestimated. Alternatively, farm and admixed individuals could exhibit lower fitness in the wild than pure common quails, slowing down the spread of farm alleles in the wild population. In simulations incorporating a variety of fitness values (1, 0.8, 0.6, 0.4 and 0.2) and assuming that between 2-3% of the potential breeders come from farms, the observed proportion of admixed (and pure farm) quails in the field was compatible with relative fitness values as low as 0.6 (Table 3). If the proportion of farm individuals among potential breeders was larger than 3%, the data would be compatible with even lower fitness values for admixed birds. These results indicate that admixed individuals may have lower chances of contributing offspring to the next generation than common quails. Interestingly, our data was not

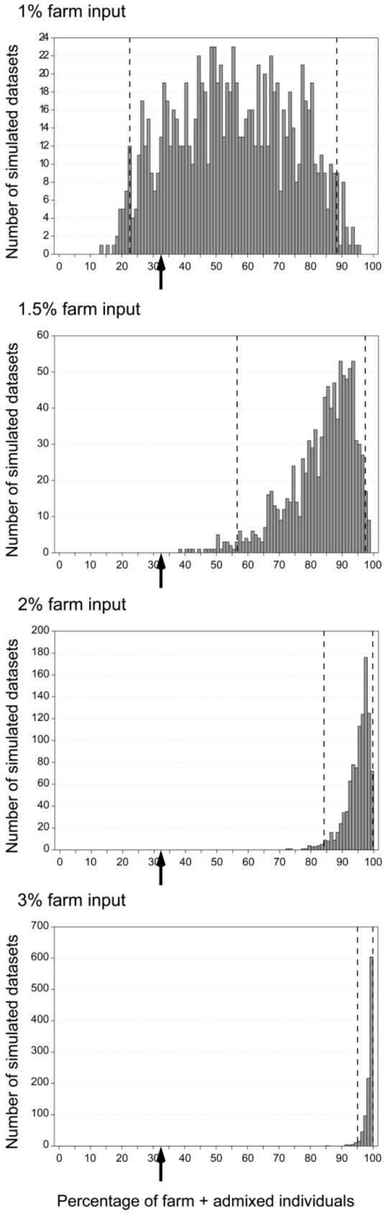

Figure 7. Distribution of the proportion of non pure wild individuals (farm or admixed, q_{farm}>0.10) in 1000 simulated datasets representing a sampling pattern analogous to that of Dataset III (2007-2010) assuming various proportions of potential breeders coming from farms: 1, 1.5, 2, and 3%. Dashed lines show 2.5th and 97.5th percentiles. Arrows indicate proportion observed in the real data: 32.4% (Fig. 4B and Supplementary Material Table S.2b).

compatible with the simulated scenarios in which fitness values of admixed individuals was 0.2. Introgression would only be completely negligible if the results were compatible with fitness=0. This implies that some introgression must be taking place.

Long-term effects

Our simulations showed that even though we did not observe a clear increase over time in the proportion of admixed individuals, the genetic analysis of field samples was compatible with slow but accumulating introgression (Table 3). In order to investigate the effects of such introgression on wild quail populations in the near future, we simulated populations evolving under two scenarios compatible with field data: (i) 1% of potential breeders deriving from restocked quails and hybrids without diminished fitness, (ii) 2% of the potential breeders coming from farms and a fitness of 0.6 for hybrids (Fig. 8A, 8B, respectively). In both cases, running the simulations for 20 more generations (40 generations in total) translated into extensive introgression of farm alleles and more than 20% of the wild individuals reached values of q_{farm} indicative of admixture ($0.10 < q_{farm} < 0.90$; Fig. 8c).

	Fitness				
Farm %	**1**	**0.8**	**0.6**	**0.4**	**0.2**
1	22.6 - 88.3	11.0 - 49.5	6.7 - 20.2	4.6 - 12.4	2.1 - 7.8
1.5	56.5 - 97.2	19.8 - 79.9	10.2 - 31.1	5.7 - 15.2	3.2 - 9.2
2	84.1 - 99.7	50.2 - 96.1	17.3 - 66.4	7.4 - 18.4	3.9 - 10.6
2.5	91.9 - 100.0	71.4 - 98.2	17.3 - 63.6	9.9 - 24.4	5.3 - 12.4
3	95.1 - 100.0	84.1 - 99.3	30.7 - 87.6	13.1 - 33.6	5.7 - 13.4
4	98.9 - 100.0	94.7 - 100.0	60.4 - 95.4	18.7 - 50.2	8.5 - 17.3
5	99.3 - 100.0	97.9 - 100.0	83.4 - 99.3	28.6 - 69.6	11.3 - 21.6

Table 3. 95% confidence intervals of the proportion of not pure wild (admixed and pure farm individuals, $q_{farm}>0.10$) for the simulated data corresponding to Dataset III. Simulations consisted of 1000 STRUCTURE runs, varying (i) the levels of fitness for admixed individuals, and (ii) the proportion of potential breeders that come from farms (restocked) each generation (35 000 STRUCTURE runs in total). Simulated data sets were built replicating the sampling pattern from the field data for datasets I and III (same number of samples per year). Grey cells represent the parameter combinations compatible with our field data, i.e. simulations where confidence intervals include the proportion of individuals with $q_{farm}>0.10$ observed in our sample (32.4%; Supplementary Material Table S.2b).

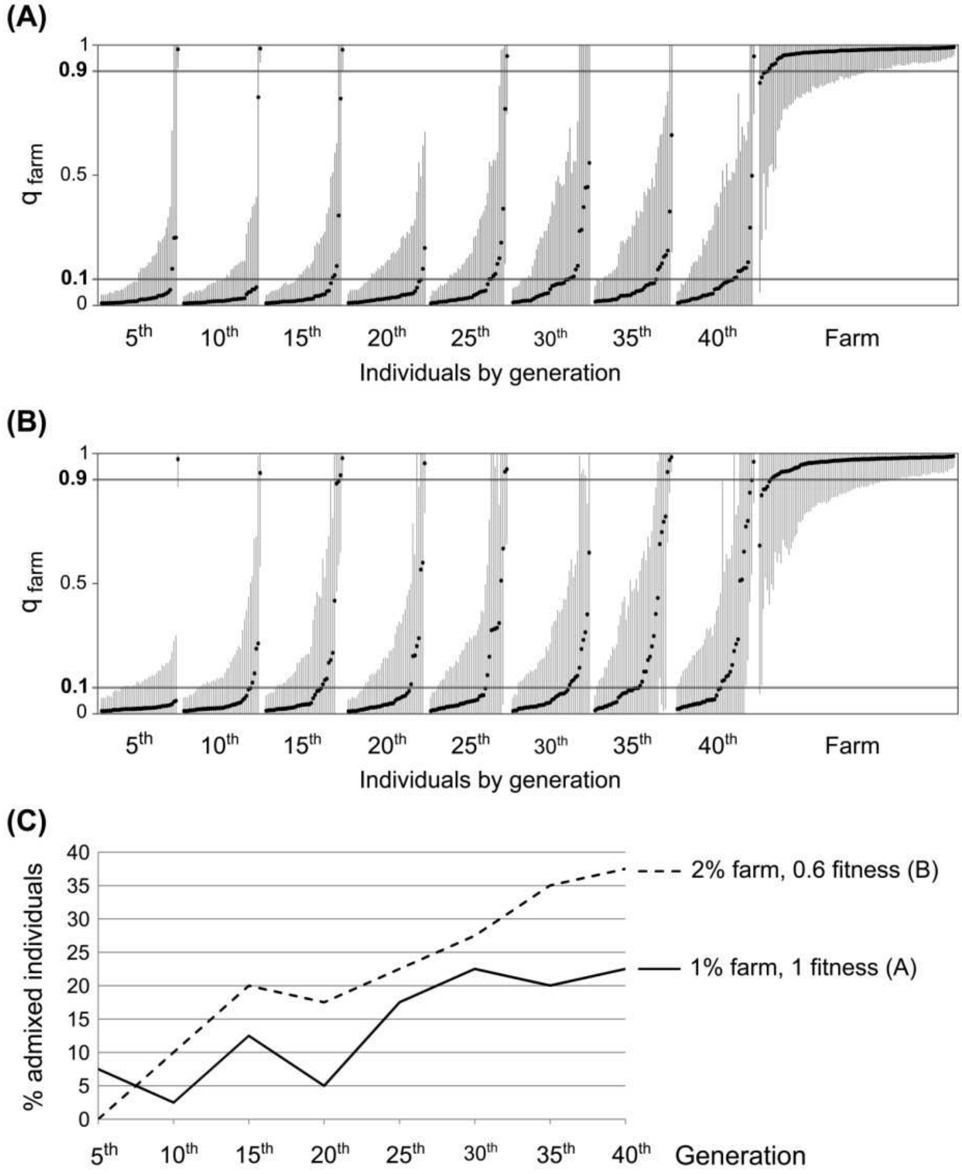

Figure 8. Examples of results from an analysis using STRUCTURE (K=2) on data simulated over 40 generations, with 50 individuals randomly sampled every 5 generations, along with 100 simulated farm individuals. **(A)** 1% of potential breeders derived from farms, fitness of 1 for all individuals. **(B)** 2% derived from farms, fitness of 0.6 for first and second generation admixed individuals. **(C)** Proportion of admixed individuals ($0.10 < q_{farm} < 0.90$) over time under scenarios (a) (continuous line) and (b) (dashed line). In both cases, a clear increase in the introgression of farm alleles into the wild population is observed.

DISCUSSION

Studies on the population-wide impact of introgression have a long history in areas like fisheries research and aquaculture, where massive restocking practices are common (see, e.g., Hedgecock & Coykendall 2007; Waples et al. 2007; Waples & Drake 2008). On the other hand, only little is known about the genetic effects of large scale releases for supportive breeding in other wildlife groups (Laikre et al. 2010). Studies on birds and mammals often focus on the individual identification of hybrids and the estimation of their proportion in the population. From this information, however, it is not straightforward to infer the rate of introgression and its potential impact on the population. In this study we integrated three separate datasets to cover a large time period of 15 years (ca. 15 generations), which could allow separating hybridization and introgression, and thus monitor the advance of introduced alleles into the focal population and assess the fitness of hybrids.

In the study region (Catalonia, NE Spain), the number of farm quails restocked every year was 3-11 times larger than the estimated wild population (from Rodríguez-Teijeiro et al. 2004 and Hunting order plans of the Regional Government of Catalonia). However, only a small proportion of the released farm quails become potential breeders: those that escape hunting and predation and those that also survive the winter, with a probably depressed immune system response (due to relaxed selection in captivity) and in breeding grounds with limited food resources, or that successfully migrate to wintering latitudes and return the next breeding season (which seems unlikely due to the reduced migratory impulse they may have inherited from domestic Japanese quails; Derégnaucourt 2000, Derégnaucourt et al. 2005). Since our results indicate that only 2-3% of the potential breeders in the study area were of farm origin, approximately only one out of every 100-500 farm quails released can potentially contribute to the wild breeding population. However, our results show that those individuals could have an important impact on the wild population.

Based on the proportion of quails with vocalizations indicating hybrid origin, Puigcerver et al. (2007) suggested that the proportion of admixed birds in the population had remained relatively constant, and proposed that those individuals could be animals of farm origin that survived in the field for some time but failed to reproduce. However, the proportion of admixed relative to pure farm in the wild is much larger than in captivity and this, together with the capture of admixed birds with common quail mtDNA, indicating that the mother was a wild quail, confirm that interbreeding has happened in the wild. Field experiments carried out by Puigcerver et al. (2014) have shown that farm and wild individuals can mate in the wild, but our study provides the first evidence that this in occurring outside the experimental conditions.

The detection of offspring from wild x farm quail crosses does not necessarily imply a progressive build-up of Japanese quail alleles in the common quail population. Continued backcrossing of the crossbred individuals with common quails is required to produce an increase in the proportion of admixed individuals and introgression of alleles. An analysis of three separated studies of field samples did not show such increase (Fig. 3). However, not seeing such a trend could either be due to its non-existence or to the lack of power to detect it, showing that the observed data is inconclusive. Our simulations pointed to the latter, as they revealed that analyses covering short time frames (as those generated when analyzing the three datasets separately) lack the power to detect an increase in introgression, especially when the proportion of potential breeders coming from farms is relatively small. In these cases, STRUCTURE analyses underestimated the degree of admixture (Fig. 5D), possibly due to an inaccurate estimation of the ancestral allele frequencies (before introgression). After a few generations of admixture, some introgressed alleles may have spread in the native population, complicating the identification of their alien origin. This is an especially important outcome of our simulations since studies of hybridization and introgression are generally based on samples collected over a short time period, and this could result in similar biases in the estimation of the rate of admixture. It is possible that increasing sample sizes during the different time periods or genotyping a larger number of genetic markers would also increase the power to detect introgression. However, this is often not possible or too expensive. Overall, our results illustrate how power tests are essential in determining if a study can robustly test the hypotheses presented.

To increase our power to detect changes through time, we jointly analyzed the first and last datasets, spanning 15 years. This implied reducing the power to individually classify samples as pure or admixed and increased confidence intervals for the proportion of farm ancestry q_{farm} (see Fig. 3 and Fig. 6A). This cannot be just result of reducing the number of genetic markers included in the analysis since it remains the same for Dataset I (9 loci) but may be indicating important admixture.

Simulations replicating the sampling pattern of datasets I and III indicated that the power of the markers and samples analyzed should be sufficient to detect increased introgression in the case that as little as just 1.5% of the potential breeders came from farms. However, the real data did not show an increase in the proportion of admixed individuals even though the average proportion of farm animals detected in the study site every generation was about twice as much. This suggests that either the proportion of farm animals contributing to the reproduction is well below 1.5%, or that farm and admixed individuals have a lower fitness and, thus, have a lower contribution to the following generations than pure common quails. This would be possible if most of the admixed individuals were not involved in the reproduction. However, this does not seem likely because field experiments have shown that female quails of farm origin are more successful than wild common quails in attracting and mating wild common quail males (Puigcerver et al. 1999, 2013; Puigcerver et al. 2014). The limited introgression of farm alleles into the wild population may be due to the lower fitness of the released birds and their descendants, which is also supported by experimental observations in the wild that point to a decreased survival and increased nest predation for females of farm origin (Puigcerver et al. 2014). Our simulations indicate that their overall fitness could easily be 40% lower than the fitness of wild common quails.

Common and Japanese quails have evolved under different selective forces and have experienced different demographic changes. Consequently, it is highly possible that alleles of Japanese quail origin present in the farm population are maladaptive and contribute to a lower fitness in common quail populations. However, probably more important than this is the change in selective forces imposed first by the domestication process in Japanese quails, and later by the captive breeding with common quails for restocking. These further promote the fixation of maladaptive alleles and traits (Lynch & O'Hely 2001; Frankham 2008; Baskett & Waples 2013) that could, for example, contribute to reduce anti-predatory behavior, flight restlessness or ability to explore alternative food sources. As a result, restocking practices often have very low success rates (Gortázar et al. 2000, Guyomarc'h 2003). This could contribute to the reduced fitness of admixed birds.

Interestingly, real data was not compatible with simulated scenarios where the fitness of hybrids was very low (0.2). When we simulated data with this very low fitness for admixed individuals, fewer individuals from the simulated datasets were identified as admixed than in the real data, suggesting that farm alleles may be slowly, but progressively, integrating into the native common quail gene pool. Our simulations were simple and relied on several assumptions (large effective population size, no population structure, generation time of 1 year, non-overlapping generations, etc); the simulations were also based on the assuming that farm quail releases were initiated at the time our group first detected them (around 1990) and took place at a constant rate (constant proportion of farm individuals added every generation), but this may not be the case. Also, fitness effects could be introduced into the simulations in a number of ways (for example, varying fitness depending on the proportion of the genome originating in the farm, or reduced survival could be modeled at different stages in the life cycle). Consequently, the fitness estimations obtained can only be taken as rough approximations, but we believe that they reflect population trends and indicate that although admixed individuals have reduced fitness some introgression is taking place every generation.

Our results highlight the possible consequences of continuing the current restocking practices. While we do not detect an increase in the proportion of admixed individuals over a

15 year time period, our simulations show that this does not guarantee that introgression is not taking place. Two simulated scenarios, compatible with estimates from real data, predict extensive introgression of farm alleles in the near future (Fig. 8). Of course, these simulations are based on estimates obtained from a very small area, and the proportion of potential breeders coming from farms is likely to vary from place to place and from year to year. However, considering the high mobility of common quails and how widespread restocking practices are, we do not have reasons to expect very different patterns in other areas within the western distribution of the common quail.

CONCLUSIONS

Extensive restocking with farm-reared quails does not yet appear to have had large impacts on the genetic integrity of common quails in Northeast Spain. However, we here provide evidence that interbreeding has already happened in the wild and that overall admixture levels may be slowly increasing over time. Although some degree of selection against introgressed alleles appears to exist, our simulations predict a marked risk of genetic swamping of the common quail population, if release practices are maintained. Our results therefore support initiatives currently being taken to control quail restocking practices across Europe.

More generally, our results highlight that the impacts of introgression on wild populations can be difficult to detect, especially when hybridization occurs over long time periods and at low frequency. In such cases, data collected over a short time period may be insufficient and lead to an underestimation of the degree of admixture. Our study therefore underlines the need for long time series to reliably assess the consequences of hybridization in natural populations and the need for power tests.

DATA ACCESSIBILITY

Newly obtained mitochondrial DNA sequences have been submitted to GenBank (accession numbers: KF410830-KF410849). Microsatellite data files are deposited in Dryad (reference number: *pending number assignation*). Scripts were set to run in a computer with a Linux operative system (Ubuntu 12.10) and are available at http://www.consevol.org.

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AUTHOR CONTRIBUTIONS

This study is part of a long-term study on quail populations led by M.P. and J.D.R.-T. It is part of the doctoral thesis of I.S.-D. (at Barcelona University, Spain), supervised by J.D.R.-T., M.P. and C.V., and the master thesis of J.H. (at Uppsala University, Sweden), supervised by C.V. and F.H. C.V. and I.S.-D. conceived the genetic study. I.S.-D., M.P. and J.D.R.-T. did field work. I.S.-D., J.H., F.H. and J.E. performed laboratory work. I.S.-D. and C.V. did genetic and statistical analyses and simulations, with input from J.H. and F.H. All authors contributed to the text.

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SUPLEMENTARY MATERIAL

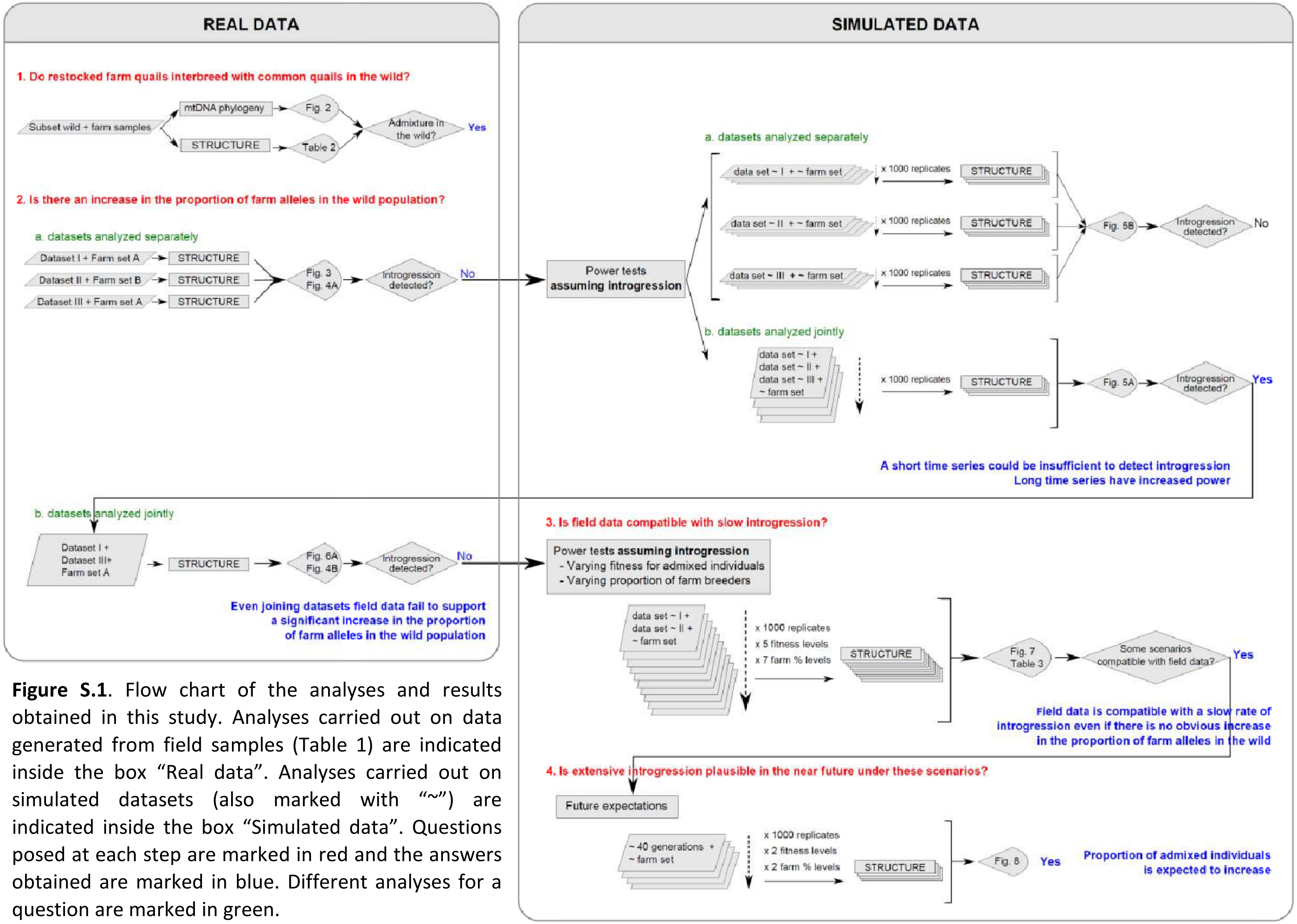


Figure S.1. Flow chart of the analyses and results obtained in this study. Analyses carried out on data generated from field samples (Table 1) are indicated inside the box “Real data”. Analyses carried out on simulated datasets (also marked with “~”) are indicated inside the box “Simulated data”. Questions posed at each step are marked in red and the answers obtained are marked in blue. Different analyses for a question are marked in green.

Table S.1. Primers from microsatellite loci described by Kayang *et al*. (2000, 2002) and modified for this study.

Locus	**Primer**	**GenBank accession number**	**Original primer (5'-3')**	**Modified primer (5'-3')**
GUJ0017	Forward	AB035827	AGAGAGATTAGAGGAGCTGC	AGGAGCTGCCTGGAGATAGA
GUJ0057	Forward	AB063125	GGAATGGAAAATATGAGAGC	GCTCTGTTTCACAGCA
GUJ0065	Forward	AB063133	GCGTGCCATTTACTTCCCGG	GAGTGAGTTACAGCACAG
GUJ0065	Reverse	AB063133	AGCCAGGATGACCAGGAAGG	AGGATGACCAGGAAGG
GUJ0097	Reverse	AB063165	GAGCAAGAGGTGAGTGTTTC	GGAACCTGTCCTATCCTAGA

Table S.2. Classification of the studied samples into pure wild, pure farm and admixed according to q_{farm} values obtained with STRUCTURE (K=2). Reference farm samples are the same for datasets I and III (farm set A), but with different number of loci. Farm set B was used as reference farm samples for Dataset II.

a. Analysis carried out separately for each dataset with its corresponding reference farm set (as in Fig. 3 and Fig. 4A).

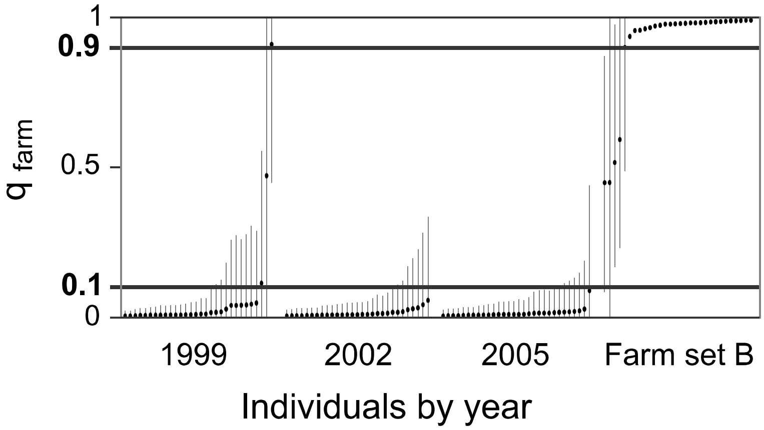
Dataset	**Year**	**N**	**Pure wild ($q_{farm} \leq 0.1$)**		**Admixed ($0.1 < q_{farm} < 0.9$)**		**Pure farm ($q_{farm} \geq 0.9$)**	
			N	**%**	**N**	**%**	**N**	**%**
I	1996	42	37	88	5	12	0	0
	1997	51	43	84	8	16	0	0
II	1999	31	29	94	1	3	1	3
	2002	30	28	93	2	7	0	0
	2005	31	25	81	3	10	3	10
III	2007	50	43	86	4	8	3	6
	2008	33	31	94	2	6	0	0
	2009	97	87	90	10	10	0	0
	2010	103	94	91	8	8	1	1
Wild, average				89.0		8.9		2.2
Farm set A (9 loci)		52	0	0	11	21	41	79
Farm set A (11 loci)		52	0	0	5	10	47	90
Farm set B		29	0	0	4	14	25	86

b. Datasets I and III analyzed together (and with farm set A) using 9 microsatellite loci (as in Fig. 6A and Fig. 4B). The reduction in the number of markers implies lower precision but allows comparisons along a longer time span.

Year	**N**	**Pure wild ($q_{farm} \leq 0.1$)**		**Admixed ($0.1 < q_{farm} < 0.9$)**		**Pure farm ($q_{farm} \geq 0.9$)**	
		N	%	N	%	N	%
1996	42	31	74	11	26	0	0
1997	51	33	65	18	35	0	0
2007	50	31	62	14	28	5	10
2008	33	24	73	8	24	1	3
2009	97	60	62	36	37	1	1
2010	103	76	74	25	24	2	2
Wild, average			68.3		29.0		2.7
Farm set A *(9 loci)*	52	0	0	2	4	50	96

Text S.1. Analysis of admixture of a different wild quail population during 6 years

During the same period monitored in Dataset II (1999, 2002, 2005), a parallel study was carried out on the breeding quail population in a different locality located in a hunting area in the Pyrenees (Alp, Spain; 42° 22′ N, 1° 53′ E; 1158 m above sea level), 107 Km north of the study location (Mas Esplugues). Samples from 89 quails were obtained (30 in 1999, 29 in 2002, and 30 in 2005) and were genotyped for the same 13 autosomic microsatellites as Dataset II. They were analyzed with STRUCTURE with the same control farm samples (farm set B). As with Dataset II, no increase in the proportion of admixed individuals was observed over the years.



Text S.2. Power to detect increasing introgression with a dataset spanning 6 years

Dataset II was the dataset spanning a largest time period (samples from 1999, 2002 and 2005). To investigate if this data series was long enough to detect the effects of introgression with the markers used, we simulated 20 generations of a large random mating population genotyped with the same 13 microsatellite markers used with this dataset. Initial allele frequencies were based on the frequencies estimated from the samples in the dataset excluding individuals whose genetic composition is clearly dominated by farm alleles (in STRUCTURE analyses in Fig. 3, $q_{\text{farm}} \geq 0.40$). Allele frequencies for simulated farm quail genotypes corresponded to those from farm set B. We assumed that each generation a proportion (1, 2, 3, 4 or 5%) of the potential breeders derived from restocked farm birds. The resulting genotypes corresponding to generations 8, 11 and 14 were sampled 1000 times simulating the sampling pattern of Dataset II (31, 30 and 31 individuals for generations 8, 11 and 14, representing years 1999, 2002 and 2005). The program STRUCTURE was run for K=2 with each one of the simulated datasets.

To check if there was an increase in the proportion of pure farm+admixed individuals ($q_{\text{farm}} > 0.10$) through time we compared the proportion for 1999 with the proportion for 2005 for each one of the 1000 simulated datasets generated for a given proportion of potential breeders originating from farms. We would expect an increase with time. However, out 1000 runs with 1% potential breeders coming from farm per generation, in 403 runs the proportion of pure farm+admixed individuals was not higher in 2005; 168 for 2%; 47 for 3%; 22 for 4% and 50 for 5%. This means that a dataset like this, spanning 6 years and including three samples of about 30 individuals, may be insufficient to clearly detect increasing arrival of farm alleles, in particular when the proportion of potential breeders from farm is lower than 3% (in our case: 2.7%, see Fig. 4B and Supplementary Material Table S.2B).

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Kayang BB, Inoue-Murayama M, Nomura A, Kimura K, Takahashi H, Mizutani M & Ito S (2000) Fifty microsatellite markers for Japanese quail. *The Journal of Heredity* 91: 502–505.

GENERAL DISCUSSION

Track among ceral crops (Sant Magí de Brufaganya, Catalonia)
by Ines Sanchez-Donoso

DISCUSSION

Restocking a wild population with captive reared individuals is a commonly used strategy for the conservation and management of animal species. Introduction objectives may be, for example, to bolster endangered populations (Massemin-Challet et al. 2006, Armstrong & Seddon 2008, Santos et al. 2009) or to supplement fisheries and game bags in order to increase productivity or maintain recreational activities (Blanco-Aguiar et al. 2008, Barbanera et al. 2010).

Restocking is a critical management strategy for species conservation when their survival is in serious danger. This is the case of the Iberian lynx (*Lynx pardinus*), for example, where restocking of individuals born and reared in captivity is being used to reinforce existing populations and maintain its depleted genetic diversity (Vargas et al. 2009). However, in less critically endangered species other management actions could be more effective than restocking for the increase of the population size, such as habitat conservation and banning of human threatening activities like poisoning, overfishing or hunting. In the case of the common quail, with large populations worldwide, restocking is not intended as a conservation tool, but solely as a way to artificially increase hunting bags.

Although restocking can have positive effects for the conservation of wild populations, it entails several aspects that can suppose a threat for those populations (Laikre et al. 2010, Marie et al. 2010):

a) **Parasites and diseases**. Restocking of farm-reared individuals constitute a point of entrance for parasites and diseases that could cause important mortality in the native populations (Peeler et al. 2006).

b) **Low effective population size**. Restocked individuals usually derive from a reduced number of breeders, what implies low genetic diversity. Interbreeding with wild individuals could result in a reduction of the effective population size and the evolutionary potential by loss of genetic diversity (Ryman & Laikre 1991), deeply altering the genetic composition of native populations (Ford 2002) and limiting their potential for population growth.

c) **Domestication**. Captive breeding and domestication represents a general relaxation of selective forces and promotes traits that are maladaptive in the wild (Lynch & O'Hely 2001, Frankham 2008, Baskett & Waples 2013). The interbreeding of farm animals with wild individuals could cause the introgression of maladaptive alleles into the native population, reducing its adaptive potential and survival, and leading to its decline.

d) **Different species**. In addition, if restocked individuals do not belong to the same species, the genetic structure of the native species would be affected and local adaptations could be disrupted (Mank et al. 2004, Eldridge & Naish 2007).

Results from this thesis have shown that quail restocking entails for the common quail at least the three last threats exposed (the first one has not been tested in this thesis but is probably also happening) because individuals used for restocking in Spain are farm-reared quails of domestic and hybrid origin, and with low genetic diversity. The degree of admixture of these individuals is variable, being the majority of them second (at least) generation hybrids.

According to our results, farm-reared quails show low genetic diversity. No differences have been detected among the Spanish game farms analyzed, which points to a very reduced pool of breeders. This agrees with a Spanish farm survey (Sánchez García-Abad et al. 2009) that shows that very few farms breed quails, while the majority only raises them. Therefore, the low genetic diverse farm population used for restocking could lead to a reduction of the effective population size of the common quail. In addition, since they show adaptation to life in captivity, restocking could likely lead to the introgression of maladaptive alleles in the wild common quail population.

It is possible to think that hunting restocked quails instead of common quail could act as a parapet for the last, since a hunted farm-reared quail would suppose one common quail not hunted. But this is not the case due to the time lag that occurs between the presence of common quails in the fields and the opening of the hunting season. Common quails do not distribute homogeneously neither across Spain nor along the breeding season. They show nomadic movements to higher latitudes and altitudes in search of suitable habitat and mates. Rodríguez-Teijeiro et al. (2009) showed that common quails that had bred in the Iberian Peninsula tend to aggregate in the Castilian plateau at the end of the breeding season. This is the last place in Spain where there is available habitat, since due to the cereal phenology, cereal fields have been already harvested in the rest of the country. Although the hunting season occurs on the same dates across Spain, quail population density varies extensively. At that time, there are lots of quails available for hunting in the Castilian plateau and restocking with farm-reared quails does not seem necessary to increase hunting bags there. Instead, at lower latitudes and altitudes there are very few common quails because the majority of them left the area when crops were harvested, and the ones that could arrive from other areas by nomadic movements would not stay there because the habitat is depleted. To compensate for this, a high number of farm-reared quails are restocked for hunting in the next days. Because of this, there is not an important overlap neither in density nor in time between common quails and restocked quails during the hunting season.

However, some overlap between both groups of quails happens during the breeding season. Farm quails can be restocked in intensive hunting grounds before and during the breeding season, so they could directly be recruited into the breeding population. On the other hand, quails restocked after the breeding season (the majority of the released quails) could be part of the breeding population only if they survive the hunting period, a migration to the south (in case they do), the winter weather (wintering most probably in European latitudes) and a second migration back to breeding areas. Because all of these impediments, it could be suggested that restocking is only an almost negligible problem for the breeding common quail population. However, the presence of quails of hybrid ancestry in the breeding population shows that it is a real threat: around 2.5% of the breeding quails have been genetically identified as farm quails. In addition, results show that not only interbreeding between restocked and native quails in the wild is possible, but that it has already happened and has caused introgression of farm alleles into the wild population: quails captured during the breeding season have been genetically identified as admixed individuals born in the wild.

Taking into account that a proportion of the restocked quails becomes part of the breeding population every year and that they can interbreed with wild quails and produce fertile offspring, an increase in the proportion of admixed individuals in the wild population would be expected. However, this increase is not easily detectable by our genetic approaches because it is happening at a low rate. The small rate of introgression could be explained by a reduced fitness for the released quails. Results of this thesis show that farm-reared quails for restocking, both males and females, show the same reproductive potential in the wild than common quails, but they suffer from a higher mortality rate (Table 1). High mortality rate has been observed in other farmed game bird species when released to the wild (Putaala & Hissa 1998, Gortazar et al. 2000, Duarte & Vargas 2004, Casas et al. 2012). It could mainly be associated to their poor adaptation to life in the wild, exemplified by their reduced predator awareness (Guyomarc'h 2003, McPhee 2003). In fact, results show that predation is an important explanation for the low fitness of restocked quails.

Results from this thesis show that restocked individuals present in the field during the breeding season could show the same reproductive potential or more than common quails if they overcome the important filter of a high mortality rate. Farm quail females have higher mortality than common quail females. However, if they survive long enough, they mate and nest with the same probability. They also lay the same number of eggs per clutch and the same proportion of fertilized ones. However, predation over farm-reared females' nests is higher than over common females' nests, what also shows the low predator awareness of farm females. From the nests that are not predated, the same proportion of eggs hatch as in common quail nests. In captivity, chicks from both kinds of females have the same chances of surviving the first three weeks of life, the most critical period for chick survival. However, since during this time chicks are more dependent on their mother's care to control their body head, get undiscovered by predators and finding food, and since females of farm origin have lower survival, one can expect these chicks to also have lower survival under natural conditions (as seen in other Galliformes, like the red-legged partridge (*Alectoris rufa*), Duarte & Vargas 2004). Although mortality for restocked males has not been studied in this thesis, it is likely to also be reduced due to the same lack of anti-predatory instinct. However, this should suppose a less accentuated effect in their fitness than in the case of females, since offspring survival does not depend on them.

Farm-reared hybrid females are more attractive for common quail males than common quail females themselves. That could imply a reduction in the mating opportunities for common females, and a higher proportion of intercrosses between the two lineages. Farm-reared hybrid males, on the other hand, do not attract as many females as common quail male do. Because of that, we would expect that the majority of the heterospecific couples in the field would be constituted by a farm female and a common quail male. That would imply offspring with domestic Japanese quail mtDNA (all farm quails have domestic Japanese quail mtDNA) although they could show nuclear DNA undistinguishable from that of common quails. This introgression could lead to malfunction of gene complexes by cytonuclear incompatibilities (Cruzan & Arnold 1999, Chou & Leu 2010) and a lower fitness.

Results from this thesis have shown that the bond establish for several days between male and female is likely to increase the probability of egg fertilization. The longer the male stays with the female, the higher proportion of eggs he would fertilize. Results show that farm-reared males need less time than common males to fertilize the same proportion of eggs. This implies that in field conditions, farm-reared males would not need to spend as much time guarding the female as common quail males do, and could use this time looking for new mates instead. This could represent a competitive disadvantage for common males.

To have a global perspective on how restocking with farm-reared hybrid quails could affect common quail population, we have to take into account also some ecological and behavioral barriers that could reduce admixture under natural conditions (See Table 1 and Box 1). Some of these barriers have not been studied in this thesis, but could be important for restocked quail survival and fitness:

1) The first and probably most intense source of mortality for released quails is hunting. Although we do not have data about the mortality rate of restocked quails during the hunting season, it seems reasonable to consider that the same lack of anti-predator behavior would favor a higher hunting pressure on hybrids, even on those that could have survived the breeding season. Moreover, growing in captivity causes poorly flying

abilities in quails when released and restocked individuals are usually released just before the opening of the hunting season (or before special hunting events), so they mortality due to hunting is expected to be very high.

2) The increased sedentariness behavior among farm quails could favor higher mortality due to exposure to hard winter conditions of northern latitudes.
3) Farm-reared quails could be more affected by diseases and parasites due to lower immune system response as a result of relaxed selection in captivity (Villanúa et al. 2008).
4) Farm-reared quails, due to their controlled rearing, could have more difficulties on finding appropriate food in the wild (Guyomarc'h 2003).

Although restocked individuals and the offspring of their interbreeding with common quails could show a lower fitness than native individuals, results from this thesis show that introgression of farm (and domestic Japanese) alleles is ongoing. We conducted power tests to assess if the panel of highly polymorphic markers used in this study would be likely to show the progressive arrival of alleles into the native population using traditional analytical approaches. Interestingly, our simulations suggest that such introgression would not be detectable and cautions about hybridization studies that do not include such power tests to assess the reliability of the conclusions. In fact, the simulations clearly illustrate that most analyses, including small panels of markers and samples spanning a short time period, are likely to underestimate introgression when this is moderate or slow.

We used our simulations to estimate sets of realistic parameters compatible with the observed data. Subsequent simulations with these parameters suggest that a gradual increase in the proportion of admixed individuals during the next generations could be expected. Consequently, the enforcement of the current laws banning the release of hybrids and domestic Japanese quails is an urgent need to prevent the further advance of alien alleles.

Event	Effect	Explanation	Study
Hunting	(-)	Due to their low anti-predator behavior and short time for adaptation to the wild before hunting, the proportion of hunted individuals for restocked quails is likely higher than for common quails.	To be studied
Female attractiveness	+	Common quail males are more attracted by game farm females than by common quail females.	2
Male attractiveness	-	Common quail females are less attracted by farm males than by common quail males.	2
Postcopulatory selection	+	Farm males show a higher fertility rate/potential than common quail males.	3
Female survival	-	Farm female survival is lower than common quail one, probably due to lack of adaptation to life in the wild. That affects all the reproductive parameters directly related with females.	2
Female mating success	=	Farm females mating probability is the same as for common quail females.	2
Nesting success	=	Farm females nesting probability is the same as for common quail females.	2
Nest predation	-	Nest from farm females suffer a higher risk of predation than common quail females, probably due to their lower anti-predator instinct.	2
Clutch size	=	Farm females that survive enough to complete the clutch lay the same number of eggs than common quail females.	2
Fertility	=	The proportion of eggs fertilized over the ones laid is the same for clutches of both kinds of females.	2

Hatching probability (in captivity)	=	The proportion of fertilized eggs that hatch is the same for clutches of both kinds of females.	2
Chick survival (in captivity)	= (-)	The proportion of chicks alive after the three first weeks of live in captivity is the same for clutches of both kinds of females. Although it has to be tested, chick survival in the wild is expected to be lower for farm female clutches, due to the high farm female mortality.	2
Crop harvesting and nomadism	(-)	Sedentariness could be accentuated in most hybrid quails. It could affect their movements in search of habitat or mates, potentially reducing their fitness.	To be studied
Migration or wintering in northern latitudes	(-)	Sedentariness could increase the fraction of hybrid quails wintering at northern latitudes. Harsh weather, continued hunt and depleted habitats are likely to reduce their survival.	To be studied
Parasites and diseases	(-)	Parasites and diseases of farm or wild origin are expected to have a strong impact on restocked quails due to their depressed immune system resulting from selection in captivity.	To be studied
Food resources	(-)	Although neither seeds nor insects seem to be scarce in cereal crops, farm-reared quails could have problems to find food resources in the wild because they are used to feed ad-libitum.	To be studied

Table 1. Ecological, reproductive and behavioral events that could have an effect on the fitness of restocked farm quails compared to common quails. Positive effects (+) would be strengthening introgression, negative effects (-) would be containing it and equal degree of effects in farm and common quails (=) would be perpetuating it. Symbols in parentheses correspond to our guess, based on previous knowledge, but have not been tested. Female survival affects all the reproductive parameters for females. On the other hand, nest predation also affects all the parameters related with offspring production. In the last column, number of the paper where the event has been studied.

Recommendations for common quail conservation

Several recommendations for common quail conservation can be derived from this thesis. To sum them up, the main recommendations are:

a) Restocking with admixed farm-reared quails is not an appropriate management tool for common quail populations. All restocking should be banned until assessment of the origin of the birds is appropriately carried out using genetic tools.

b) There is a need for an extensive survey of the diversity in wild quails to assess the impact that these releases may have had across its distribution range.

c) Although the possibility of restocking with pure common quails should be assessed, it is also potentially damaging.

d) Instead of restocking, limiting the number of quails hunted according to the breeding population size would contribute to a more sustainable management.

Box 1. Future research

Some of the events that could affect fitness of restocked quails should be studied to better understand how the introgression process is taking place and how to improve quail conservation. Some of these are already under study by our research team (marked with an asterisk):

- **Survival to hunting***. Probably, hunting has the biggest effect on restocked quail survival. By marking and releasing farm and wild birds and conducting a mark-recapture study with the information provided by hunters it may be possible to estimate the relative survival for birds from both origins. Our hypothesis is that the lack of anti-predator behavior in restocked quails makes them more vulnerable to hunters.
- **Migration or sedentarism***. Genetic analyses of individuals captured at the beginning of the outbound migration in autumn will allow us to assess if admixed quails engage in migration to spend the wintering season in southern latitudes. Analyses of individuals during the inbound migration would tell us the relative survival of farm and common quails to migration and wintering in Africa. If farm origin and admixed quails have lower survival, their proportion should be smaller among samples obtained during the inbound migration.
- **Wintering in northern latitudes***. It has been suggested that the presence of quails during winter could be consequence of their hybrid origin, corresponding to individuals that has lost their migratory drive. Genetic analyses of individuals hunted during the winter in northern latitudes could be used to estimate the proportion of sedentary quails that show genetic admixture.
- **Habitat selection** could have strong consequences on individual survival. Restocked quails could show a lower capacity to find appropriate spots for mating, eating or nesting without being detected by predators.
- **Parasites and diseases.** Wild diseases and parasites could strongly affect restocked individuals, which have a depressed immune system due to captivity.
- **Food resources**. The capability of finding high quality food in the wild could affect the survival of restocked individuals and their chicks. Probably, adaptation to captivity makes it more difficult for farm-reared quails.

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CONCLUSIONS

Ceral crop detail (Punta Blava, Catalonia)
by Ines Sanchez-Donoso

CONCLUSIONS

- Most quails reared in Spanish game farms and sold for game restocking are not pure common quail but have domestic Japanese quail ancestry. The majority of them show a degree of admixture similar to second generation hybrids or backcrosses with common quail.

- Farm-reared hybrid quails and wild common quails can potentially interbreed in the wild. In fact, interbreeding in the wild has already occurred.

- Restocked farm-reared quail females and common quail females can reach similar reproductive success in the wild. They have similar mating probability, nesting success, clutch size and fertility. Egg hatching probability and chick survival in captivity are also similar. In addition, in semi-natural experimental settings, farm-reared hybrid females are more successful at attracting wild common males than common quail females themselves, what may strengthen interspecific interbreeding.

- Postcopulatory sexual selection between farm-reared hybrid males and common quail males could modulate the admixture between these two lineages. Farm males have higher fertilization success than wild males. Consequently, a relatively small number of successful interspecific matings could have a higher impact on the native population than initially suspected by just considering the number of crosses between wild and farm quails.

- Despite their fertilization success, reduced fitness of restocked and admixed individuals, due to their reduced anti-predatory behavior, results in selection against alien alleles. Restocked hybrid females show higher mortality rates in the wild than common quail females, and their nests suffer more predation.

- Genetic data is compatible with a slow rate of ongoing introgression of domestic Japanese quail alleles into the common quail gene pool. Although extensive restocking with farm-reared quails does not yet appear to have had a large impact on the genetic integrity of common quails in Northeast Spain, overall admixture levels may be slowly increasing over time and simulations predict this could result in extensive admixture in the near future if release practices are maintained.

- The genetic impact of introgression on wild populations can be difficult to detect, especially when hybridization occurs over long time periods and at a low rate. In such cases, data collected over a short time period may be insufficient and lead to an underestimation of the degree of admixture. Instead, samples obtained over several generations can help to assess temporal changes in the level of admixture and can offer a long-term view on the introgression of invasive alleles, more meaningful from a conservation and evolutionary point of view. The robust characterization of slow introgression requires the use of long time series to allow tracking the arrival of new alleles into the population.

- Genetic methods used are useful to distinguish the genetic origin of quails. Intensive control and genetic monitoring of individuals for restocking is needed as massive release of farm-reared hybrids represents a severe threat for the long term survival of the common quail.

RESUMEN EN CASTELLANO

Quail’s nest in a cereal crop (Mas Esplugues, Catalonia)
by Ines Sanchez-Donoso

INTRODUCCIÓN GENERAL

Hibridación e introgresión

La hibridación es un proceso por el cual los individuos de dos especies o linajes diferentes se entrecruzan. Es un evento común en la naturaleza, pero sólo cuando los descendientes del entrecruzamiento - llamados híbridos - son fértiles y se reproducen con éxito, la hibridación tiene un impacto en la evolución de la especie.

Cuando estos descendientes se cruzan con una de las especies parentales por retrocruzamiento repetido, se produce un flujo de alelos de una de las especies parentales a la otra. Este proceso se llama introgresión. La introgresión difiere de la hibridación en que en la primera los alelos de una especie se introducen en el acerbo genético de la otra, resultando en una mezcla compleja de alelos de las especies parentales. Puede producirse hibridación incluso cuando los genomas de las dos especies se mantienen perfectamente separados, lo cual ocurre cuando los híbridos no producen descendencia. Solamente si los individuos híbridos sobreviven y se retrocruzan con éxito con las especies parentales, el entrecruzamiento de las dos especies implica introgresión de alelos de una especie a la otra. Así, una introgresión exitosa es un proceso a largo plazo que no siempre ocurre después de un proceso de hibridación. Si no son eliminados por deriva genética o mediante selección purificadora, estos alelos pueden permanecer indefinidamente en la población, modificando la identidad genética de la especie (Rhymer & Simberloff 1996). Este cambio en la composición genética de una población puede ser de gran importancia evolutiva, ya que puede originar nuevas especies o adaptaciones (Arnold 1997). Sin embargo, la hibridación y la introgresión también pueden resultar en la pérdida de oportunidades de apareamientos intraespecíficos y en la disfunción de adaptaciones locales o complejos de genes coadaptados (Frankham et al. 2010).

La hibridación y la introgresión antropogénicas, sin embargo, son por lo general procesos recientes, resultado de la introducción de especies o de la invasión de especies exóticas como consecuencia de actividades humanas, los cuales, muy a menudo, conducen a graves problemas de conservación (Levin et al. 1996, Rhymer & Simberloff 1996). Los alelos alóctonos pueden no ser adaptativos en un nuevo hábitat y reducir la eficacia biológica de los individuos que los presenten. Si la reducción de la densidad poblacional se produce a un ritmo más rápido que la adaptación y evolución de la especie, la introgresión podría llevar a la población a la extinción.

Recuadro 1. Glosario

Hibridación: cruce entre dos especies o linajes diferentes.

Híbrido: descendiente del cruce entre dos especies o linajes diferentes.

Especie parental: especie de la que desciende el híbrido.

Retrocruzamiento: cruce entre un híbrido y una de las especies parentales. También, descendiente de un evento de retrocruzamiento.

Flujo genético: movimiento de alelos entre grupos de individuos.

Acerbo genético: conjunto de todos los genes, o información genética, en una población.

Introgresión: flujo de genes de una especie al acerbo genético de otra mediante el retrocruzamiento reiterado de los híbridos.

Deriva genética: Cambio en las frecuencias alélicas en una población debido al azar en la reproducción.

Eficacia biológica: Contribución genética de un genotipo o de un individuo en las generaciones futuras.

La codorniz común

La codorniz común (*Coturnix coturnix*) (Linnaeus, 1758) es un Galliforme migratoria (Fam. Phasianidae) cuyas poblaciones podrían estar en peligro debido a introgresión antropogénica (Fig. 1).

Figura 1. Macho de codorniz común

Esta es una especie ampliamente distribuida durante la época de cría (febrero - agosto) en toda Europa, norte de África y oeste de Asia (Moreau & Wayre 1968). Al final de ése periodo, la mayoría de individuos migran miles de kilómetros para invernar principalmente al sur del Sahel, en África, y en el subcontinente indio (Guyomarc'h et al. 1998, Rodríguez-Teijeiro et al. 2012). Algunos individuos invernan en el sur de la Península Ibérica (Delibes 1972) y en el norte de Marruecos. Del mismo modo, existen pequeñas poblaciones reproductoras en Sudáfrica, que migran hacia el Norte para pasar el invierno en Angola, Zaire, Namibia y Zambia (BirdLife International 2012a, Del Hoyo et al 1994.) (Figura 2).

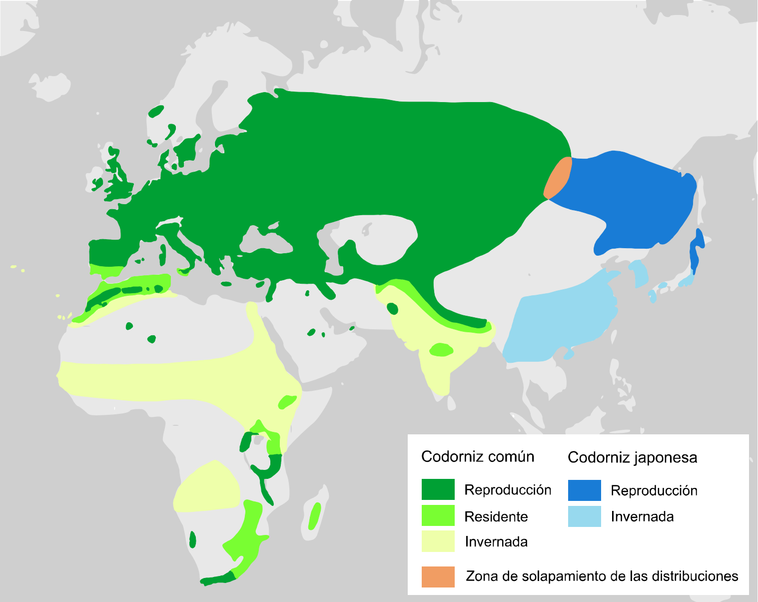


Figura 2. Distribución de la codorniz común y de la codorniz japonesa (adaptado de BirdLife International 2012a y Del Hoyo et al. 1994).

Durante la temporada de cría habita pastizales y tierras de cultivo (principalmente campos de cereales de invierno) (Guyomarc'h 2003). Muestra algunas adaptaciones características asociadas a este hábitat: plumaje críptico de tonos pardos, tiende a caminar sigilosamente entre el cereal para los desplazamientos cortos, y sólo alza el vuelo cuando se siente realmente amenazada.

Su ciclo vital está determinado en gran medida por las limitaciones impuestas por el hábitat, el cual está sometido a fuertes fluctuaciones debido a la cosecha del cereal, que destruye por completo la cobertura vegetal. Cuando esto sucede, comenzando en el sur y avanzando paulatinamente hacia el norte, las codornices se mueven en busca de nuevos hábitats disponibles (Rodríguez-Teijeiro et al. 2009).

Es una especie muy móvil, que presenta tres tipos de movimientos: (a) movimientos migratorios de miles de kilómetros de distancia (Guyomarc'h et al 1998), (b) movimientos nomádicos durante la temporada de cría en busca hábitats disponibles (Puigcerver et al. 1989, Rodríguez-Teijeiro et al. 2009), (c) y movimientos en busca de apareamientos dentro de un mismo parche de hábitat (Rodríguez-Teijeiro et al. 2006). Estudios previos han demostrado que en la zona de donde provienen los datos de esta, el 95% de la población de machos se renueva en menos de 15 días (Rodríguez-Teijeiro et al. 1992). En consecuencia, esta es una población abierta, con continuas y asincrónicas entradas y salidas de individuos.

Esta especie presenta un complejo sistema de apareamiento. Los machos se agregan en “pseudo-leks” (Sardà-Palomera et al. 2011) y cantan para hacerse detectables por las hembras y otros machos. Las hembras sólo se hacen detectables cuando responden a las llamadas de los machos. Macho y hembra crean un vínculo que dura varios días (Rodríguez-Teijeiro et al. 2003). Durante ese tiempo, a menudo se producen cópulas oportunistas fuera de la pareja, así como cambios de pareja (Rodrigo-Rueda et al. 1997). Se han observado machos satélites alrededor de la pareja en busca de cópulas con hembras apareadas (Sardà-Palomera et al. 2011).

Las hembras son poliándricas y generalmente se aparean con varios machos de forma seriada en el tiempo, tanto en un intento reproductivo como durante toda la temporada de reproducción (Rodríguez-Teijeiro et al. 2003). Los machos son también polígamos y se aparean secuencialmente con varias hembras durante la temporada de cría (Rodríguez-Teijeiro et al. 1992). Después de que el macho guarde a la hembra durante unos días, el vínculo de pareja se rompe. Las hembras permanecen en el área cuidando la puesta, mientras que los machos se mueven hacia el norte en busca de nuevos apareamientos (Puigcerver et al 1984, Rodríguez-Teijeiro et al. 1992). Debido a esta diferencia en el comportamiento y en la movilidad entre los sexos, la proporción de sexos se inclina a favor de los machos a lo largo de un gradiente latitudinal (Schleidt 1983, Hemon et al. 1988, Puigcerver 1990, Rodríguez-Teijeiro et al. 1992). Los nidos se establecen en el suelo, el número promedio de huevos por nidada es de 10,6 (Gallego et al. 1993) y los polluelos son nidífugos. Las hembras pueden poner varias puestas durante una temporada de cría, variando entre 1 y 3 dependiendo de la disponibilidad de hábitat y la latitud (Puigcerver et al. 1997). También pueden hacer puestas de reposición cuando el nido es depredado. La esperanza de vida de la codorniz es de unos 0.8 años (Puigcerver et al. 1992).

Una especie cinegética

Se estima que el tamaño poblacional es globalmente grande, entre 35 y 300 millones de individuos según la Unión Internacional para la Conservación de la Naturaleza (IUCN, BirdLife International 2012a), con importantes fluctuaciones interanuales (Puigcerver et al. 2012). Debido a que la detección visual es difícil, el método de censo más comúnmente utilizado implica la detección de los cantos de los machos durante la época de reproducción cuando son estimulados con un canto de hembra (Gallego et al. 1993). Sin embargo, este sistema está lejos de ser perfecto y no es posible realizar estimas del tamaño poblacional con pequeños intervalos de confianza. Por esta razón, la evaluación de las tendencias de la población y su estado de conservación es motivo de controversia. Según algunos autores (Burfield 2004), la especie ha sufrido un descenso en las últimas décadas y presenta un estado de conservación desfavorable. Sin embargo, otros investigadores consideran que su estado de conservación es "de menor preocupación" (BirdLife International 2014). En cualquier caso, se trata de una especie cinegética muy popular, con una larga tradición e importante impacto socioeconómico en los países del Mediterráneo y Oriente Próximo, donde millones de codornices son cazadas anualmente. Sólo en España, donde se encuentra la población reproductiva más grande de Europa Occidental (Rodríguez-Teijeiro et al. 2006), más de 1,3 millones de individuos fueron cazados anualmente durante los años 1973-2010 (Anuarios de Estadística Agroalimentaria del Ministerio Español de Agricultura, Pesca y Alimentación).

Debido al interés cinegético de esta especie y con el objetivo de aumentar el número de individuos para su caza, las poblaciones en muchas de esas zonas de caza (como en Italia, Grecia, la República de Serbia, Montenegro, Rumanía y España) son incrementadas anualmente mediante la suelta de miles de codornices criadas en granjas (Puigcerver et al. 2007, Rodríguez-Teijeiro et al. 1993, Tsiompanoudis et al. 2011). Por ejemplo, en Cataluña (noreste de España), de donde provienen los datos de esta tesis, más de 68.000 codornices criadas en granja fueron liberadas anualmente durante el período 1990-2006 (comunicación personal del Departamento de Agricultura, Ganadería, Pesca, Alimentación y Medio Ambiente de la Generalitat de Catalunya), mientras que la población reproductora silvestre se estima que es de aproximadamente entre 5.000 y 21.000 machos (Rodríguez-Teijeiro et al. 2004). Al menos en esta zona, que podría ser representativa de la situación en muchas otras, el número de codornices soltadas es varias veces más grande que el tamaño de la población reproductora.

La codorniz japonesa

La codorniz japonesa (*Coturnix japonica*, Temminck & Schlegel, 1849) es una especie cercana a la codorniz común. También es un ave migratoria con fenotipo y requisitos de hábitat muy similares a los de la codorniz común (Taka-Tsukasa, 1941).

La codorniz japonesa se distribuye en el este de Asia, incluyendo Japón (Johnsgard 1988). Las distribuciones naturales de la codorniz común y de la japonesa sólo se solapan en la zona del lago Baikal en Rusia (Fefelov 1998), y en la región de Kentei en Mongolia (Kozlova, 1932) (Fig. 2). Aunque esta superposición se produce durante el período reproductivo no se conoce una extensa hibridación natural (Del Hoyo et al. 1994, Guyomarc'h et al. 1998, Moreau & Wayre 1968), a pesar de cierta evidencia anecdótica (Barilani et al. 2005).

Durante varios siglos, la codorniz japonesa ha sido domesticada mediante cría en cautividad y seleccionada para la producción y comercialización de carne y de huevos (Yamashina 1961) (Figura 3). La codorniz japonesa doméstica fue introducida por primera vez Europa (Francia e Italia) en los años 1950 (Rizzoni & Lucchetti 1957, 1972). Hoy en día se cría comúnmente en cautividad en toda Europa como ave de corral. Ha sido seleccionada artificialmente para mostrar, entre otras características, un reducido impulso migrador (Derégnaucourt et al. 2005b). Como consecuencia de la selección para la vida en cautividad, también muestra dificultades para moverse y volar, así como un disminuido instinto anti-predatorio.

Figura 3. Hembra de codorniz japonesa doméstica

Sospechas sobre la identidad de las codornices soltadas

La cría en cautividad de codorniz común es difícil debido a su inquietud motora (Caballero de la Calle & Peña Montañés 1997) y debido a que la captura de hembras es muy poco probable (los machos son fácilmente atraídos por la llamada de la hembra, pero las hembras son difíciles de atraer). Esto ha dado lugar a ciertas sospechas sobre el origen genético de las codornices criadas en granjas para las sueltas cinegéticas. Una posible explicación del gran éxito reproductor en las granjas es que las codornices podrían ser de origen híbrido, resultado de cruces entre codornices comunes silvestres y codornices japonesas domésticas.

Dado que la reproducción en cautividad representa una relajación general de las fuerzas selectivas naturales y una adaptación a unas condiciones de vida muy particulares, las codornices japonesas domésticas podrían tener conjuntos de alelos seleccionados favorablemente para la cautividad pero no adaptativos en el medio silvestre. Estudios en cautividad han demostrado que los fenotipos migratorios no son comunes entre las codornices híbridas (Derégnaucourt et al. 2005a, b), las cuales han heredado la reducida inquietud migradora de la codorniz japonesa doméstica (Derégnaucourt 2000, en Barilani et al. 2005). Este es un punto clave para la conservación de la codorniz común: si los individuos soltados se entrecruzan en la naturaleza con codornices comunes, la población nativa podría perder gradualmente la movilidad necesaria para superar los cambios que se producen en el hábitat. El sedentarismo podría afectar drásticamente la viabilidad de codorniz común, conduciendo a una disminución de la fracción de codornices migradoras y a una disminución de la densidad de población (Guyomarc'h 2003). Los individuos sedentarios pasarían el invierno en un hábitat destruido (cultivos de cereales cosechados), con recursos alimentarios reducidos, donde el riesgo de depredación probablemente sería más alto, y sufrirían condiciones climáticas adversas. Es de suponer que ello conduciría a una mayor tasa de mortalidad (Rodríguez-Teijeiro et al. 1992, Guyomarc'h 2003). Los individuos probablemente también cambiarían su comportamiento nómada en busca de apareamientos y hábitat disponible, lo que podría afectar su eficacia biológica. Incluso en el caso que la eficacia biológica de los individuos mezclados no disminuyera, las sueltas masivas de codornices criadas en granja podrían dar lugar a una contra-intuitiva reducción del tamaño efectivo de la población mediante efectos de Ryman-Laikre: una gran parte de la población reproductora potencialmente podría derivar de un pequeño número de individuos reproductores de granja, lo que resultaría en una reducción de la diversidad genética y del potencial evolutivo (Ryman & Laikre 1991). Incluso si los individuos soltados no mostraran un comportamiento migratorio reducido, se esperaría un cambio en el acerbo genético de la codorniz común, con consecuencias imprevisibles.

Al menos en teoría, la hibridación entre la codorniz común y la de granja es probable que conduzca a un rápido incremento en la proporción de híbridos y de individuos con distinto grado de mezcla en las poblaciones silvestres. Una vez la hibridación ha comenzado, es difícil detenerla si los híbridos son fértiles y se aparean tanto con otros híbridos como con las dos especies parentales (Allendorf et al. 2001). En pocas generaciones, este proceso podría resultar en una situación en la que casi todos los individuos fueran de origen híbrido (Allendorf et al. 2001, Huxel 1999).

Legislación

Como respuesta a esta situación de riesgo, el Plan de Gestión de la Unión Europea para la codorniz común (período 2009-2011) ha insistido en la necesidad de prohibir las sueltas de codorniz japonesa o híbridos en toda Europa y en el resto de la distribución de la codorniz común (Perennou 2009). Varios países y regiones de Europa ya han prohibido o regulado las sueltas cinegéticas, haciendo ilegal la suelta de codornices japonesas y híbridos (Portugal y Francia desde 2002, Grecia desde 2007). En Italia, la codorniz japonesa se considera una especie introducida (BirdLife International 2012b). En España, el país donde el estudio se ha llevado a cabo, la codorniz japonesa también se considera en la actualidad una especie introducida (español Real Decreto 630/2013). En este mismo país, la Ley 42/2007 sobre Patrimonio Natural y Biodiversidad permite las sueltas de individuos de la especie autóctona, pero no de codorniz japonesa ni híbridos; ningún reglamento especial había sido aplicado antes de la aprobación de esta ley.

Si bien el marco legal actual parece apropiado para evitar la introgresión de alelos no adaptativos, su ejecución actual no es suficiente. Muchos individuos criados en granja están siendo todavía soltados asumiendo que son codornices comunes puras. Los granjeros las venden como codornices comunes amparándose en un certificado veterinario que sólo se basa en su morfología para diagnosticar su identidad. Pero el diagnóstico en base al fenotipo no permite diferenciar inequívocamente entre codornices comunes puras y individuos mezclados, ya que son muy similares, incluso indistinguibles en algunos casos (Derégnaucourt 2000, en Barilani et al. 2005). La correcta evaluación de la identidad de los individuos de granja es fundamental para el diseño de planes de conservación y gestión de la codorniz común apropiados. Los análisis genéticos son útiles para este propósito.

Las sueltas cinegéticas: una amenaza real

Varios autores ya han advertido que la suelta cinegéticas de codorniz japonesa doméstica e híbridos puede suponer una grave amenaza para la integridad genética y la supervivencia de la codorniz común (Guyomarc'h 2003, Barilani et al. 2005, Chazara et al. 2006, 2010, Puigcerver et al. 2007). Ello puede no ser sólo una amenaza teórica. Por un lado, en Europa se han detectado individuos híbridos en el medio natural (Guyomarc'h 2003, Rodríguez-Teijeiro et al. 1993). Sin embargo, estudios anteriores (Puigcerver et al. 2007, 2013) han demostrado que la proporción de codornices identificadas como híbridas en zonas cercanas a la estudiada en esta tesis (en Cataluña) no ha aumentado significativamente en un período de 20 años desde la primera detección de híbridos en 1990 (Figura 4). En estos estudios, éstos fueron identificados por el canto de apareamiento de los machos, el cual puede conducir a subestimar el número de individuos mezclados (Derégnaucourt et al. 2001), por lo que no es un método fiable para el censo de híbridos. Por otro lado, algunos estudios han demostrado que, en cautividad, no existen barreras pre- ni post-cigóticas que impidan el cruce entre codornices comunes y japonesas domésticas o retrocruzamientos de híbridos con codornices comunes (Taka-Tsukasa 1941, Derégnaucourt et al. 2002, Derégnaucourt & Guyomarc'h 2003). Sin embargo, se sabe muy poco sobre el destino de las codornices soltadas y su impacto en las poblaciones nativas.

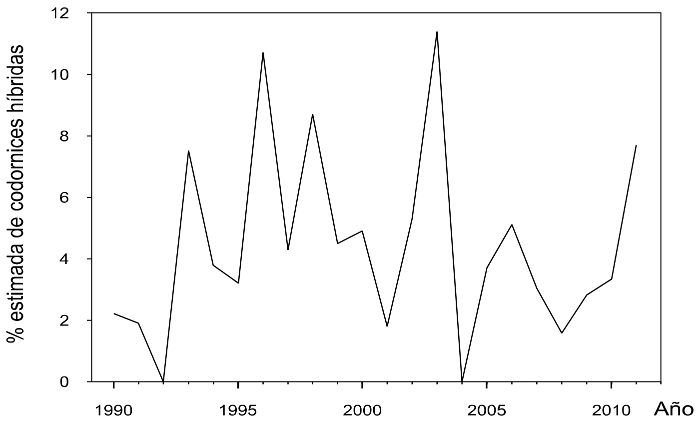


Figura 4. Estimación del porcentaje de codornices híbridas detectadas durante el período 1990-2011 en tres zonas de Cataluña (NE España) (adaptado de Puigcerver et al. 2013).

Los marcadores genéticos

Dado que el fenotipo de las codornices comunes puras no es claramente diferenciable del de los individuos híbridos (con un amplio grado de mezcla genética), los análisis genéticos parecen ser el único método para caracterizar la identidad de los individuos de manera fiable. El estudio de la genética de poblaciones ha resultado muy útil para comprender mejor la ecología y el comportamiento de las especies silvestres, así como para resolver problemas de conservación. Las herramientas genéticas se han utilizado con distintos objetivos, como la caracterización de la estructura poblacional (Latch et al. 2011), la identificación de individuos migrantes (Reddy et al. 2012), el estudio de los sistemas de apareamiento mediante análisis de paternidad (Huck et al. 2014) o la detección de individuos híbridos (Barbanera et al. 2011, Mirimin et al. 2014).

Uno de los métodos más comúnmente utilizados en la actualidad para caracterizar la estructura genética de las poblaciones es el genotipado de individuos mediante microsatélites (Allendorf et al. 2013). Los microsatélites son marcadores moleculares situados en el ADN nuclear consistentes en secuencias de nucleótidos que contienen repeticiones en tándem (Ellegren 2004). El número de repeticiones es generalmente muy fácil de caracterizar y tiende a ser altamente polimórfico, por lo que diferentes alelos pueden observarse en una misma población, proporcionando una poderosa herramienta para la identificación individual. Varios microsatélites (por lo general entre 8 y 25) son caracterizados en cada individuo, proporcionando su genotipo: un código de identificación individual constituido por la combinación de diferentes alelos en cada uno de los microsatélites. Los microsatélites más comúnmente utilizados no codifican ninguna proteína y tienen herencia simple (en organismos diploides, un alelo se hereda del padre y el otro de la madre). Todas estas características los hacen muy útiles para los estudios de genética de poblaciones.

Los alelos presentes exclusivamente en una especie son los más útiles para la identificación de individuos híbridos. La detección únicamente de estos alelos en un individuo sería una prueba inequívoca de la pertenencia a ésa especie, mientras que los híbridos y los individuos con introgresión tendrían alelos de varios orígenes. Sin embargo, cuando se comparan especies estrechamente relacionadas, como son la codorniz común y la codorniz japonesa, lo más habitual es observar que los alelos en un locus (un cierto lugar en el genoma) sólo difieren en sus frecuencias relativas. En este caso, los grupos de individuos (poblaciones o especies) se caracterizan por la probabilidad de encontrar un determinado genotipo en cada uno de los grupos. Por lo tanto, el estudio de frecuencias alélicas mediante el análisis de un gran número de individuos permite detectar la estructura poblacional. Procedimientos bayesianos son utilizados para ello (Vähä & Primmer 2006). Mediante el análisis de una muestra de genotipos y de las frecuencias alélicas en ésa muestra, algunos procedimientos, como los implementados en el programa STRUCTURE (Pritchard et al. 2000), permiten inferir la proporción del genotipo de cada individuo perteneciente a un grupo genético. Basándose en la variabilidad genética de la muestra analizada, este software agrega los individuos en grupos basados en su similitud genética. Otros métodos bayesianos, como los implementadas en NEWHYBRIDS (Anderson & Thompson 2002), permiten calcular la probabilidad individual de pertenencia a una categoría u otra en un suceso de hibridación (especie pura A, especie pura B, híbrido de primera generación,...).

Sin embargo, el poder de estos análisis se haya limitado al número de microsatélites utilizados y a su variabilidad (Vähä & Primmer 2006). Además, por lo general, la identidad genética asignada es incierta más allá de los híbridos de segunda generación y primer retrocruzamiento, debido a la variabilidad inherente en la composición genética resultante del cruzamiento de individuos de diferente linaje (Figura 5). La fiabilidad de la identificación de las diferentes categorías puede maximizarse aumentando la cantidad de marcadores genéticos no ligados utilizados y su variabilidad.

La asignación de paternidades también es posible mediante el análisis de microsatélites (Xue et al. 2014, Li et al. 2014). Básicamente, se calcula la probabilidad para cada genotipo de ser el progenitor de otro genotipo observando los alelos que comparten, después de corregir por la frecuencia de estos alelos en toda la población (un alelo podría ser compartido entre los dos individuos no por estar emparentados sino porque ése alelo es muy común en la población). Programas informáticos implementados para la asignación de paternidades, como CERVUS (Kalinowski et al. 2007), comparan pares de genotipos asignados como descendiente y padre putativo, y establecen su probabilidad con un cierto nivel de confianza.

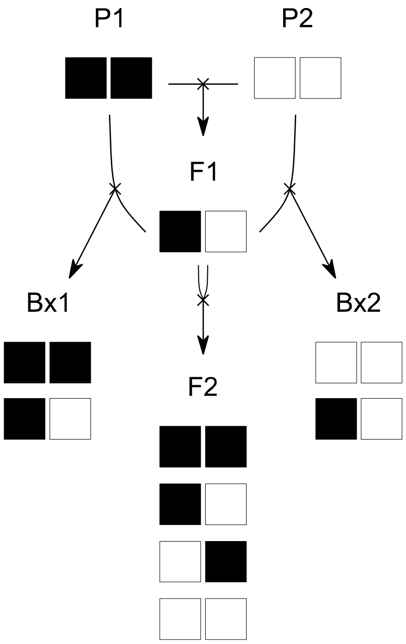


Figura 5. Genotipos esperados en un locus como resultado del cruzamiento de dos especies distintas. P1: especie parental 1; P2: especie parental 2; F1: híbrido de primera generación; F2: híbrido de segunda generación; Bx1: retrocruzamiento con P1; Bx2: retrocruzamiento con P2. Los cuadrados blancos y negros representan alelos procedentes de las dos especies parentales. Un genotipo está constituido por la combinación de dos cuadrados. Mientras los híbridos F1 tienen un alelo de cada especie parental en todos los loci, los retrocruzamientos tienen los dos alelos de una de las especies parentales o un alelo de cada una, pero nunca dos alelos de las dos especies parentales. Cruces más allá de este punto (como F2) podrían resultar en cualquier combinación de alelos en cada locus, por lo que la clasificación de ésos individuos en una categoría u otra es mucho más difícil y depende de la probabilidad de cada combinación de alelos en cada locus. La información de varios microsatélites puede aumentar el poder para determinar la categoría a la que un individuo pertenece.

Otro marcador molecular comúnmente utilizado en los estudios de genética de poblaciones es el ADN mitocondrial. Este marcador presenta algunas características que lo hacen fácil de amplificar y estudiar: es de herencia materna, no sufre recombinación y existen muchas copias por célula (algunas veces miles de copias). Estas características lo han convertido en el marcador elegido tradicionalmente para estudios de filogenia y filogeografía (Avise 2000). Dado que este marcador es uniparental (se hereda sólo de un progenitor), puede ser útil para identificar la direccionalidad en los apareamientos híbridos. Si los linajes de ADN mitocondrial están claramente separados en dos especies, su análisis en un híbrido informará acerca de la especie de la madre de este individuo (por ejemplo, véase Muñoz-Fuentes et al. 2007).

Recuadro 2. Acontecimientos en el ciclo de vida de la población de codorniz común sometida a sueltas de codornices de granja para fines cinegéticos

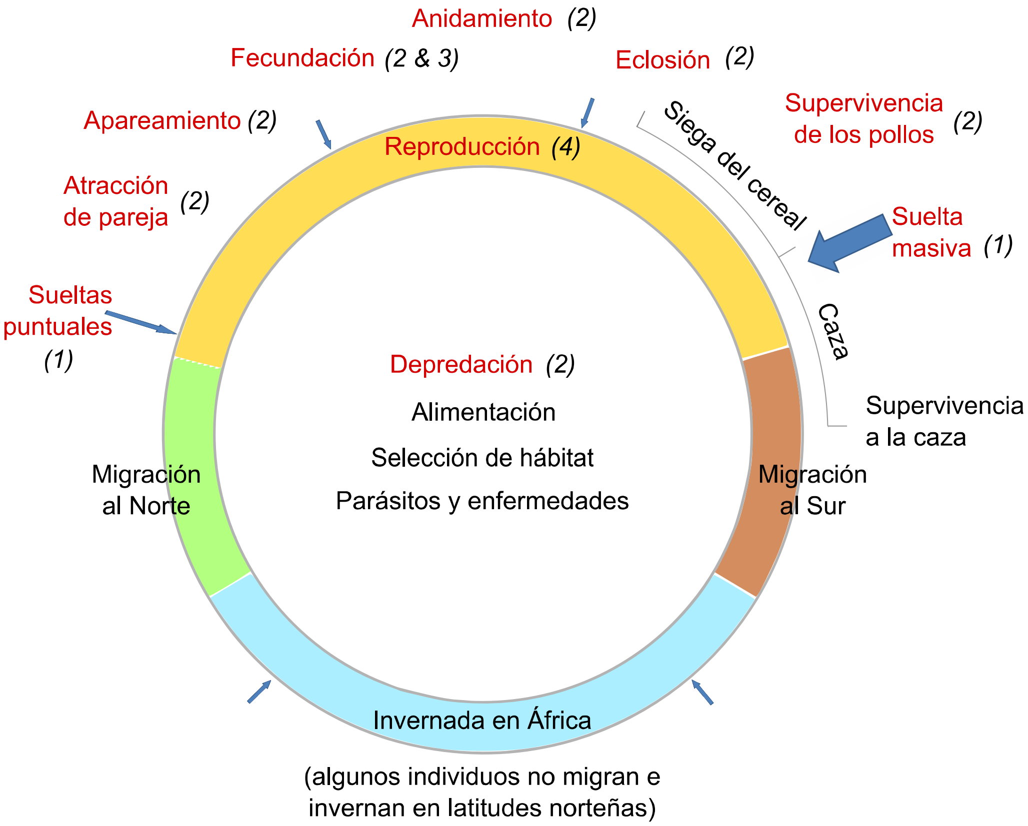
Durante su ciclo de vida, y en las zonas donde se sueltan codornices de granja, las codornices comunes tienen que superar varios acontecimientos que modulan su eficacia biológica. En esta tesis, se estudia cómo algunos de los acontecimientos que se producen durante la temporada de cría están afectando a la eficacia biológica relativa de los dos tipos de codornices. Aunque la fecha exacta de estos acontecimientos depende de la fenología local del hábitat y de los movimientos de la codorniz común (tiendo lugar más tardíamente en lugares de mayor latitud y altitud), ocurren en el mismo orden en todas la poblaciones.

Después de invernar en latitudes más bajas, las codornices llegan gradualmente a Europa y comienzan la búsqueda de apareamientos. El complejo sistema de apareamiento de la codorniz incluye varios momentos claves donde la coexistencia de codornices comunes con codornices soltadas de granja podría afectar su eficacia biológica relativa: la atracción de la pareja, la creación del vínculo de pareja, el apareamiento y la fecundación de los óvulos.

Cuando las hembras comienzan a incubar, el vínculo de pareja ya se ha roto y los machos se han ido en busca de nuevos apareamientos en la misma o diferente área (Rodrigo-Rueda et al. 1997). No todos los óvulos son fecundados, ni todos ellos se desarrollan en embriones que eclosionan, pero la eclosión de los huevos se produce casi de forma sincrónica en una misma puesta (en un período de 24 horas de tiempo). Los pollos abandonan el nido justo después de la eclosión, pero permanecen cerca de la hembra durante al menos tres semanas: tiempo en que no regulan su temperatura corporal, por lo que dependen de la protección de su madre. Es en estos momentos, hasta que desarrollan su primer plumaje, cuando la supervivencia de los pollos puede ser menor. En poco tiempo, los juveniles se desarrollan lo suficiente para volar perfectamente. Cuando los pollos ya no dependen de ella, la hembra puede realizar un segundo intento de reproducción. A veces esto se precipita debido a la depredación del nido. En otros casos, la cosecha del cereal hace que la hembra tenga que desplazarse a otras zonas en busca de nuevos apareamientos.

En España (donde parte de los datos de esta tesis han sido tomados), la temporada de caza (la media veda) comienza algún tiempo después de la cosecha del cereal. Justo antes y durante la temporada de caza, se lleva a cabo la suelta de miles de codornices criadas en granja con el fin de aumentar el número de individuos para la caza. Hay también cotos intensivos donde se permite la caza y la suelta de codornices durante todo el año. Estas áreas están distribuidas por toda España, pero constituyen una pequeña parte del total. El origen genético de los individuos soltados para la caza es uno de los puntos clave para la conservación y gestión de las poblaciones de codorniz común que se tratan en esta tesis.

Durante la época de reproducción, las codornices también están bajo la presión de la depredación, la búsqueda de alimentos y la disponibilidad de hábitat (Guyomarc'h 2003), así como de parásitos y enfermedades, que modulan su supervivencia y eficacia biológica. Después de la temporada de cría, la mayoría de las codornices, incluidos los individuos nacidos ése año, migran de vuelta a África para invernar.



Ciclo anual con los principales acontecimientos en la historia de vida de la codorniz común que pueden determinar su eficacia biológica. Estos podrían afectar también a las codornices de granja soltadas con fines cinegéticos, por lo que tendrían un papel importante en la tasa de introgresión de alelos de codorniz japonesa doméstica en la codorniz común. En rojo, los acontecimientos que son objeto de estudio en esta tesis; entre paréntesis, el número del artículo científico en el que se tratan en esta tesis.

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OBJETIVOS

El objetivo principal de esta tesis doctoral es entender los efectos que las sueltas con fines cinegéticos tienen sobre las poblaciones de codorniz común. Así como obtener conocimientos aplicables en mejorar la conservación y gestión de la codorniz común.

Estos objetivos generales se abordan mediante el estudio de **objetivos específicos**:

- Evaluar el origen genético de las codornices criadas en granjas cinegéticas españolas para su posterior suelta. ¿Son codornices comunes puras? (Artículo 1)
- Evaluar el poder de los métodos genéticos utilizados en esta tesis para la identificación de codornices de origen híbrido. (Artículo 1 y Artículo 4).
- Evaluar la eficacia biológica de la codorniz de granja cinegética en comparación con la de la codorniz común:
    - Comprobando si el entrecruzamiento en la naturaleza es posible. (Artículo 2)
    - Estudiando el efecto de diferentes parámetros reproductivos sobre la eficacia biológica de las codornices de suelta en comparación con la de codornices comunes. (Artículo 2)
    - Estudiando si la selección sexual post-copulatoria entre machos híbridos de granja y machos de codorniz común podría modular la hibridación de estos dos linajes en la naturaleza y tener un efecto en la tasa de introgresión. (Artículo 3)
- Evaluar en qué medida las sueltas de codornices de granja están afectando a la composición genética de la población nativa. ¿Hay introgresión de alelos de codorniz japonesa doméstica en las poblaciones de codorniz común? (Artículo 4)
- Prever cambios en la composición genética de la población local en un futuro próximo bajo diferentes escenarios de introgresión. (Artículo 4).

RESUMEN DE LOS ARTÍCULOS

Harvesting (Figuerola del Camp, Catalonia)
by Ines Sanchez-Donoso

RESUMEN ARTÍCULO 1

Son las codornices criadas en granja para repoblaciones cinegéticas realmente codornices comunes (*Coturnix coturnix*)?: Una aproximación genética.

Sanchez-Donoso I, Vilà C, Puigcerver M, Butkauskas D, Caballero de la Calle JR, Morales-Rodríguez PA, Rodríguez-Teijeiro JD (2012) Are Farm-Reared Quails for Game Restocking Really Common Quails (*Coturnix coturnix*)?: A Genetic Approach. *PLoS ONE* 7 (6): e39031.

La codorniz común (*Coturnix coturnix*) es una popular especie cinegética. La suelta de individuos criados en granjas para su caza es una práctica común. En algunas áreas, el número de codornices soltadas supera ampliamente el número de codornices nativas reproductoras. Sin embargo, la cría de codorniz común en cautividad es difícil, lo cual hace sospechar sobre un posible origen híbrido de los individuos criados en granja, los cuales procederían de cruces entre codornices comunes y codornices japonesas domésticas (*C. japonica*).

En este estudio se utilizaron marcadores microsatélites autosómicos para caracterizar el origen genético de las codornices criadas en granjas españolas para fines cinegéticos, y el de codornices de una granja cinegética experimental fundada a partir de individuos híbridos, los cuales han sido retrocruzados sistemáticamente con codornices comunes silvestres. Los genotipos de las codornices de granja cinegética fueron comparados con los de codornices comunes silvestres y codornices japonesas domésticas.

Nuestros resultados muestran que más del 85% de las codornices de granja cinegética no eran codornices comunes puras sino individuos híbridos con distintos grados de mezcla, así como codornices japonesas domésticas. En la granja experimental la proporción de codornices con genotipos similares a los de codorniz común fue mayor.

Llegamos a la conclusión de que la mayoría de las codornices criadas en granjas españolas y vendidas para sueltas cinegéticas no eran codornices comunes.

El seguimiento genético de los individuos criados para sueltas cinegéticas es indispensable, ya que la liberación masiva de híbridos de granja podría representar una grave amenaza para la supervivencia a largo plazo de la especie nativa.

RESUMEN ARTÍCULO 2

La reducida eficacia biológica de las codornices híbridas soltadas previene una rápida mezcla genética con las codornices comunes

Puigcerver M, **Sanchez-Donoso I**, Vilà C, Sardà-Palomera F, García-Galea E, Rodríguez-Teijeiro JD (2014) Decreased fitness of restocked hybrid quails prevents fast admixture with wild common quails. *Biological Conservation* 171: 74-81.

Las sueltas con fines cinegéticos de individuos de especies no nativas es una práctica muy habitual en algunas especies de Galliformes, aunque puedan resultar en la introgresión de alelos no adaptativos en las poblaciones nativas. Las codornices de granja cinegética son el resultado del cruzamiento de codornices japonesas domésticas (*Coturnix japonica*) con codornices comunes (*Coturnix coturnix*). Por ello, las sueltas masivas de estos individuos podrían representar una amenaza para la codorniz común.
En este estudio se siguió por radiotelemetría un total de 16 hembras de codorniz común y 51 hembras criadas en granja cinegética durante cuatro temporadas reproductoras en una sola localidad.

Nuestros resultados muestran que las hembras híbridas de granja atrajeron más machos salvajes de codorniz común que las propias hembras comunes, probablemente debido a que las primeras produjeron más llamadas de apareamiento.

En este estudio se muestra, por primera vez, la evidencia empírica de que las codornices comunes y las codornices de granja pueden aparearse en condiciones silvestres. Además, las hembras híbridas de granja y las hembras comunes mostraron similar probabilidad de apareamiento, éxito de anidación, tamaño de puesta, fecundidad, probabilidad de eclosión y probabilidad de supervivencia de los polluelos. Sin embargo, las hembras de granja tuvieron mayor tasa de mortalidad que las hembras de codorniz común, y sus nidos sufrieron una mayor tasa de depredación. Estos últimos resultados podrían explicar por qué los dos linajes no aparecen completamente mezclados en el campo después de más de 20 años de sueltas cinegéticas masivas. Sin embargo, nuestros resultados muestran claramente la falta de barreras reproductivas en condiciones silvestres, por lo que la introgresión sería imposible. Por lo tanto, la prohibición de las sueltas cinegéticas de codorniz japonesa doméstica o de híbridos es indispensable para prevenir la introgresión progresiva de alelos no adaptativos en las poblaciones de codorniz común.

RESUMEN ARTÍCULO 3

La selección sexual post-copulatoria favorece el éxito de fecundación de las codornices híbridas usadas en sueltas cinegéticas sobre el de las codornices comunes nativas

Sanchez-Donoso I, Morales-Rodriguez PA, Puigcerver M, Caballero de la Calle JR, Vilà C, Rodríguez-Teijeiro JD. Postcopulatory sexual selection favors fertilization success of restocking hybrid quails over native common quails. Enviado a *Ibis*.

La selección sexual post-copulatoria juega un importante papel en el éxito reproductor de los machos en muchas especies. Por lo tanto, las diferencias en el éxito de fecundación podrían afectar las tasas de hibridación e introgresión genética entre linajes divergentes.

Investigamos la precedencia del esperma en apareamientos en cautividad entre codornices comunes (*Coturnix coturnix*) y codornices de granja de origen híbrido (*C. coturnix* x codorniz japonesa doméstica, *C. japonica*), las últimas ampliamente utilizadas en prácticas de sueltas cinegéticas para aumentar el número de individuos para caza. Estos apareamientos en condiciones naturales representan una amenaza importante para las poblaciones de codorniz común.

Los resultados mostraron que el éxito de fertilización de cada macho dependía de 1) el tiempo que pasó con la hembra, 2) la presencia de esperma de un macho anterior en el oviducto de la hembra, 3) el tiempo que el macho anterior había estado copulando con la hembra, y, lo más importante, 4) el origen genético del macho (común o híbrido). Los machos híbridos mostraron mayor éxito en la fecundación que los machos comunes, necesitaron permanecer menos tiempo con la hembra para fecundar la misma proporción de huevos. La presencia de esperma alóctono en el oviducto de la hembra redujo el porcentaje de huevos fertilizados por el macho. Sin embargo, esta reducción fue mayor para los machos comunes cuando el macho precedente era un macho híbrido. En resumen, el esperma de los machos híbridos puede ser más competitivo que el esperma de los machos comunes y esto podría favorecer la introgresión de alelos no adaptativos en la población de codorniz común, constituyendo una amenaza grave para la conservación de las poblaciones de codorniz común.

RESUMEN ARTÍCULO 4

Detectando la lenta introgresión de alelos alóctonos en una ave cinegética masivamente repoblada

Sanchez-Donoso I, Huisman J, Echegaray J, Puigcerver M, Rodríguez-Teijeiro JD, Hailer F, Vilà C (2014) Detecting slow introgression of invasive alleles in an extensively restocked game bird. *Frontiers in Ecology and Evolution* 2: 00015.

El cruce de dos especies en la naturaleza implica la introgresión alélica de una especie en el acerbo genético de la otra sólo cuando los individuos híbridos resultantes sobreviven y se retrocruzan con las especies parentales. En consecuencia, la estimación de la proporción de individuos híbridos de primera generación en una población no resulta informativa sobre la evolución del proceso de hibridación. Las muestras obtenidas en un período largo de tiempo pueden ofrecer una visión más precisa de la propagación de la introgresión alélica en el acerbo genético de una especie. La población de codorniz común (*Coturnix coturnix*) en Europa ha sido extensamente repoblada con codornices de granja de origen híbrido (descendientes de cruces con codornices japonesas domésticas, *C. japonica*). Se identificó genéticamente una población de codorniz común durante más de 15 años para investigar si se está produciendo introgresión genética y se usaron simulaciones para investigar nuestro alcance para detectarla.

Nuestros resultados revelaron que se ha producido introgresión, pero no se observó un aumento significativo de la proporción de individuos mezclados a lo largo del tiempo. Sin embargo, las simulaciones mostraron que el grado de mezcla puede ser mayor de lo previsto debido a la limitada potencia de los análisis sobre datos tomados en un corto lapso de tiempo, y a que los datos observados eran compatibles con una baja tasa de introgresión genética, probablemente como resultado de la reducción de la eficacia biológica de los individuos mezclados. Las simulaciones predicen que esto podría dar lugar a una situación de gran introgresión en un futuro próximo.

DISCUSIÓN GENERAL

Field crops (Alp, Catalonia)
by Ines Sanchez-Donoso

DISCUSIÓN GENERAL

La introducción de individuos criados en cautividad en una población silvestre es una estrategia comúnmente utilizada para la conservación y gestión de distintas especies. Estas introducciones pueden tener como objetivo, por ejemplo, reforzar las poblaciones en peligro de extinción (Massemin-Challet et al. 2006, Armstrong & Seddon 2008, Santos et al. 2009) o aumentar la pesca y la caza con el fin de aumentar la productividad o mantener actividades recreativas (Blanco -Aguiar et al. 2008, Barbanera et al. 2010).

La introducción de individuos es una estrategia de gestión fundamental para la conservación de las especies cuando su supervivencia está en grave peligro. Este es el caso del lince ibérico (*Lynx pardinus*), por ejemplo, en el que la introducción de ejemplares nacidos y criados en cautividad se usa para reforzar las poblaciones existentes y mantener su empobrecida diversidad genética (Vargas et al. 2009). Sin embargo, para la conservación de especies en una situación de riesgo menor, otras acciones de gestión podrían ser más eficaces que las introducciones, tales como la conservación del hábitat y la prohibición de las actividades humanas que supongan una amenaza, como el envenenamiento, la sobrepesca o la caza. En el caso de la codorniz común, con un gran tamaño poblacional, la introducción de individuos no pretende ser una herramienta de conservación, sino únicamente un modo de aumentar artificialmente el número de individuos disponibles para cazar.

Aunque la introducción de individuos puede tener efectos positivos para la conservación de las poblaciones silvestres, también implica varias posibles amenazas (Laikre et al. 2010, Marie et al. 2010):

a) **Parásitos y enfermedades**. La introducción de individuos criados en granjas constituye un punto de entrada de parásitos y enfermedades que podrían causar una importante mortalidad en las poblaciones silvestres (Peeler et al. 2006).

b) **Bajo tamaño efectivo de la población**. Los individuos introducidos descienden normalmente de un reducido número de progenitores, lo cual supone una baja diversidad genética. Su entrecruzamiento con individuos silvestres podría resultar en una reducción del tamaño efectivo de la población y del potencial evolutivo mediante pérdida de diversidad genética (Ryman & Laikre 1991), alterando profundamente la composición genética de las poblaciones nativas (Ford 2002) y limitando su potencial de crecimiento poblacional.

c) **La domesticación**. La cría en cautividad y la domesticación suponen una relajación general de las fuerzas selectivas y promueven rasgos que no son adaptativos en libertad (Lynch & O'Hely 2001, Frankham 2008, Baskett & Waples 2013). El cruzamiento de animales de granja con individuos silvestres podría causar la introgresión de alelos no adaptativos en la población nativa, reduciendo su potencial de adaptación y supervivencia, lo que conduciría a un declive poblacional.

d) **Especies distintas**. Además, si los individuos introducidos no pertenecen a la misma especie que los silvestres, la estructura genética de la especie nativa se vería afectada y las adaptaciones locales también podrían verse afectadas (Mank et al. 2004, Eldridge & Naish 2007).

Los resultados de esta tesis han demostrado que las sueltas cinegéticas de codorniz conllevan, para la codorniz común, las tres últimas amenazas expuestas (la primera no ha sido estudiada en esta tesis, pero es probable que también suceda) ya que los individuos soltados con finalidades cinegéticas en España son codornices híbridas criadas en granjas que presentan una baja diversidad genética. El grado de mezcla de estos individuos es variable, siendo la mayoría de ellos híbridos de segunda generación (por lo menos).

De acuerdo con nuestros resultados, las codornices de granja muestran una baja diversidad genética. No se han detectado diferencias entre las codornices de las distintas granjas cinegéticas españolas analizadas, lo que apunta a un pequeño número de progenitores. Ello está de acuerdo con un sondeo realizado de las granjas españolas (Sánchez García-Abad et al. 2009), el cual muestra que muy pocas granjas producen codornices, mientras que la mayoría sólo las crían hasta venderlas. Por lo tanto, la baja diversidad genética de estos individuos soltados podría provocar una reducción del tamaño efectivo de la población de codorniz común. Además, debido a que las codornices e granja muestran adaptaciones a la vida en cautividad, su suelta podría conducir a la introgresión de alelos no adaptativos en las poblaciones de codorniz común.

Es posible llegar a pensar que cazar codornices de granja introducidas en vez de codorniz común podría actuar como un parapeto para esta última, ya que una codorniz de granja cazada supondría una codorniz común cazada menos. Pero este no es el caso debido al desfase temporal y físico que se produce entre que las codornices comunes están en los campos y la apertura de la temporada de caza. La codorniz común no se distribuye de forma homogénea, ni en toda España, ni a lo largo de la temporada reproductiva. Muestra movimientos nómadas en busca de hábitat disponible y de apareamientos hacia zonas de mayor latitud y altitud. Rodríguez-Teijeiro et al. (2009) demostraron que las codornices comunes que crían en la Península Ibérica tienden a agregarse en la meseta castellana al final de la temporada de cría. Este es el último lugar en España donde hay hábitat disponible, ya que debido a la fenología del cereal, los cultivos ya han sido cosechados en el resto del país. Aunque la temporada de caza ocurre en las mismas fechas en toda España, la densidad de codorniz varía ampliamente. En ese momento, hay muchas codornices disponibles para la caza en la meseta castellana, por lo que la suelta de codornices de granja no parece necesaria. En cambio, en latitudes y altitudes más bajas en ése momento hay muy pocas codornices comunes porque la mayoría de ellas abandonó la zona cuando se cosecharon los cultivos, y los que pudieran llegar de otras áreas no se quedaría allí porque el hábitat es inexistente. Para compensar esta situación, se suelta un gran número de codornices de granja para ser cazadas en los días siguientes. Debido a esto, no se produce un solapamiento importante durante la época de caza ni en la densidad ni en el tiempo entre codornices comunes y codornices de granja.

Sin embargo, cierta superposición entre los dos grupos de codornices sucede durante la época de reproducción. Las codornices de granja pueden ser soltadas en cotos intensivos antes y durante la época de reproducción, por lo que podrían ser reclutadas directamente en la población reproductora. Por otra parte, las codornices soltadas después de la temporada de reproducción (la mayoría de las codornices liberadas) podrían formar parte de la población reproductora sólo si sobreviven el período de caza, una migración hacia el sur (en caso de que la hagan), el clima de invierno (muy probablemente en latitudes europeas) y una segunda migración de regreso a las zonas de reproducción. Debido a todos estos obstáculos, se podría sugerir que las sueltas cinegéticas suponen un problema casi insignificante para la población de codorniz común. Sin embargo, la presencia de codornices híbridas entre la población reproductora muestra que se trata de una amenaza real: alrededor del 2,5% de las codornices en época de cría han sido genéticamente identificadas como codornices de granja. Además, los resultados muestran que no sólo entrecruzamiento en el campo entre codornices introducidas y nativas es posible, sino que ya ha ocurrido y ha causado la introgresión de alelos de granja en la población silvestre: codornices capturadas durante la temporada de cría se han identificado genéticamente como individuos híbridos nacidos en libertad.

Teniendo en cuenta que una parte de las codornices soltadas se convierte en parte de la población reproductora cada año y que pueden cruzarse con codornices salvajes y producir descendencia fértil, esperaríamos un aumento en la proporción de codornices con cierto grado de hibridación en la población silvestre. Sin embargo, este aumento no es fácilmente detectable mediante los métodos comúnmente usados ya que está ocurriendo a baja intensidad. Esta baja tasa de introgresión podría explicarse por una eficacia biológica reducida de las codornices soltadas. Los resultados de esta tesis muestran que las codornices de granja cinegética, tanto machos como hembras, presentan el mismo potencial reproductor en libertad que las codornices comunes, pero sufren una mayor mortalidad (Tabla 1). Esta alta tasa de mortalidad se ha observado también en otras aves de granja cuando se liberan para fines cinegéticos en el medio natural (Putaala y Hissa 1998, Gortázar et al. 2000, Duarte y Vargas 2004, Casas et al. 2012). Ello podría estar asociado a su pobre adaptación a la vida en libertad, ejemplificada por su reducido comportamiento anti-predatorio (Guyomarc'h 2003, McPhee 2003). De hecho, los resultados muestran que la depredación es uno de los motivos por los cuales las codornices soltadas de granja tienen una menor eficacia biológica.

Los resultados de esta tesis muestran que los individuos de granja soltados al campo y presentes en él durante la época reproductora pueden tener el mismo o incluso mayor potencial reproductivo que las codornices comunes si pueden superar el importante filtro de su elevada mortalidad. Las hembras de codorniz de granja sufren mayor mortalidad que las hembras de codorniz común. Sin embargo, si sobreviven el tiempo suficiente, pueden aparearse y anidar con la misma probabilidad. También tienen el mismo número de huevos por puesta y la misma proporción de ellos son fecundados. Sin embargo, la depredación sobre sus nidos es mayor que sobre los de hembras de codorniz común, lo que también muestra el poco instinto anti-predatorio de las hembras de granja. De aquellos nidos que no son depredados, la misma proporción de huevos eclosionan. En cautividad, los pollos de ambos tipos de hembras tienen la misma probabilidad de sobrevivir a las primeras tres semanas de vida, el período más crítico para su supervivencia. Sin embargo, ya que durante este tiempo los polluelos son más dependientes del cuidado de su madre, y estas presentan alta mortalidad, es de esperar que estos pollos también presenten menor probabilidad de supervivencia en condiciones naturales (como también sucede en otras Galliformes, como la perdiz roja (*Alectoris rufa*), Duarte & Vargas 2004). Aunque la mortalidad en los machos de granja liberados no se ha estudiado en esta tesis, es probable que también sea elevada debido a la misma falta de instinto anti-depredador. Sin embargo, ello debería suponer un efecto menos acentuado sobre su eficacia biológica que en el caso de las hembras, ya que la supervivencia de los pollos no depende de ellos.

Las hembras híbridas son más atractivas para los machos de codorniz común que las propias hembras de codorniz común. Esto implicaría una reducción en las posibilidades de apareamiento de las hembras comunes, y una mayor proporción de cruces entre los dos linajes. Los machos híbridos, por su parte, no atraen tantas hembras comunes como lo hacen los machos de codorniz común. Debido a ello, es de esperar que la mayoría de las parejas heteroespecíficas en el campo estarían constituidas por una hembra de granja y un macho común. Eso implicaría que los descendientes tendrían ADN mitocondrial de codorniz japonesa doméstica (todas las codornices de granja tienen ADN mitocondrial de codorniz japonesa doméstica) aunque podrían mostrar ADN nuclear indistinguible del de las codornices comunes. Esta introgresión podría provocar el mal funcionamiento de complejos de genes debido a incompatibilidades citonucleares (Cruzan & Arnold 1999, Chou & Leu 2010) y una menor eficacia biológica.

Los resultados de esta tesis han demostrado que el vínculo que se establece durante varios días entre macho y hembra aumenta la probabilidad de fertilización de los óvulos. Cuanto más tiempo guarda el macho a la hembra, mayor proporción de huevos consigue fecundar. Los resultados muestran que los machos híbridos necesitan menos tiempo que los machos comunes para fertilizar la misma proporción de huevos. Ello podría implicar que en condiciones de libertad los machos híbridos no tendrían que pasar tanto tiempo guardando a la hembra como los machos comunes, y podrían utilizar ese tiempo en buscar nuevos apareamientos. Esto podría representar una desventaja competitiva para los machos comunes.

Para tener una perspectiva global sobre los efectos que las sueltas cinegéticas pueden tener sobre las poblaciones de codorniz común, hay que tener en cuenta también algunas barreras ecológicas y comportamentales que podrían reducir la introgresión en condiciones naturales (véase Tabla 1 y Recuadro 1). Algunas de estas barreras no se han estudiado en esta tesis, pero podrían ser importantes para la supervivencia y la eficacia biológica de las codornices de granja introducidas:

1) La primera y, probablemente, más importante barrera para las codornices soltadas es la caza. Aunque no disponemos de datos sobre la tasa de mortalidad de codornices soltadas durante la temporada de caza, parece razonable considerar que la misma falta de comportamiento anti-predatorio favorecería una mayor presión de caza sobre los animales soltados, incluso sobre aquellos que podrían haber sobrevivido la temporada de cría. Además, la vida en cautividad reduce las habilidades para el vuelo, y no tienen tiempo de acostumbrarse al medio en el tiempo entre que son soltadas y cazadas, por lo que se prevé que la mortalidad debida a la caza sea muy alta.

2) El acusado comportamiento sedentario entre las codornices de granja favorecería una mayor mortalidad debido a la exposición de estos animales a duras condiciones invernales en altas latitudes.

3) Las codornices de granja introducidas podrían verse más afectadas por enfermedades y parásitos debido a una respuesta debilitada del sistema inmunológico, resultado de la selección artificial en cautividad (Villanúa et al. 2008).

4) Las codornices de granja, debido a su cría controlada, pueden tener más dificultades para encontrar la comida adecuada en el medio silvestre (Guyomarc'h 2003).

Aunque los individuos introducidos y sus descendientes podrían mostrar una menor eficacia biológica que las codornices comunes, los resultados de esta tesis demuestran que la introgresión de alelos de codorniz doméstica japonesa está sucediendo. Hemos comprobado el poder de los marcadores genéticos altamente polimórficos utilizados según los métodos tradicionales en este estudio para mostrar la posible introgresión progresiva de alelos alóctonos en la población nativa. Curiosamente, nuestras simulaciones sugieren que tal introgresión no sería detectable, lo cual sirve de advertencia a los numerosos estudios sobre hibridación que no incluyen este tipo de pruebas para evaluar la fiabilidad de sus conclusiones. De hecho, las simulaciones ilustran claramente que es probable que la mayoría de análisis, los cuales incluyen un número pequeño de marcadores y de tiempo abarcado, subestimen la introgresión cuando esta se produce a un ritmo moderado o lento.

Utilizamos nuestras simulaciones para estimar parámetros compatibles con los datos observados. Simulaciones posteriores con estos mismos parámetros sugieren que se espera un aumento gradual de la proporción de individuos con algún grado de hibridación en la población en las próximas generaciones. En consecuencia, es urgente la aplicación más firme de las leyes actuales que prohíben las sueltas de híbridos y codornices japonesas domésticas para evitar un mayor avance de alelos alóctonos.

Recomendaciones para la conservación de la codorniz común

Varias recomendaciones para la conservación de la codorniz común pueden derivarse de esta tesis. A modo de resumen:

a) La suelta de codornices híbridas o japonesas domésticas criadas en granja no es una herramienta de gestión adecuada para las poblaciones de codorniz común. Cualquier introducción debería ser prohibida hasta que la certificación de la identidad genética de las aves se realice adecuadamente mediante el uso de métodos genéticos.

b) Existe la necesidad de realizar una prospección más amplia de la diversidad genética de las codornices comunes para evaluar el impacto que las sueltas cinegéticas pueden haber tenido en toda su área de distribución.

c) A pesar de que la posibilidad de soltar codornices comunes puras con fines cinegéticos debe ser evaluada, esta estrategia de gestión es también potencialmente dañina.

d) En lugar de realizar sueltas cinegéticas, limitar el número de codornices cazadas en función del tamaño de la población reproductora contribuiría a una gestión más sostenible de la especie.

Recuadro 1. Próxima investigación

Algunos de los acontecimientos que podrían afectar la eficacia biológica de las codornices liberadas deben ser estudiados para comprender mejor cómo está teniendo lugar el proceso de introgresión y cuál es la forma de mejorar la conservación de la codorniz. Algunos de ellos se encuentran en estudio por nuestro equipo de investigación (marcados con un asterisco):

- **Supervivencia a la caza ***. Probablemente, la caza tiene el mayor efecto sobre la supervivencia de la codorniz soltada. Mediante el marcaje y la suelta de codornices de granja y comunes, y el estudio de las recapturas realizadas por los cazadores, sería posible estimar la supervivencia relativa para ambos tipos de codornices. Nuestra hipótesis es que la falta de un comportamiento anti-predatorio en las codornices de suelta las hace más vulnerables a la caza.

- **Migración o sedentarismo ***. Análisis genéticos de individuos capturados en migración hacia el sur nos permitirán evaluar si las codornices híbridas migran también para pasar la temporada de invierno en latitudes del sur. Análisis de individuos capturados en migración hacia las zonas de reproducción servirán para estimar la supervivencia relativa de las codornices híbridas y las codornices comunes a la migración y la invernada en África. Si las codornices de granja y sus descendientes tienen una menor supervivencia, la proporción en la que se encuentren entre las muestras obtenidas debería ser menor que en el caso de las comunes.

- **Invernada en las áreas de reproducción ***. Se ha sugerido que la presencia de codornices durante el invierno en latitudes donde se da reproducción podría ser consecuencia de su origen híbrido, siendo éstos individuos que han perdido su impulso migrador. Análisis genéticos de individuos cazados durante el invierno en esas latitudes servirían para estimar la proporción de codornices sedentarias que muestran algún grado de hibridación.

- La **selección de hábitat** podría tener fuertes consecuencias en la supervivencia de los individuos. Las codornices soltadas podrían mostrar menor capacidad de encontrar lugares adecuados para aparearse, comer o anidar sin ser detectadas por los depredadores.

- **Parásitos y enfermedades** podrían afectar intensamente a las codornices soltadas debido a un sistema inmunológico deprimido por la selección en cautividad.

- **Recursos alimentarios**. Probablemente, la adaptación a la cautividad haga que para las codornices criadas en granja sea más difícil encontrar comida de alta calidad en la naturaleza, lo cual podría afectar a su supervivencia y a la de sus pollos.

Acontecimiento	Efecto	Explicación	Artículo
Caza	(-)	Debido al reducido comportamiento anti-predatorio y al poco tiempo de adaptación a las condiciones naturales antes de la caza, la proporción de individuos cazados entre las codornices soltadas probablemente sea mayor que entre las codornices comunes.	Por estudiar
Atractivo femenino	+	Los machos de codorniz común son atraídos en mayor medida por hembras híbridas que por hembras comunes.	2
Atractivo masculino	-	Las hembras comunes son atraídas en menor medida por machos híbridos que por machos comunes.	2
Selección post-copulatoria	+	Los machos híbridos tienen un mayor potencial para fecundar que los machos comunes.	3
Supervivencia de las hembras	-	La supervivencia de las hembras híbridas es menor que la de las comunes, probablemente debido a su reducidas adaptaciones a la vida silvestre. Ello afecta todos los parámetros reproductivos relacionados directamente con la hembra.	2
Apareamiento de la hembra	=	La probabilidad de apareamiento es la misma para ambos tipos de hembras.	2
Anidamiento	=	La probabilidad de anidamiento es la misma para ambos tipos de hembras.	2
Depredación de nidos	-	Los nidos de las hembras híbridas sufren un mayor riesgo de depredación que los de las hembras comunes, probablemente debido al reducido instinto anti-predatorio.	2
Tamaño de la puesta	=	Las hembras híbridas que sobreviven suficiente como para completar la puesta ponen el mismo número de huevos que las hembras comunes.	2
Fecundidad	=	La proporción de huevos fecundados sobre el total de huevos puestos es la misma en las puestas de ambos tipos de hembras.	2

Probabilidad de eclosión (en cautividad)	=	La proporción de huevos fertilizados que eclosionan es la misma en las puestas de ambos tipos de hembras.	2
Supervivencia de los pollos (en cautividad)	= (-)	La proporción de pollos que sobreviven las tres primeras semanas de vida en cautividad es la misma para las puestas de ambos tipos de hembras. A falta de ser comprobado, esperamos que la supervivencia de los pollos en el campo sea menor para los de las hembras híbridas, debido a la alta mortalidad que estas presentan.	2
Siega del cereal y nomadismo	(-)	El comportamiento sedentario estaría acentuado en la mayoría de codornices híbridas. Ello puede afectar sus movimientos en busca de hábitat y apareamientos, reduciendo su eficacia biológica.	Por estudiar
Migración o invernación en elevadas latitudes	(-)	El comportamiento sedentario incrementaría la fracción de híbridos invernando en latitudes superiores. El mal tiempo, la caza continuada y la falta de hábitat disminuirían su supervivencia.	Por estudiar
Parásitos y enfermedades	(-)	Probablemente, los parásitos y enfermedades de la granja o del campo tendrían un efecto importante en las codornices liberadas de la granja, debido a su sistema inmunitario deprimido, resultado de la selección en cautividad.	Por estudiar
Alimentación	(-)	Aunque no es de esperar que las semillas ni los insectos escaseen en los campos de cereal, las codornices introducidas podrían tener problemas para encontrar alimento ya que están acostumbradas a la alimentación ad-libitum.	Por estudiar

Tabla 1. Acontecimientos ecológicos, reproductivos y comportamentales que podrían tener un efecto sobre la eficacia biológica de las codornices soltadas para fines cinegéticos en comparación con codornices comunes. Los efectos positivos (+) fortalecerían la introgresión, los efectos negativos (-) la contendrían, y la misma intensidad de efecto en codornices de suelta y comunes (=) la perpetuarían. Los símbolos que aparecen entre paréntesis corresponden a nuestra suposición basada en conocimientos previos, pero los efectos no han sido probados. La supervivencia de las hembras afecta todos los parámetros reproductivos directamente relacionados con ellas. Por otro lado, la depredación de de los nidos afecta a todos los parámetros relacionados con los pollos. En la última columna, se indica el número del artículo de la tesis en el que se ha estudiado ése acontecimiento.

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CONCLUSIONES

- La mayoría de las codornices criadas en granjas españolas y vendidas para sueltas cinegéticas no son codorniz común pura sino que tienen carga genética de codorniz doméstica japonesa. La mayoría de ellas muestran un grado de hibridación similar a híbridos de segunda generación o retrocruzamientos con codorniz común.

- Las codornices híbridas de granja y las codornices comunes silvestres pueden potencialmente cruzarse en la naturaleza. De hecho, el cruzamiento en la naturaleza ya se ha producido.

- Las hembras híbridas de granja y las hembras de codorniz común pueden tener un éxito reproductivo similar en condiciones naturales. Presentan similar probabilidad de apareamiento, probabilidad de apareamiento, nidificación, tamaño de puesta y fertilidad. La probabilidad de eclosión de sus huevos y la supervivencia de sus pollos en cautividad son también similares. Además, en condiciones experimentales de semi-naturalidad, las hembras híbridas de granja atraen más a los machos comunes que lo que lo hacen las hembras, lo que puede fortalecer el apareamiento interespecífico.

- La selección sexual post-copulatoria entre machos híbridos de granja y machos comunes podría modular la mezcla entre estos dos linajes. Los machos de granja tienen mayor capacidad de fecundación que los machos comunes. En consecuencia, un número relativamente pequeño de apareamientos interespecíficos exitosos podría tener un mayor impacto en la población nativa del que se sospechaba inicialmente teniendo sólo en cuenta el número de cruces entre ambos tipos de codornices.

- A pesar de su éxito en la fecundación, una menor eficacia biológica de los individuos soltados y sus descendientes, debido a un menor instinto anti-predatorio, resultaría en una selección negativa sobre los alelos no adaptativos. Las hembras de granja soltadas presentan tasas de mortalidad mayores que las comunes, y sus nidos tienen mayor probabilidad de ser depredados.

- Los datos genéticos son compatibles con una lenta introgresión de alelos de codorniz japonesa doméstica en el acerbo genético de la codorniz común. Aunque las sueltas masivas de codornices de granja no parecen haber tenido un gran impacto en la integridad genética de la codorniz común en Cataluña, los niveles generales de mezcla genética pueden estar incrementando lentamente a lo largo del tiempo. Simulaciones predicen que ello podría resultar en un enjambre híbrido en un futuro próximo si las sueltas cinegéticas siguen realizándose.

- El impacto genético de la introgresión en las poblaciones silvestres puede ser difícil de detectar, especialmente cuando la hibridación se produce durante períodos de tiempo largos y a un ritmo lento. En tales casos, los datos recopilados a lo largo de un período corto de tiempo pueden ser insuficientes y conducir a la subestimación del grado de mezcla. En cambio, las muestras obtenidas durante varias generaciones pueden ayudar a evaluar los cambios temporales en el grado de hibridación y pueden ofrecer una visión a largo plazo de la introgresión de alelos alóctonos de una forma más significativa desde un punto de vista conservacionista y evolutivo. La clara caracterización de un proceso de introgresión lento requiere el uso de datos tomados a lo largo de largas series de tiempo que permitan la detección de la entrada de nuevos alelos en la población.

- Los métodos genéticos utilizados son útiles para distinguir la identidad genética de las codornices. Un control intensivo y la identificación genética de los individuos para sueltas cinegéticas son indispensables, ya que las sueltas masivas de individuos híbridos de granja representan una grave amenaza para la supervivencia a largo plazo de la codorniz común.

APPENDIX

Rearing quail chicks in the lab
by Ines Sanchez-Donoso

Are Farm-Reared Quails for Game Restocking Really Common Quails (*Coturnix coturnix*)?: A Genetic Approach

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Abstract

The common quail (*Coturnix coturnix*) is a popular game species for which restocking with farm-reared individuals is a common practice. In some areas, the number of released quails greatly surpasses the number of wild breeding common quail. However, common quail are difficult to raise in captivity and this casts suspicion about a possible hybrid origin of the farmed individuals from crosses with domestic Japanese quail (*C. japonica*). In this study we used a panel of autosomal microsatellite markers to characterize the genetic origin of quails reared for hunting purposes in game farms in Spain and of quails from an experimental game farm which was founded with hybrids that have been systematically backcrossed with wild common quails. The genotypes of these quail were compared to those of wild common quail and domestic strains of Japanese quail. Our results show that more than 85% of the game farm birds were not common quail but had domestic Japanese quail ancestry. In the experimental farm a larger proportion of individuals could not be clearly separated from pure common quails. We conclude that the majority of quail sold for restocking purposes were not common quail. Genetic monitoring of individuals raised for restocking is indispensable as the massive release of farm-reared hybrids could represent a severe threat for the long term survival of the native species.

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Introduction

Restocking has become one of the most commonly used practices for the conservation and management of endangered and game species. One important reason for restocking is to supplement fisheries and game bags in order to increase productivity or maintain recreational activities [1]. In some cases this may threaten local gene pools [2]. Restocking for hunting purposes has been carried out with diverse species such as deer [3], marmot [4], wild boar [5], waterfowl [6] and different Galliform species [7,8], including partridges [9–11], pheasants [12] and quails [13–15].

The common quail (*Coturnix coturnix*) is a migratory Galliformes species distributed across Eurasia during its breeding period [16] and currently has an unfavourable conservation status in Europe due to a large historical decline [17]. Even so, the common quail is a popular game species with an important socio-economic impact. Only in Spain, more than 1,300,000 quails have been hunted annually during the period 1973–2010 (*Yearbook of Agro-alimentary Statistics* of the Spanish Ministry of Agriculture, Fishing and Food). Restocking with farm-reared quails to increase bag numbers is a very common practice in several European countries, such as Italy [18], Greece [14,19], The Republic of Serbia, Montenegro, Romania and Spain [13,15]. According to Guyomarc'h [16], 430,000–480,000 released quails were shot in France during the 1983–1984 hunting season, corresponding to 67%–75% of the 640,000 total individuals hunted. In Catalonia (Northeast Spain), an average of more than 68,000 farm-reared quails have been released annually during the period of 1990–2006 (Hunting order plans of the Regional Government of Catalonia). Taking into account that the estimated wild male quail population in Catalonia ranges between 5,347 and 20,847 individuals [20], the number of quails restocked greatly exceeds the breeding population.

However, rearing common quails in captivity for restocking is difficult because of their restlessness [21]. This has led to some concern about the genetic origin of farm-reared quails. A possible explanation for the great reproductive success in farms is that those individuals could be of hybrid origin, resulting from crosses between wild common quails and domestic Japanese quails (*Coturnix japonica*). The Japanese quail is sister species to the common quail and is distributed across eastern Asia [22]. For several centuries Japanese quails have been bred in captivity and selected for meat and egg productivity [23]. These domestic Japanese quail first entered Europe (France and Italy) in the 1950 s [24,25]. As a result of the selection for life in captivity, these birds have lost their migratory restlessness [26], show some reluctance to move and fly, and have lower anti-predatory instinct.

These differences in behaviour suggest that restocking with farm-reared domestic Japanese quails or hybrids between the two species (using this term to refer to all admixed individuals and not just first generation hybrids) would result in a conservation problem. In fact, several authors have warned about the risk that restocking can represent for common quails when carried out with domestic Japanese quails and hybrids [14–16,27,28]. It entails a potential threat to the native common quail because it could lead to the introgression of domestic Japanese quail alleles and a subsequent loss of migratory behaviour and decline in fitness in the native common quail population [16,29]. Individuals that do not migrate or that show low mobility could be more exposed to adverse climatic conditions, loss of habitat after the harvesting of cereal crops (the usual habitat of the species), predation, or a lack of food resources [16]. Thus, the arrival of maladaptive genes from domestic Japanese quail could reduce the survival and adaptive potential of wild common quail.

This conservation concern seems to be a real one: hybrid individuals have already been detected in common quail breeding areas in different European countries such as Portugal, France, Italy and Spain [13–16]. Moreover, recent field experiments with released radio-tagged farm-reared individuals of hybrid origin have shown that they can mate with wild common quails and produce fertile offspring (unpub. data) as happens in captivity [30]. Other closely related native Galliformes, the red-legged partridge (*Alectoris rufa*) [31,32] and the rock partridge (*A. graeca*) [33], are threatened by hybridization resulting from game restocking with sister species or hybrids. As a result of the concern about hybridization, the release of Japanese quails and hybrids is illegal in Spain, Portugal, France and Greece. However, the diagnosis of the specific origin of farmed quails used for restocking is usually based on their morphology despite the difficulty in unequivocally differentiating between pure common quails and admixed individuals on the basis of their phenotype ([34] in [14]). Thus, genetic analyses are needed to assess species identity of restocked farm-reared individuals.

The aim of this study was to identify the genetic origin of quails reared for restocking and hunting purposes in five different Spanish game farms by using a panel of nuclear microsatellite markers. We also analysed quails from an experimental farm that managed to reduce the genetic contribution of the founders, which had domestic Japanese quail ancestry, by crossing farm-born individuals with wild common quails. We hypothesize that a large proportion of the released quails are of hybrid ancestry due the easy breeding of domestic Japanese quails in captivity. If this is the case, the release of these birds could negatively affect the long term survival of natural common quail populations.

Materials and Methods

Ethics Statement

All work related with animals in this study has been conducted according to the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA). It fulfills the ethic recommendations of the European Union and the Spanish legislation and has been approved by the Ethics Committee on Animal Experimentation from the University of Barcelona and from the University of Castilla-La Mancha.

Samples origin and collection

One hundred and fifty-two quails were sampled for this study. They were collected from four different origins (Table 1). They consist of: 1) Males and females randomly sampled from five different Spanish game farms in 2009 and 2010, purchased for restocking and hunting purposes. 2) Quails from an experimental farm from the University of Castilla-La Mancha (Spain) managed for about 20 years to reduce the genetic contribution of founders. These individuals are descendants of crosses between admixed females with wild common quail males whose offspring has been backcrossed with wild common quail males in successive generations. 3) Wild quail males captured (see [15]) during 1996–2009 around Seville (South Spain), Barcelona (Northeast Spain) and Drenthe (The Netherlands) which were identified as common quails on the basis of their song, phenotype and preliminary genetic analyses. 4) Domestic Japanese quails from two laboratory lines from the Laboratory of Molecular Ecology of the Institute of Ecology of the Nature Research Centre, Vilnius University (Lithuania) [35], and from four different Spanish meat farms.

Blood (100 μl) or muscle samples were individually stored at -20°C in 95% ethanol until DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen) following manufacturer's protocols.

Typing of microsatellite loci

Individuals were genotyped for 11 unlinked autosomal microsatellite loci originally developed for Japanese quail [36,37]: GUJ0001, GUJ0017, GUJ0028, GUJ0039, GUJ0044, GUJ0057, GUJ0065, GUJ0074, GUJ0085, GUJ0093 and GUJ0097. Loci were amplified by polymerase chain reaction (PCR). While some markers were PCR-amplified in a multiplex, others were amplified separately and subsequently pooled before electrophoresis. Detailed protocols are available upon request. All PCR products were electrophoresed on an ABI 3730 sequencer (Applied Biosystems) following manufacturer's protocols. Alleles were sized and scored using the software GeneMapper v3.5 (Applied Biosystems).

Analysis of microsatellite data

To measure genetic variation, average number of alleles per locus and allelic richness (mean number of alleles per locus corrected for minimum sample size, in this case nine successfully genotyped individuals per population and locus) [38] were calculated using FSTAT version 2.9.3.2 [39]. In order to measure the marker informativeness we calculated the Polymorphic Information Content (PIC) [40], which takes into account the number of alleles per locus and the frequency of these alleles, using EXCEL MICROSATELLITE TOOLKIT 3.1.1 [41]. We used the same software to calculate observed (H_O) and expected (gene diversity, H_E) heterozygosities [42].

Patterns of genetic differentiation were visualized by plotting the individuals in a two-dimensional space according to their microsatellite composition, independently of any *a priori* classification, using a factorial correspondence analysis (FCA) in GENETIX [43].

To identify genetically distinct clusters present in the data we used a Bayesian clustering procedure implemented in STRUCTURE 2.3.2 [44]. STRUCTURE identifies the number (K) of genetically distinct clusters that maximizes the likelihood of the data and estimates, for each individual, the fraction of the genome (q) that belongs to each one of the clusters. Analyses were performed using all individuals under the "admixture model" (as

Table 1. Quail samples studied.

Group	N_G	Sampling origin	N
Game farm quails	52	Game farm 1	13
		Game farm 2	7
		Game farm 3	20
		Game farm 4	6
		Game farm 5	6
Experimental quails	19	Experimental farm	19
Wild common quails	42	Seville (S Spain)	5
		Barcelona (NE Spain)	25
		Drenthe (The Netherlands)	12
Domestic Japanese quails	39	Meat farm (4 farms, 4 samples from each)	16
		Laboratory lines (2 lines with 9 and 14 samples)	23
Total			152

N_G: number of individuals per group. N: number of individuals per sampling origin.
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each individual may have ancestry in more than one parental population), with correlated allele frequencies and without population or sampling location information (USEPOPINFO and LOCPRIOR inactives). Simulations were run for 100,000 steps following a burn-in period of 30,000 steps, considering values of K ranging between two and 10, and were replicated five times [45] after verifying that results did not vary significantly with longer runs of iterations. Likelihood values were observed to converge during the runs. For $K=2$, for each individual we estimated the 90% probability interval for the proportion of membership to each cluster (q). The best value of K was chosen following the method proposed by Evanno et al. (2005) [46], with STRUCTURE HARVESTER [47], which takes into account the rate of change in the log likelihood between successive K values. Each individual was assigned exclusively to one of the inferred clusters when its q to that cluster was equal or larger than a threshold corresponding to the minimum value observed among the non-admixed individuals used as reference (see below). Alternatively, individuals that showed lower q values for all clusters could not be assigned exclusively to one of them and were considered putatively admixed.

After confirming with STRUCTURE that none of the wild common quails and domestic Japanese quails had a genome that seemed admixed, we considered them purebred and used them as reference in analysis with NEWHYBRIDS 1.1 [48]. With this software, we computed the posterior probability (P) for each individual to belong to each of the following genotypic classes: parental purebred 1 (P_1), parental purebred 2 (P_2), first generation hybrid (F_1), second generation hybrid (F_2, offspring of crosses between F_1 hybrids), backcross of F_1 with P_1 (Bx_1) and backcross of F_1 with P_2 (Bx_2). Posterior distributions were evaluated after running five independent analyses to confirm convergence, starting with different random seeds, for 10^5 Monte Carlo Markov Chain iterations after 10^4 burn-in steps, without using prior allele frequency information. Analyses were run for four combinations of prior distributions (JEFFREYS or UNIFORM for Theta and Pi) to explore the robustness of the results, as recommended by the software authors [48]. The affinity of an individual to the genotype classes was assessed by its posterior probability values (P): those that showed $P \geq 0.85$ to one genotype class were assigned to that class; if no value reached 0.85, but the sum of all hybrid classes was above this threshold, individuals were identified as hybrids of unknown generation [45]; individuals whose origin could not be identified under these criteria were left unclassified.

Results

Loci and population genetic characteristics

All 152 individuals studied were successfully genotyped at seven or more of the 11 markers, and more than 95% of them were typed for eight or more loci. All loci were polymorphic in the four groups of quails studied. A total of 224 alleles were found, 145 of which were exclusive to the reference wild common quails and eight to the reference domestic Japanese quails (excluding individuals from game and experimental farms), implying great power for hybrid identification.

Domestic Japanese quails showed the lowest average number of alleles per locus, allelic richness and PIC while the highest values were found in wild common quails (Table 2). Values for game and experimental farm quails were intermediate. Observed heterozygosity was higher than expected in experimental farm quails ($p=0.0003$). On the other hand, observed heterozygosity was lower than expected in domestic Japanese quails ($p=0.0012$) as could be expected considering that the samples originated from separate breeding lines.

Population differentiation

Wild common quails appeared completely separate from domestic Japanese quails along the first factorial component (FA-I) of the FCA (Figure 1). Game farm quails occupied an intermediate position between common quails and domestic Japanese quails along the same axis showing almost no overlap with either of them. Individuals from the experimental farm occupied the same range of values compared to wild common quails along the first axis, but showed a clear separation along the second axis (FA-II), with some individual values overlapping the range observed for wild common quails.

According to the approach of Evanno et al. (2005) [46], the total sample could be subdivided in four clusters (Figure 2). Likelihood values converged during the runs and results did not change across replicates. Common quails and domestic Japanese quails were completely separated in two different clusters. All wild common quails ($N=42$) were unequivocally assigned to cluster 1 (C_1), with $q_1 \geq 0.84$, while all domestic Japanese quails ($N=39$) were assigned

Table 2. Genetic diversity for each group of samples.

Group	N	Average number of alleles	Allelic richness	PIC	H_E	H_O
Game farm quails	52	14.00	7.65	0.80	0.83	0.80
Experimental farm quails	19	8.64	6.93	0.77	0.82	0.84**
Wild common quails	42	17.73	9.82	0.87	0.90	0.90
Domestic Japanese quails	39	5.27	4.11	0.59	0.66	0.60*

N: number of individuals. PIC: Polymorphic Information Content [40]. H_E: expected heterozygosity [42]; H_O: observed heterozygosity [42]. Significant differences between H_E and H_O are indicated by * ($p \leq 0.05$) and ** ($p \leq 0.001$).
doi:10.1371/journal.pone.0039031.t002

to cluster 2 (C_2), with $q_2 \geq 0.92$. Considering these results, we established that q values under a threshold value of 0.84 could be suggesting admixed ancestry. Of the game farm quails (N = 52), 73% grouped in a separate cluster (C_3) while 27% appeared as admixed. Experimental farm quails (N = 19) split between their own cluster (C_4) (48%), and C_1 (26%) (with common quails), while 26% of them were identified as admixed. These results show that none of the game farm quails were assigned to the same cluster as common quails, and this was also the case for 74% of the experimental farm quails.

Hybrid identification

The partition of the sample in four groups was the result of the relative isolation between the different groups. However, since the sample included two evolutionarily distinct lineages (common and Japanese quails) and perhaps admixed individuals, we also investigated the partition of the sample into two groups ($K = 2$). The likelihood values converged during the runs and results did not change between replicates. Common quails and domestic Japanese quails appeared completely differentiated in separate clusters, as in the previous analysis (Figure 2). All wild common quails (N = 42) were unequivocally assigned to cluster 1 (C1), with $q_1 \geq 0.87$, while all domestic Japanese quails (N = 39) were assigned to cluster 2 (C_2) with $q_2 \geq 0.83$ (Figure 2). In this case and considering these results, we established the value of 0.83 as the threshold below which individuals could be classified as admixed. Forty-three of 52 (83%) game farm quails had admixed genotypes, eight (15%) clustered in C_1 with common quails and one (2%) with domestic Japanese quails in C_2. Sixteen (84%) of the experimental farm quails clustered in C_1, while the remaining three individuals (16%) had admixed genotypes (Figure 2).

We also evaluated the width of the 90% probability interval for each q for $K = 2$. While none of the wild common quails or domestic Japanese quails had probability intervals that excluded the possibility of being pure ($q_1 = 1$ or 0, respectively; Figure 3), purebred ancestry could be excluded in 71% of the game farm quails. On the contrary, probability intervals did not exclude the possibility of being a pure common quail for any of the individuals from the experimental farm (Figure 3).

After defining wild common quails and domestic Japanese quails as reference purebred groups, we used NEWHYBRIDS to identify the origin of all farm quails. More than 95% of wild common quails and domestic Japanese quails showed $P \geq 0.85$ to their genotypic class (P_1 and P_2, respectively), independently of the prior combinations used. This confirmed that these samples could be used as reference groups. Game farm individuals that were classified as F_2 hybrids using a Jeffreys distributed prior for Theta were classified as domestic Japanese quails using a uniform prior. This last classification was considered unlikely due to the previously known information regarding the management of game farms (see discussion) and due to the results obtained with STRUCTURE when $K = 2$. For this reason, we only took into account results obtained using Jeffreys prior for Theta (individual classification did not show significant differences depending on the

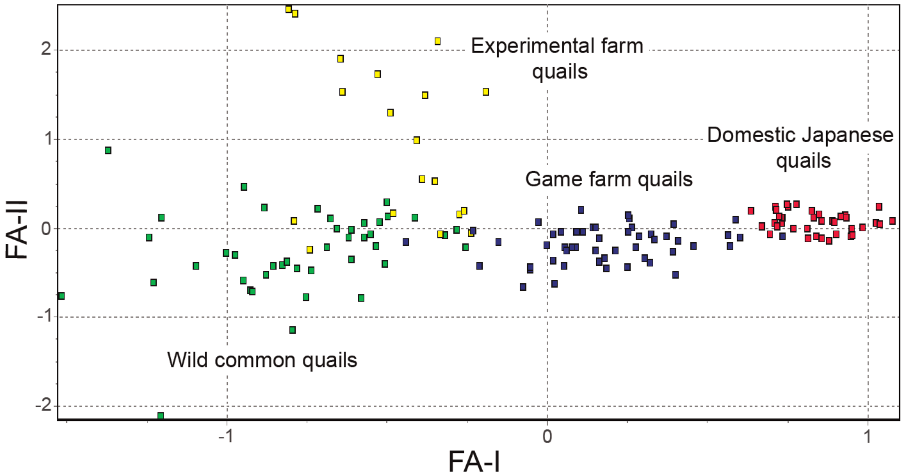

Figure 1. Factorial correspondence analysis. Green: wild common quails; red: domestic Japanese quails; blue: game farm quails; yellow: experimental farm quails.
doi:10.1371/journal.pone.0039031.g001

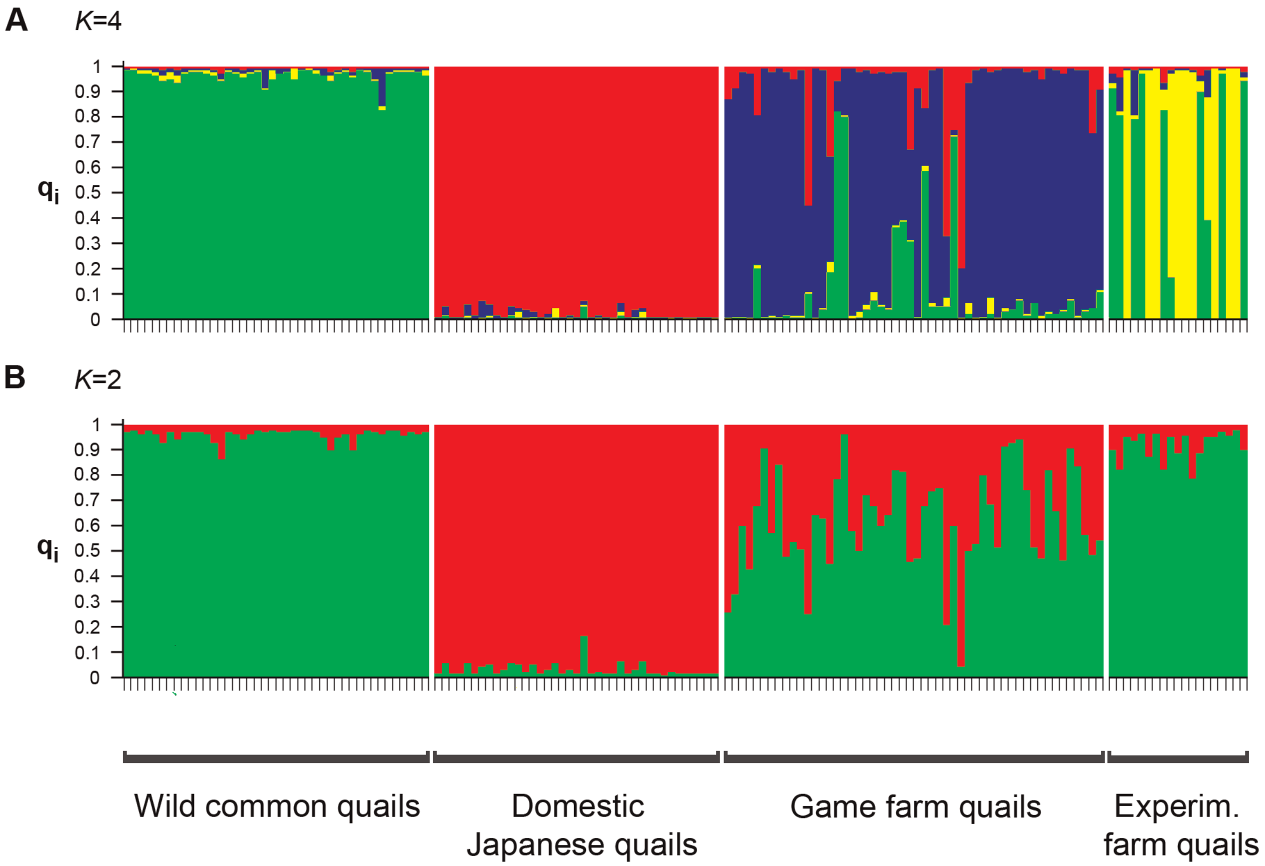

Figure 2. Clustering of individual genotypes into $K=4$ (A) or $K=2$ (B) clusters according to STRUCTURE. Each vertical bar represents one individual and clusters are represented by colours. The extent of the colours within each column represents the individual proportion of membership (q) to each one of the clusters.
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prior used for Pi). The analyses indicated that 46 out of 52 (88%) game farm quails were admixed (Figure 4). Thirty of these 46 could be identified as F_2 hybrids, while 16 of them were hybrids of unknown generation (probably indicating older admixture). Three of the game farm quails (6%) could not be classified into a unique class, two were assigned as common quails and one as domestic Japanese quail. On the other hand, 10 out of 19 of the experimental farm quails (53%) were identified as common quails, seven (37%) could not be classified and two (10%) were hybrids of an unknown generation.

Comparing the results regarding game farm and experimental farm quails obtained with STRUCTURE for $K=2$ and with NEWHYBRIDS, we observed assignment inconsistencies in only three individuals. For the three cases (three game farm quails) individuals were classified as common quails by STRUCTURE but as hybrids of unknown generation by NEWHYBRIDS. This is likely a result from ancient admixture with multiple generations of backcrossing with pure common quails. While all individuals were classified as either pure or admixed by the criteria that we defined in STRUCTURE, the classification with NEWHYBRIDS was more conservative and some of the individuals were left unclassified.

Discussion

Our results showed that each one of the groups of quails studied had a unique identity that allowed its separation in genetic analyses. Thus, wild common quails, domestic Japanese quails, game farm quails and experimental farm quails formed four well separated groups, as suggested by STRUCTURE (Figure 2). Among these, wild common quails had the largest genetic diversity, as should be expected since the other groups represent smaller captive lineages that were more or less reproductively isolated from each other. Probably, the arrival of domestic Japanese quails from Asia into Europe did not involve many different lines [24,25]. This bottleneck could have produced the low genetic diversity present in the domestic Japanese quail lines analysed. Similarly, the number of breeders in game farms is expected to be relatively small. On the other hand, domestic Japanese quails do not constitute a uniform population but are fragmented (with separate meat and laboratory lines, for example), as shown by the difference between observed and expected heterozygosity resulting from a Wahlund effect [49]. The opposite pattern is observed in the experimental farm, where outbred matings are regularly imposed.

The differentiation was largest between wild common quails and domestic Japanese quails since both groups appeared in the two extremes of the first axis of the FCA without any overlap (Figure 1). All STRUCTURE analyses clustered them in separate groups, including those with $K=2$, which allowed separating the two evolutionary lineages. Also, a large number of alleles were not shared between the two groups, although this can be greatly affected by the sample size and by the fact that domestic Japanese quails were represented by inbred lines. This clear separation for

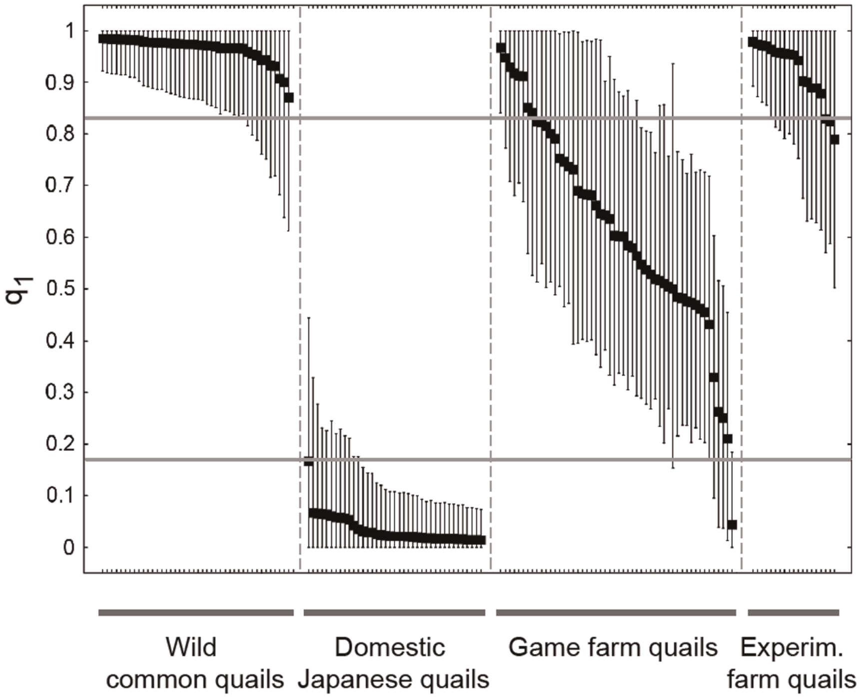

Figure 3. Individual proportion of membership to cluster 1 (q_1) and 90% individual probability intervals according to STRUCTURE for *K* = 2. The value of q_1 indicates membership to the same cluster as wild common quails. Individuals are sorted by group and by q_1 value. Probability intervals excluding 0 and 1 are indicative of admixed ancestry. Horizontal lines indicate threshold values for q used for a first separation of pure and admixed individuals (see text).
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the studied markers facilitates the identification of admixed individuals, overcoming the difficulties derived from their similar phenotypes.

Our analyses suggest that, contrary to the claims commonly expressed by farm managers, at least 85 to 90% (depending on the approach used) of the analysed game farm quails purchased for restocking purposes were not pure common quails and showed obvious signs of admixture with domestic Japanese quails. This estimate is based on the assumption that the reference individuals indeed represent purebred common and domestic Japanese quails. However, it is possible that some of them could have slightly admixed ancestry. Nevertheless, if this was the case, our numbers

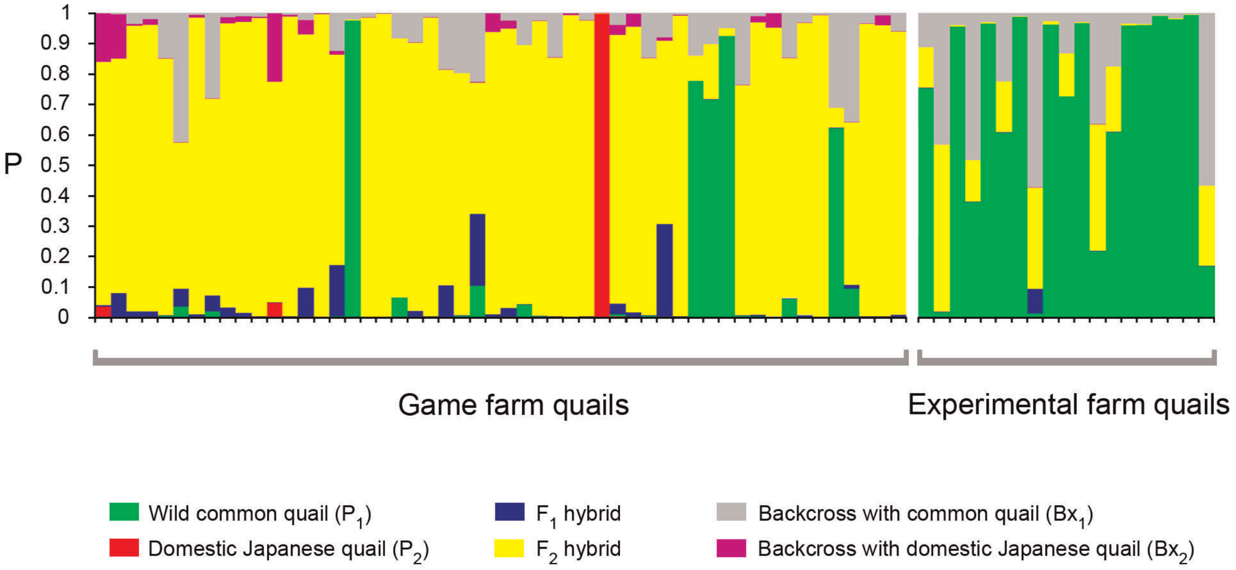

Figure 4. Individual genotype classification according to NEWHYBRIDS. Each vertical bar represents one individual. Each colour represents the posterior probability (P) of each individual to belong to the six different genotypic classes. Game farm and experimental farm individuals are sorted as in Figure 2.
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would be an underestimate of the proportion of admixed individuals in game farms, and the real proportion would be even larger.

This large proportion of admixed individuals does not seem exclusive to Spanish game farms. Previous genetic studies included a few game farm-reared quails and suggested hybrid origin in Portugal [50], France, Italy and Spain [14]. Most of the time, farm managers avoid revealing their procedures but indirect reports have suggested that they may often interbreed individuals of hybrid origin for many generations. We investigated the probability of farm individuals being pure or offspring of two generations of intercrossing or less (F_1, F_2 or a backcross of F_1 to one of the two parental classes). It is likely that many of the individuals studied are the result of a larger number of generations of intercrossing, but in order to estimate the ancestry of each individual with higher precision we would require a larger number of markers with high PIC, which would allow a better separation between hybrid classes [45]. Nevertheless, our results clearly show the admixed ancestry of the studied game farm birds.

The analyses with NEWHYBRIDS indicated that, depending on the priors used, most game farm individuals were classified as domestic Japanese or F_2 hybrids. We assumed that identification as F_2 was more likely because of the results obtained with STRUCTURE with $K = 2$ and because of the mating strategies that may have been taking place in game farms to obtain individuals for hunting purposes [16]. Domestic Japanese quails and common quails [26,51] could have been crossed, most likely domestic Japanese females with wild common quail males trapped in the field [50], and the offspring could have been subsequently intercrossed. With this procedure, fertile hybrids [30] may easily be obtained showing a ''wilder'' instinct, flying better and being smaller than the domestic Japanese quails [16], and thus being more attractive for sportive hunting. However, these hybrids inherit the reduced restlessness of domestic Japanese quails ([34] in [14]). Among the game farm quails analysed, between 4% and 15% were identified as common quails, depending on the approach used. This could reflect the occasional introduction of wild quails into the captive populations to act as breeders in order to decrease inbreeding and genetic load.

In the case of the experimental farm, common quail males have been regularly introduced to the farm breeding population during the last 20 years. In the last 10 years (about 10 generations) only females that are descendants of a common quail father have been used as breeders. Consequently, we expect a high frequency of common quail alleles in the genomes of quails from the experimental farm: 53 to 84% (depending on the methodological approach used) of the birds were classified as common quail. This implies that, after 20 generations of experimental crossing, between 10% and 16% of them are still identified as hybrids and 37% cannot be classified into any of the genotypic classes. In addition, we expect all individuals from this farm to carry Japanese quail mitochondrial DNA (see also [14]). The management strategy implemented in the experimental farm, trying to obtain individuals genetically more similar to the common quail than individuals from ordinary game farms, achieves its goal. However, we do not have any information about the possible cytonuclear conflicts deriving from the different origin of their mitochondrial and nuclear genomes, or about the relative fitness, behaviour and survival of these individuals in the wild compared to pure common quails. We do not know to which extent this management could minimise the impact of released admixed individuals on the native populations.

Due to the concern about the risk of restocking hybrid farm-reared quails, several European countries and regions have banned or regulated restocking practices (Portugal and France since 2002, Greece since 2007). In Spain, although different regions have different policies, the national law allows restocking with common quail, but not with Japanese quail or hybrids (Spanish Law 42/2007, Natural heritage and Biodiversity). Even so, many farm-reared individuals are still being released assuming that, in fact, they are pure common quails. Authorities allow quail releases trusting on the diagnosis of veterinarians that identify them as common quails on the only basis of their phenotype despite the fact that this identification method is usually ambiguous ([34] in [14]). Genetic analyses should be required to certify the origin of individuals used for restocking. Similarly, there is a need for an extensive survey of the diversity in wild quails to assess the impact that these releases may have had across its distribution range.

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Author Contributions

Conceived and designed the experiments: ISD CV MP JDRT. Performed the experiments: ISD CV MP JRCC PAMR JDRT. Analyzed the data: ISD CV. Contributed reagents/materials/analysis tools: ISD CV MP DB JRCC PAMR JDRT. Wrote the paper: ISD CV MP JDRT.

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Decreased fitness of restocked hybrid quails prevents fast admixture with wild European quails

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ABSTRACT

Restocking with non-native species for hunting purposes is a widespread practice in some Galliformes species that may result in the introgression of maladaptive alleles into native populations. Quails farmed for restocking are produced by interbreeding domestic Japanese quails (*Coturnix japonica*) with European quails (*Coturnix coturnix*). Massive releases of these animals could represent a threat to native European quails. In this study we radio-track 16 female native European quails and 51 female farm-reared quails over four breeding seasons in a single locality. Our results show that farmed female quails attracted more wild common quail males than European quail females, probably because they produce more rally calls. Here for the first time we show empirical evidence that European quails and restocked farmed quails interbreed in the wild. Further, hybrid farmed females and European females had similar probabilities of mating, nesting success, clutch size, fertility, hatching probability and chicks' survival probability. However, female farmed quail had higher mortality than European quail females, and their nests had a higher rate of predation. These last observations could explain why the two lineages do not appear completely admixed after more than 20 years of massive restocking practices. However, our results clearly show a lack of reproductive barriers in the wild and that introgression may not be completely prevented. An immediate ban of the release of non-native quails is necessary to preserve their genetic integrity. Thus, banning restocking with Japanese quail or hybrids is necessary to prevent the progressive introduction of maladaptive alleles into the European quail populations.

1. Introduction

Anthropogenic hybridization, either resulting from the introduction of species or from the invasion of alien species due to the modification of habitats, may create serious conservation problems (Levin et al., 1996; Rhymer and Simberloff, 1996). Introductions may be accidental (Kidd et al., 2009; McDowell, 2002) or part of a management plan, for example, to bolster endangered populations (Armstrong and Seddon, 2008; Massemin-Challet et al., 2006; Santos et al., 2009) or to increase hunting bags in the case of game species (Barbanera et al., 2010; Blanco-Aguiar et al., 2008). Restocking with non-native species for hunting purposes is a widespread management action for Galliformes (Barbanera et al., 2005, 2007, 2008; Blanco-Aguiar et al., 2008; Casas et al., 2012; Derégnaucourt et al., 2002; Derégnaucourt et al., 2005a,b; Porkert et al., 1997; Puigcerver et al., 2007; Tejedor et al., 2007). These restocking practices are carried out mainly with farm-reared birds, in most cases of doubtful genetic origin (Perennou, 2009), and thus constitute a real danger for native populations (Chazara et al., 2010; Laikre et al., 2010; Olden et al., 2004).

The European quail (*Coturnix coturnix*), also called common quail in Europe, is a migratory Galliform with a distribution from the British Islands to Lake Baïkal and from the Arctic Circle to the tropics (Del Hoyo et al., 1994; Guyomarc'h et al., 1998; Moreau and Wayre, 1968). It is of conservation concern (SPEC 3) due to a large historical decline (Burfield, 2004). Even so, it is a very popular game bird with a high socio-economic value, especially in some Mediterranean countries. In Spain alone, which hosts the largest breeding population in Western Europe (Gallego et al., 1997; Rodríguez-Teijeiro et al., 2006), more than 1,300,000 individuals

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were hunted annually during the period 1973–2010 (Yearbooks of Agro-alimentary Statistics of the Spanish Ministry of Agriculture, Fisheries and Food). Because of hunting interest in this species, restocking with farm-reared quails is a very common practice in several European countries such as Italy, Greece, the Republic of Serbia, Montenegro, Romania and Spain (Puigcerver et al., 2007; Rodríguez-Teijeiro et al., 1993; Tsiompanoudis et al., 2011). For example, in Catalonia (Northeast Spain), more than 68,000 farm-reared quails have been restocked annually during the period 1990–2006 (personal communication from the Catalan Department of Agriculture, Farming, Fisheries, Food and Environment) in an area with a wild population of between about 5000 and 21,000 males (Rodríguez-Teijeiro et al., 2004).

Recent genetic studies show that these farm-reared quails are hybrids of European and domesticated Japanese (*Coturnix japonica*) quails (Amaral et al., 2007; Barilani et al., 2005; Chazara et al., 2010; Sanchez-Donoso et al., 2012) (in this manuscript we use the term "hybrid" to refer to all individuals whose genomes show extensive admixture of the two lineages, without limiting the definition to first generation hybrids). The Japanese quail is distributed across East Asia, including Japan. The natural ranges of the two species only overlap in the surroundings of Lake Baïkal in Russia (Fefelov, 1998) and in the Kentei region in Mongolia (Kozlova, 1932). No natural hybridization has been reported (Del Hoyo et al., 1994; Guyomarc'h et al., 1998; Moreau and Wayre, 1968). However, hybrids of the two species generated in captivity are or have been used for releases in different European countries, and, as a result, the Japanese quail is considered an introduced species in Italy by the IUCN (http://maps.iucnredlist.org/map.html?id=100600195; accessed on November 5 2013) and in Spain by the Spanish Government (Royal Decree 630/2013). Thus, there is a real possibility that restocking could lead to the extensive introgression of domestic Japanese quail alleles into the European quail gene pool.

The European quail and the Japanese quail have almost identical habitat requirements (Taka-Tsukasa, 1941), and experiments in captivity show that they present only partial mate discrimination (Derégnaucourt and Guyomarc'h, 2003). Derégnaucourt et al. (2002) have also shown that hybridization between European and domestic Japanese quail, and backcrosses between hybrids and the parental species readily occurs in captivity and no differences have been found in average fertility, hatch and chick survival rates between hybrid pairs and European quails.

However, the arrival of Japanese quail alleles may represent a threat for European quails if they represent different adaptation to environmental conditions, co-adapted gene complexes or even sets of maladaptive alleles favorably selected in captivity. The European quail is a partially migratory species, whereas the domestic Japanese quail lost its migratory impulse during the domestication process (Derégnaucourt et al., 2005a,b; Guyomarc'h, 2003). As released hybrid quails have a lower frequency of migratory phenotypes (Derégnaucourt et al., 2005a,b), the introgression of their genes into wild populations could cause a decrease in the migratory tendency of European quail populations. This could lead to a decrease of the European quail population density, as non-migratory quails are more likely to suffer seasonal depletion of food resources in winter and a longer hunting season.

At least in theory, hybridization between European and farmed quails is likely to lead to a rapid increase in the proportion of hybrids and admixed individuals in wild populations. Once admixture has begun it is difficult to stop, especially if hybrids are fertile and mate both with other hybrids and with both parental species (Allendorf et al., 2001). As a result, in a few generations this process might result in a hybrid swarm in which almost all individuals are of hybrid origin (Allendorf et al., 2001; Huxel, 1999), leading to the collapse of the European quail population. But this may not be just a theoretical threat. Hybrid individuals have been detected across Europe (Guyomarc'h, 2003; Rodríguez-Teijeiro et al., 1993), and genetic evidence for introgressive hybridization has been reported in Portugal (Amaral et al., 2007), Spain (Barilani et al., 2005; Sanchez-Donoso et al., 2012) and France (Chazara et al., 2010). However, these studies do not provide evidence of hybridization occurring in the wild. Consequently, Guyomarc'h (2003) called for changes in European Union law to stop the introgression of Japanese quail genes by banning the production and commercialization of Japanese quails as well as the breeding of hybrid quails for restocking purposes. Finally, the European Union Management Plan for the European quail (period 2009–2011) has stressed the need to ban Japanese/hybrid quail releases throughout Europe and across the rest of the European quail distribution (Perennou, 2009).

However, in spite of this persistent threat, Puigcerver et al. (2007, 2012) monitored the phenotype and song in wild quail populations in Catalonia over a period of 28 years (from 1984 to 2011) and failed to detect an increase in the proportion of hybrids. At the same time, genetic analyses over a 15 year period (1996–2010) showed that an increase over time in the proportion of admixed individuals cannot be detected (Sanchez-Donoso et al., submitted for publication). These data strongly suggest that despite the annual release of tens of thousands of hybrids, the introgression of Japanese quail alleles into the wild population is lower than initially expected.

The lack of an obvious increase in the proportion of admixed individuals can be explained by different factors:

(a) A very high mortality rate of restocked hybrids, which could be poorly adapted to life in the wild, because they lack the ability to protect themselves against cold weather, antipredator behavior and the ability to find appropriate food (Guyomarc'h, 2003). High mortality is probably also due to hunting, since restocked individuals are usually released just before the opening of the hunting season, which starts before the birds are habituated to the new environment.
(b) In spite of the lack of reproductive barriers in captivity, ecological or behavioral barriers could prevent admixture in natural conditions. There is no direct evidence of mating between European quails and farmed birds in natural conditions.
(c) Mixed pairs (or pairs of farmed animals mating in the wild) could suffer high chick mortality (this could be due to causes such as lack of parental care resulting from adaptation to captivity, or susceptibility to parasites), as has been observed for other Galliformes. For example, Duarte and Vargas (2004) found a chick mortality rate of 91% in restocked farm-reared females of red-legged partridge (*Alectoris rufa*).

The aims of this study were to investigate whether massive restocking with farm quails of hybrid origin quails in one of the European countries where restocking with farm-reared quails is legal (Spain) may lead to extensive admixture with European quails in the wild, and to study mating and productivity differences between female wild European quails and females restocked from game farms. Finally, we investigate how these differences could contribute to explain why the proportion of admixed individuals is not clearly increasing through time in the wild population. Our results showed decreased survival and fitness for the released birds. The results also emphasized how the putative consequences of restocking practices cannot be directly inferred from just the number of individuals released.

2. Material and methods

In Spain, the country where the study has been carried out, the Law 42/2007 of Natural Heritage and Biodiversity allows restocking with native species but not with non-native ones or hybrids. In this case, restocking with European quail is allowed, but not with Japanese quail or hybrids. In our study, we have used farm-reared quails intended for restocking that were certified as European quails by the regional Administration based on a veterinarian analysis. However, these farm-reared individuals turned out to be hybrids (Sanchez-Donoso et al., 2012). As indicated below (see “Field Experiment Design”) we obtained all necessary permits required to carry out this study, and the released farmed birds that survived to the end of the study as well as their chicks were recaptured.

2.1. Study area

The study was carried out in a 1 km^2 area in Northeast Spain (41°25′N, 1°23′E, 628 m above sea level), in a short and narrow valley with winter cereals (mainly barley and wheat) which constitutes a suitable habitat for European quail reproduction. The study area is surrounded by pine forest patches unsuitable for quails, but other cereal fields with quails are located less than 2 km away.

2.2. Field experiment design

The study was conducted during the breeding seasons of the period 2007–2010. The field work started around April 23rd, coinciding with the arrival of the first migrants, and finished around August 4th with the end of the breeding season due to the harvest of cereals, which implies the loss of the breeding habitat. On average, the field work lasted 102.5 days ($SD = 7.2$) per breeding season.

During the period 2007–2009, 20 walk-in funnel traps (144 cm × 67 cm × 87 cm) were randomly distributed along the edge of the cereal plots facing the cereal. Inside these traps we put a smaller cage containing a sexual decoy (Rodríguez-Teijeiro et al., 2003; Sardà-Palomera et al., 2011). In ten of these traps, the sexual decoy was a hybrid female from a game farm close to the study area, in Catalonia (Spain). These farm-reared hybrids showed small phenotypic differences when compared to their wild counterpart (as in Guyomarc’h, 2003): they had shorter wing and longer tarsus.

Groups of male European quail have been shown to attract both males and females (Sardà-Palomera et al., 2011). For this reason, the remaining ten traps were set in five pairs to replicate the aggregations of males observed in the field (normally involving 2 to 4 males, pers. obs.). Each pair was set in parallel separated by about 5 m. One trap of the pair contained one cage and the other one contained two cages, each cage containing one male decoy. Thus, each pair of traps represented an aggregation of 3 males (hereafter, male aggregation trap). Two of the male aggregation traps contained male European quail captured in neighboring areas and the remaining three contained male hybrid quail (Table 1).

In 2010, 19 walk-in funnel traps were placed in the study area. Six traps contained hybrid females as sexual decoy, while for comparative purposes three contained European quail females (captured in neighboring areas and kept in a pen for several months). No changes were made with regard to male aggregation traps (Table 1).

Traps were checked every 2–3 days to keep disturbance caused by researchers to a minimum. Water was supplied from an aseptic 8-l tank; food trays (with vitamin-supplemented wheat) had a capacity of 3 kg which allowed individuals to eat “ad libitum”. Cages had double wire to protect the quails against predators, and trap, which was protected against sunlight and rainfall, was sprayed at every visit with a mammal repellent. At every visit, food and water supplies were checked.

When a free-ranging male entered a funnel trap containing a female (either European quail or hybrid), we attached backpack radio transmitters (PIP-3 Biotrack button cell tags) to the females and we released them. The released female was replaced in the cage by another one of the same origin. Three days per week, the released individuals were monitored with a telemetry receiver (R1000, 148–174 MHz; Communications Specialists, Inc., Orange, CA) and a three-element Yagi antenna to locate them and to check if they had mated and nested, following Rodríguez-Teijeiro et al. (2003). We consider that a female mated successfully when she succeeded in establishing a pair bond with a male, no matter whether this female would succeed in nesting or not (she could be predated before that). For nesting success we understand the success in nesting by a female, no matter whether this nest was finally predated or not. At the end of the study, hybrid radio-tagged individuals were captured and returned to captivity to avoid the risk of genetic pollution. A total of 67 individuals were radio-tagged (51 hybrid females and 16 European quail females). Table 2 shows the number of radio-tagged females and the number of nests found in each year of study.

Once a nest was located, we determined the clutch size. After 10–12 days of incubation (more than 60% of the incubation period), we took the eggs from the nest to put them in a Masalles G-180 THP incubator (Sant Cugat del Vallès, Barcelona, Spain) and raised the chicks in captivity. This procedure allowed us to know the number of fertilized eggs, the number of hatched eggs and the number of chicks surviving in captivity during the critical period of the first three weeks of life (while flying abilities are not yet developed) for European quail and hybrid females. Also, it allowed us to know the number of nests preyed upon during the 10 days of the incubation period that were monitored. After the study was completed, these chicks remained in captivity in a large pen.

In addition, telemetry also allowed finding dead individuals. Since females tend to stay in the same area after their release, we defined the known survival time for each female as the time from the release until its death or until the study period reached its end due to harvest (at that time we proceeded to the recapture of the radio-tagged individuals).

Blood samples (100 μl) from the jugular vein were taken and used to genetically identify the origin of all individuals used in this study as European quails or hybrids following Sanchez-Donoso et al. (2012). All individuals were genotyped for 11 unlinked autosomal microsatellite loci (from Kayang et al., 2002, 2004) and genotypes were analyzed with STRUCTURE 2.3.2 (Pritchard et al., 2000) and NEWHYBRIDS 1.1 (Anderson and Thompson, 2002).

The study strictly followed the requirements of the Spanish Law 5/1995 of protection of animals used in experimentation and for

Table 1
Number and types of traps used each breeding season.

Sexual decoy in the trap	2007	2008	2009	2010	Total
One hybrid female	10	10	10	6	36
One European quail female	0	0	0	3	3
Hybrid male aggregation (3 males)[a]	3	3	3	3	12
European quail male aggregation (3 males)[a]	2	2	2	2	8
Total	15	15	15	14	59

[a] Each one of these is composed by two traps and were intended to imitate groups of males found in European quail populations.

Table 2
Number of females radio-tagged in each year of study. In brackets: number of nests found.

Type of female	2007	2008	2009	2010	Total
European quail females	1 (1)	2 (2)	2 (1)	11 (7)	16 (11)
Hybrid females	11 (8)	15 (4)	17 (8)	8 (1)	51 (21)

other research goals, and the Decree 214/1997, which regulates the use of animals for experimentation and for other research goals. The animal experimentation procedure for this study (number B9900035) was approved by the Ethics Committee on Animal Experimentation of the University of Barcelona and by the Autonomic Government of Catalonia ("Direcció General del Medi Natural", "Departament de Medi Ambient i Habitatge"), which has all the competences in this subject. Moreover, the University of Barcelona institutional code of good research practices was followed (Vicerectorat de Recerca, 2010).

2.3. Data analysis

2.3.1. Attraction ability

The attraction of wild quails to traps was analyzed with regard to the sex and origin (European quail or hybrid) of the individuals used as decoy. For each kind of trap (with a female decoy or a male aggregation decoy), the number of captures was modeled with Generalised Linear Models (GLM) as a function of the origin of the decoy in the trap. The number of captured wild quails was the response variable and the origin of the individuals acting as decoy was the independent variable of interest in both models. GLM models were constructed assuming a Poisson error distribution and log link function. They were tested for overdispersion (residual deviance/residual $df > 1$) and the heterogeneity of residuals was assessed by visual examination of the figures. The number of traps with each kind of decoy was also added as an offset term because they were not equally represented not only between years, but also within years (Table 1). As female decoys do not attract wild females, an additional independent variable was the sex of the captured quails only for traps simulating aggregations of males. In this model, "male aggregation trap" was also added as a random factor because males and females captured in the same male aggregation trap are not independent observations. Thus, this model was a Generalised Linear Mixed Model (GLMM). Since only in 2010 hybrid females and European quail females were simultaneously used as decoys, the comparison of their mate attraction was carried out only in this year. The interaction between explanatory variables was initially added to the model and removed if not significant. To establish the significance of the full model we used a likelihood ratio test, comparing its deviance with that of the null model including only the intercept. To test the significance of the interaction between explanatory variables we compared the deviance of the full model with that of a corresponding reduced model without interactions. These models allowed us to test whether there were differences in the number of quails captured per trap and breeding season considering the two different decoys. We could also investigate differences between sexes in the degree they are attracted by male aggregations.

2.3.2. Female mating and breeding success

A GLM was fitted for each variable related to female breeding success: (1) mating success (binary); (2) nesting success (binary); (3) clutch size (count); (4) fertilized eggs per laid egg (proportion); (5) nest predation (binary); (6) hatched eggs per fertilized egg (proportion); and (7) surviving chicks per hatched egg (proportion). Binary and proportion variables were fitted assuming a binomial error distribution and logit link function and clutch size was fitted assuming a Poisson error distribution and log link function. If a model presented overdispersion (residual deviance/$df > 1$), the corresponding variable was refitted using, depending of the case, a quasi-Poisson or a quasi-binomial error distribution.

A factor representing years and its interaction with origin (hybrid or European) were also initially added to the models to take into account changing conditions across years. These additional factors were removed from the final models if no significant effect was found.

Since female mating and nesting success are not only influenced by female ability to mate and nest but also by their survival probability, when modeling those variables, we added as covariate the female known survival time. Thus, this covariate allowed the models to test for differences in female mating and nesting success due to their origin independently of possible differences due to different mortality. These models also allowed us to estimate how long it takes for a female to achieve 95% probability of mating or nesting.

2.3.3. Survival

We used a Cox proportional hazards regression model (Cox, 1972) to assess the effect of female origin on its survival during the breeding season. This model assumed that the relative risk of death for both types of females (hybrid or European quail) remained constant over time. This risk was estimated by the model by considering the known survival periods. The model took into account if the end of the known survival period of each female was due to death or to the beginning of harvest. We also took into account the possible differences across years, and the interaction origin-year. If the interaction was not significant, it was removed from the models.

All analyses were conducted in R ver. 2.15.2 (R Core Team, 2012). GLM's models were fitted using the `glm()` function. Cox proportional hazards model was fitted using the function `coxph()` present in the package survival (Therneau, 2012). The significance of the factors added into a model were evaluated performing an analysis of deviance with the function `Anova()` available in the package car (Fox and Weisberg, 2011). In not overdispersed, the test for the analysis of deviance for GLM and Cox models was a likelihood-ratio (χ^2). In overdispersed GLM, an F-test was used. The differences between the levels of a significant factor were tested with the function `esticon()` from the package doBy (Højsgaard et al., 2013), The p values obtained from `esticon()` function were corrected using the `p.adjust()` function which controlled type I errors performing a false discovery rate correction (Benjamini and Hochberg, 1995). Confidence intervals (CI) of the estimated parameters were calculated for a 95% confidence level.

3. Results

Genetic origin of all individuals was unambiguously assigned and according to the expectations for wild (European quails) and farm (hybrid) quails (results not shown). Analyses with STRUCTURE AND NEWHYBRIDS produced consistent results.

3.1. Attraction ability

In 2010, hybrid females acting as sexual decoys in walk-in funnel traps attracted 2.58 times more wild European quail males than did female European quails (confidence interval, CI: 1.16–6.87, $\chi^2_1 = 5.52$, $p = 0.019$, Fig. 1a).

Male aggregation traps attracted 5.75 times more wild males than females (CI: 2.87–13.16, $\chi^2_1 = 20.85$, $p < 0.001$, Fig. 1b). European quail male aggregation traps were 2.55 times more visited, by both wild males and females (interaction not significant), than hybrid male aggregation traps (CI: 1.48–4.58, $\chi_1^2 = 11.03$, $p < 0.001$). The number of individuals entering the traps varied between years ($\chi_3^2 = 12.68$, $p = 0.005$); thus, in 2008 only 0.5 individuals were captured per trap, whereas in the rest of years 3.5 individuals were captured. These results strongly suggest that: (a) female hybrids are able to attract wild European quail males more effectively than female European quail; (b) male aggregations attract more males than females (although we do not have precise information about the sex ratio in the study area; males are known to be several times more numerous than females in the area); (c) hybrid male aggregations attract less wild quails (males and females) than European quail aggregations.

3.2. Female mating and breeding success

During the four years of the study, a total of 51 hybrid females and 16 European quail females were radio-tagged and monitored. The majority of the matings (94%) took place with a common quail male. For hybrid females, 61% (31) succeeded in mating, whereas 81% of European quail females (13) mated. Two hybrid females (4%) paired with freely roaming hybrid males. Mating probability was first modeled including "female origin" and "year" as factors, together with the "known survival period" of each female as covariate. Due to the low frequency of matings with free-roaming male hybrids, male origin was not included as a factor. This model showed a significant effect of year in female mating probability ($\chi_3^2 = 8.24$, $p = 0.041$). Mating probability in 2009 was higher than in 2007 ($\chi_1^2 = 4.16$, $p = 0.041$) and 2008 ($\chi_3^2 = 4.55$, $p = 0.033$). This mating probability also depended on the female known survival period ($\chi_1^2 = 37.68$, $p < 0.001$, Fig. 2a). But the model failed to show differences in the probability of mating between hybrid and European quail females ($\chi_1^2 = 0.93$, $p = 0.335$). On average, the model shows that mating probability reached 95% 40.8 days (CI: 25.3–72.8) after the release of the female (Fig. 2a).

Radio-tracking of the 51 hybrid females monitored showed that 21 of them (41%) nested, whereas of the 16 European quail females monitored, 11 of them (69%) succeeded in nesting. In the model for nesting success, neither female origin nor year had a significant effect. However, covariate "known survival period" had a significant effect. None of the interactions were significant. For these reasons, the final model was constructed using "female origin" as independent variable and "known survival period" as a covariate. According to this model, female origin did not have a significant effect on nesting success ($\chi_1^2 = 0.43$, $p = 0.510$), whereas known survival period showed a strong influence ($\chi_1^2 = 25.94$, $p < 0.001$, Fig. 2b). Females reached a 95% probability of nesting 49.6 days (CI: 27.7–99.2) after their release.

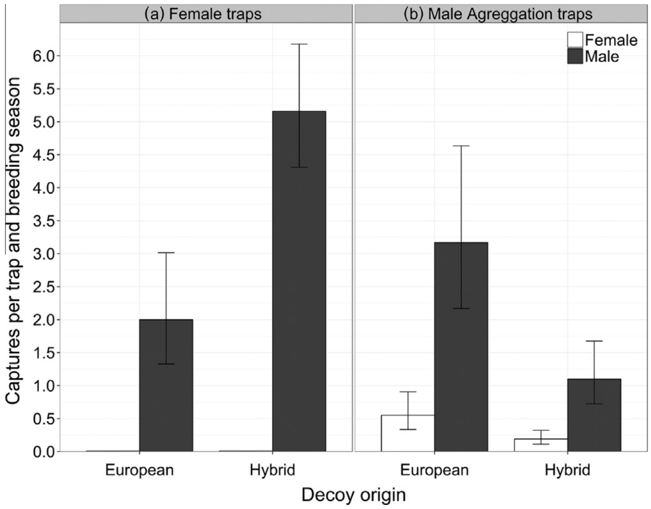

Fig. 1. Female and male wild quails captured per trap and breeding. season (mean ± se) as a function of decoy origin (European quail or hybrid originating from game farms) for two types of trap: (a) one female used as decoy (only 2010 data) and (b) a group of males used as decoy (from 2007 to 2010 data).

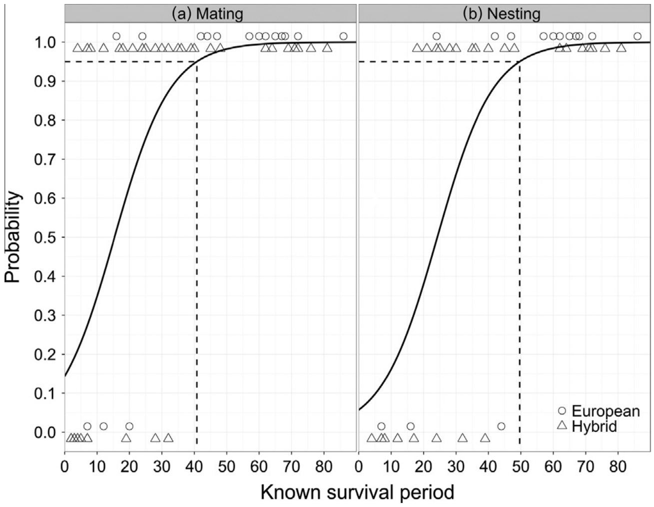

Fig. 2. Female mating (a) and nesting (b) probabilities as a function of the number of days in which females remained alive in the study area (known survival period). Adjusted functions: (a) $y = \text{inv.logit}(-1.79 + 0.12 \cdot x)$; (b) $y = \text{inv.logit}(-2.81 + 0.12 \cdot x)$. No differences were observed in these variables depending on female origin (European quail or hybrid). Dashed lines in both graphs show the time at which each Generalised Lineal Model predicts a mating or nesting probability of 95%. Observations of both European and hybrid female quails, from which the models were built, are represented by circles and triangles respectively.

Factor "year" showed a significant effect only on fertility ($\chi_3^2 = 10.11$, $p = 0.0182$). Fertility in 2009 was lower than in 2007 ($\chi_1^2 = 6.61$, $p = 0.010$). Since factor "year" did not have a significant effect on clutch size, hatching success or chick survival, it was excluded from these models. The final models did not show differences between hybrid and European quail females regarding clutch size ($\chi_1^2 = 2.74$, $p = 0.098$, Table 3), fertility ($\chi_1^2 = 0.01$, $p = 0.915$, Table 3), hatching success ($F_{(1,20)} = 2.67$, $p = 0.102$, Table 3), or chicks survival ($F_{(1,19)} = 0.46$, $p = 0.496$, Table 3).

Finally, the probability of having the nest preyed upon was higher for female hybrids ($\chi_1^2 = 4.31$, $p = 0.038$, Table 3), and did not change from year to year (initial model: $\chi_3^2 = 7.45$, $p = 0.059$).

3.3. Survival

The mortality of the released hybrid females was 73% by the end of the annual study season; 23 of them were killed by predators (62% of the dead hybrid females) and 14 were found dead from unknown causes. This mortality rate is much higher than for European quail females (31%, 3 of them killed by predators and 2 found dead from unknown causes), clearly indicating that hybrid females

Table 3
Nests variables. Means and 95% confidence intervals (in parentheses) for clutch size, fertility, hatching success, chick survival during the first three weeks of life, and nest predation probability for both hybrid and European quail female nests.

Variables studied	Hybrid female	European quail female
Clutch size	9.14 (7.30–11.50)	11.09 (9.24–13.18)
Fertility	0.97 (0.88–0.99)	0.97 (0.88–0.99)
Hatching success	0.93 (0.80–0.98)	0.83 (0.72–0.92)
Chick survival	0.85 (0.73–0.93)	0.79 (0.69–0.87)
Nest predation[a]	0.22 (0.08–0.41)	0[b]

[a] Significant differences depending on female origin.
[b] Confidence intervals cannot be calculated due to the lack of variability.

have lower survival than European quail females. The Cox proportional hazards regression model revealed that there is a significant difference in the relative risk of death for females of different origin ($\chi_1^2 = 17.55$, $p < 0.001$) and from different year ($\chi_3^2 = 12.47$, $p = 0.006$). More specifically, 2010 was the worse year. In 2010, the risk of death for a female was 5.47 (CI: 1.89–15.84) times higher than in 2007 ($\chi_1^2 = 9.84$, $p = 0.010$) and 3.54 (CI: 1.43–8.77) times higher than in 2009 ($\chi_1^2 = 7.5$, $p = 0.018$). The risk of death of a hybrid female, once released, was 7.22 (CI: 2.52–20.65) times higher than that of a European quail female. Fig. 3 shows the survival curves for European and hybrid quail females once released into the field. These curves show that 40.8 days after the release (time at which 95% of females have already mated, see Section 3.2) survival probability was higher for European quails (0.7, CI: 0.51–0.96) than for hybrids (0.29, CI: 0.19–0.44). Similarly, by the time 95% of females had nested (49.7 days after release, see Section 3.2), survival probability for female European quails was 0.68 (CI: 0.49–0.95), while for hybrids it was 0.27 (CI: 0.17–0.42).

4. Discussion

Previous studies have shown that, in captivity, there are not pre- or post-zygotic barriers preventing the interbreeding between European and Japanese quails or between European quails and hybrids (Derégnaucourt et al., 2002; Derégnaucourt and Guyomarc'h, 2003; Taka-Tsukasa, 1941). However, other studies suggest that the massive releases of farm bred quails have not resulted in a hybrid swarm (Puigcerver et al., 2007, 2012), indicating that perhaps ecological or behavioral barriers acting in the wild maintain separation (Pierotti and Annet, 1993). Nevertheless, this does not seem to be the case. Our results show, for the first time, that European quails and hybrids interbreed in the wild. Thus, the presence of admixed individuals in European quail populations in different countries during the breeding season may be explained not just as a result of restocking practices (as suggested by Puigcerver et al. (2007)), but also as the result of released hybrids reproducing in wild European quail populations.

Our results showed that female hybrids attract more wild common quail males than common quail females (Fig. 1). This could be due to the observation that they produced more rally calls from the trap, attracting more males (pers. obs.). This contrasts with the results by Derégnaucourt and Guyomarc'h (2003) who have shown that, in captivity, European quail females are very selective, emitting the greatest number of rally calls in response to mating calls from conspecific males, a lower number in response to mating calls produced by Japanese quail and an intermediate number responding to mating calls from hybrid males. A possible explanation for this difference could be that hybrid females produced more vocalizations because they were less stressed in a cage. Although we tried to minimize this effect by keeping wild-caught females in captivity for some months before starting the experiments, we cannot assess if the differences in behavior are associated to the hybrid origin or are result of adaptation to life in captivity. In addition, female hybrids and European quails (Fig. 2a) had a similar probability of mating. These matings occurred mostly with European quail males (94%), which were much more abundant than hybrids in the area (Puigcerver et al., 2007). Therefore, mixed pairs formed in the wild are most likely formed by a hybrid female and a European quail male.

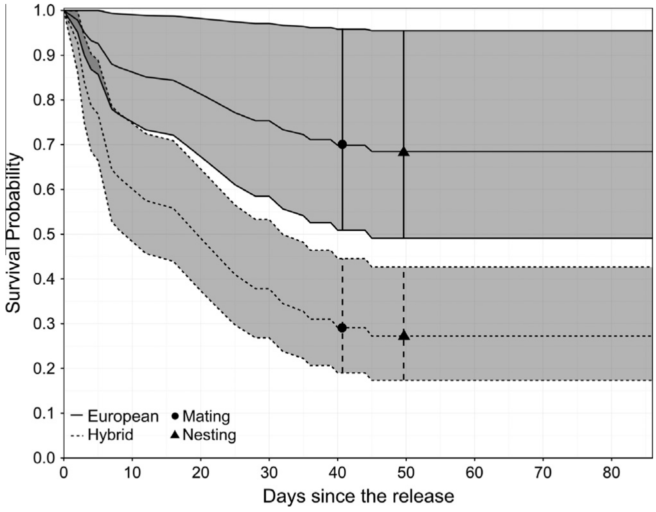

Fig. 3. Estimated survival probabilities (mean and 95% confidence intervals, based on a Cox proportional hazards regression model) for European (solid lines) and hybrid (dashed) female quails. Survival probabilities when mating (circles) and nesting (triangles) probabilities reach 95% (see Fig. 2) are represented to show that the probability of surviving long enough to mate and nest is higher for European quail females than for hybrid females.

We did not know in advance whether our artificial hybrid male aggregations could attract wild European quails of both sexes, but we expected so because Sardà-Palomera et al. (2011) showed that European male aggregations attracted both males and females as a consequence of the mating system of the species. Our results show that wild European quail males, which have a complex polygamous mating system (Rodríguez-Teijeiro et al., 2003), are more attracted by artificial European quail male aggregations than to those of hybrid males, suggesting that natural European quail male aggregations found in the wild (Guyomarc'h et al., 1998) may be reasonably preserved despite the release of hybrids.

With regard to the breeding success of the females released from the traps together with a male, restocked hybrid females had similar mating and nesting success rates to European quail females. As in captivity (Derégnaucourt et al., 2002), fertility, hatching rate and chick survival rate (within the first three weeks of life) are also very similar between hybrid and European females (Table 3), although we evaluated chick survival after hatching the eggs in captivity, and this may not reflect survival rates in the wild. As farm-raised hybrid quails have been selected for life in captivity, their chicks should be expected to survive better in captivity than chicks coming from wild common quails. Therefore, the lack of differences in survival in captivity of chicks from hybrid and common quail does not imply similar survival in the wild. Since mating preferences do not show the existence of strong pre-zygotic barriers and the breeding success does not seem obviously lower, other mechanisms must explain why the proportion of hybrids has not been increasing in European quail populations (Puigcerver et al., 2007, 2012).

The reason for this may be found, at least in part, in the lower probability for female hybrids to survive long enough to mate and nest (Fig. 3). In addition, the nests of hybrid females are preyed upon more often (Table 3). These differences could contribute to explain why the massive releases of farmed quail have not resulted in a hybrid swarm. Mortality rates of restocked farm-reared birds are usually higher than for native populations because they are poorly adapted to the natural environment and lack the ability to defend themselves against cold and to forage and select food in those conditions (Guyomarc'h, 2003). After hunting, predation is probably one of the main causes of mortality for farm-reared hybrid quails, as happens for other farmed game bird species (Casas et al., 2012; Duarte and Vargas, 2004; Gortazar et al., 2000; Putaala and Hissa, 1998) due to the lack of an antipredatory behavior (Guyomarc'h, 2003; McPhee, 2003). The same poor knowledge about the local conditions might explain the higher nest predation rate for hybrid females.

It is unsurprising that hunting is the main cause of mortality for restocked farm quails since these tend to be released immediately before the hunting season or before special hunting events. We do not have data about the mortality during the hunting season of hybrid quails that were present during the breeding season compared to that of European quails. However, it seems reasonable to consider that the same lack of anti-predatory behavior would favor a higher hunting pressure on hybrids, thus increasing their mortality rates, as suggested by Guyomarc'h (2003), who reported 75% of restocked individuals from a total of 4959 hunted quails in a large hunting estate (64,000 ha) in France.

Previous field surveys spanning a long time period (Puigcerver et al., 2007, 2012) did not show an increase in the proportion of hybrids detected in nature. This could apparently suggest that restocking European quail populations with hybrid quails does not represent a conservation problem. However, our results show that the two groups are not genetically isolated and that interbreeding occurs in the wild. These observations seem incompatible, but can be explained by the lower chances of survival and nesting of hybrid females in the wild, as well as their higher rate of nest predation. This translates into a lower fitness for the quails of hybrid origin. This lower fitness could be due either to carrying maladaptive alleles of Japanese quail origin or to the adaptation to life in captivity. This result is in agreement with the results of a 15-year-long genetic study showing that the proportion of hybrid individuals in the population was not increasing over time, but also emphasizing that the results were compatible with a slow rate of introgression if hybrids had reduced fitness (Sanchez-Donoso et al., submitted for publication). In this case, a slow introgression of farm alleles could be expected, which would translate into extensive admixture in some generations. Our results confirm reproduction and also lower fitness for the hybrids, and so we expect a progressive build-up of alleles of farm origin into the wild population. This is likely to affect the genetic composition of European quail populations, which could result in a decrease in the adaptive and evolutionary potential of the species.

Our results show that in the case of small populations of European quails, the interbreeding with restocked hybrids could represent a loss of homospecific matings and a reduction of the potential for population growth. It is paradoxical that restocking practices for this species, instead of increasing the long-term chances for survival, could result in a reduction of the genetic effective population size and evolutionary potential (Ryman and Laikre, 1991) and could limit population growth. In addition, captive breeding of European quails for restocking could make sense if the species was under conservation concern; however, it is currently classified as “least concern” by the IUCN. In this case, restocking practices make no sense from the conservation point of view, especially considering that the introduction of farm-reared individuals in wild populations may lead to other threats, such as transmission of pathogens, that could cause substantial mortality in the native populations (Peeler et al., 2006).

The results obtained also emphasize how the putative consequences of restocking practices cannot be directly inferred from just the number of individuals released.

As reported in Casas et al. (2012) for the red-legged partridge (*A. rufa*), public Administrations should ensure that releases for hunting purposes do not represent a threat for the conservation of native species and populations. These authors suggest some management strategies to control genetic introgression and help the original population structure to recover: (1) stop releases of hybrid birds; (2) implement effective inspection procedures for farms providing individuals for restocking; (3) promote management plans avoiding releases in areas where restocking programmes have not yet been performed, and where there is reliable evidence that populations consist of non-admixed individuals.

It is important to highlight that the results of this study suggest that some restocked individuals could have been interbreeding with native European quails during the last decades. Until we better understand the long-term consequences of the release of quails with – potentially – maladaptive genes, we believe all restocking programs involving Japanese quails or their hybrids within the natural distribution range of the European quail should be banned.

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Detecting slow introgression of invasive alleles in an extensively restocked game bird

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Interbreeding of two species in the wild implies introgression of alleles from one species into the other only when admixed individuals survive and successfully backcross with the parental species. Consequently, estimating the proportion of first generation hybrids in a population may not inform about the evolutionary impact of hybridization. Samples obtained over a long time span may offer a more accurate view of the spreading of introgressed alleles in a species' gene pool. Common quail (*Coturnix coturnix*) populations in Europe have been restocked extensively with farm quails of hybrid origin (crosses with Japanese quails, *C. japonica*). We genetically monitored a common quail population over 15 years to investigate whether genetic introgression is occurring and used simulations to investigate our power to detect it. Our results revealed that some introgression has occurred, but we did not observe a significant increase over time in the proportion of admixed individuals. However, simulations showed that the degree of admixture may be larger than anticipated due to the limited power of analyses over a short time span, and that observed data was compatible with a low rate of introgression, probably resulting from reduced fitness of admixed individuals. Simulations predicted this could result in extensive admixture in the near future.

Keywords: *Coturnix*, hybridization, introgression, modeling, restocking, temporal sampling

INTRODUCTION

Interspecific hybridization and its evolutionary consequences are often studied by analysis of samples obtained over a relatively short time period, without aiming to monitor genetic changes over time. This approach may allow identification of admixed individuals, but it may not be informative about the evolutionary impact of introgressive hybridization. For instance, the presence of first generation hybrids does not necessarily imply the spread of invasive alleles in the native population (introgression). When hybrids have reduced fitness or are infertile, hybridization might not have a large impact on the genetic composition of the parental species. Hybridization in such cases will still imply a loss of intraspecific mating opportunities, reducing the effective breeding population (accelerating genetic drift). Alternatively, if alien alleles do introgress into the gene pool of the native population, hybridization can have long-term fitness consequences (Rhymer and Simberloff, 1996; Haygood et al., 2003).

To estimate the impact and rate of admixture, the proportion of first generation hybrids and subsequent backcrosses can be assessed from analysis with a large panel of molecular markers (Vähä and Primmer, 2006). If the analyzed population sample includes individuals with varying degrees of admixture, this implies that first generation hybrids are interbreeding with the parental species, leading to alleles from one species introgressing into the gene pool of the other (Rhymer and Simberloff, 1996). However, such data can only offer a snapshot of the evolutionary process, without informing about changes over time. Furthermore, inference about long-term effects are susceptible to limitations due to small sample sizes (is the sample representative of the entire population?) and low frequency of interbreeding (is it possible to estimate the frequency of rare interbreeding events?). In addition, an important and often overlooked source of error is associated with varying levels of admixture from year to year (is the level of admixture changing over time?).

Samples obtained over several generations can help to assess temporal changes in the level of admixture and can offer a long-term view on the introgression of invasive alleles, more meaningful from a conservation and evolutionary point of view. The robust characterization of slow introgression requires the use of long time series to allow tracking the arrival of new alleles into the population.

Common quail (*Coturnix coturnix*) populations offer the opportunity to obtain long temporal series of samples to investigate the consequences of admixture of divergent lineages. Its European breeding population is estimated to consist of several millions of individuals (8,400,000–14,100,000, BirdLife

International, 2004) that move nomadically across the breeding range looking for suitable habitat (Puigcerver et al., 1989; Rodríguez-Teijeiro et al., 2009) and mates (Rodríguez-Teijeiro et al., 2006). This game species is of high economic and recreational interest in southern Europe; millions of quails are killed every hunting season (Guyomarc'h, 2003). Due to this interest, every year wild populations are extensively restocked prior to the game season with farm-reared quails. For example, in Catalonia (Northeast Spain), the wild common quail population has been estimated to range between 5000 and 20,000 males (Rodríguez-Teijeiro et al., 2004), and about 57,000 farm-reared individuals have been released yearly from 1990 to 2011 (Hunting order plans of the Regional Government of Catalonia). The number, size, timing, and location of the releases change from year to year and very limited information is available for most of them. While quails for game restocking are sold as be common quail, recent genetic analyses have shown that they are, actually, hybrids between common quails and domestic Japanese quails (*C. japonica*) that have been interbreeding for a number of generations in game farms (Sanchez-Donoso et al., 2012).

Captive breeding represents a general relaxation of selective forces and promotes traits that are maladaptive in the wild (Lynch and O'Hely, 2001; Frankham, 2008; Baskett and Waples, 2013). Thus, supplementation programs based on the release of captive-bred individuals have the potential to deeply alter the genetic composition of native populations (Ford, 2002). These large-scale releases can lead to loss of genetic diversity, breakdown of adaptations and change in the population genetic structure (Eldridge and Naish, 2007; Laikre et al., 2010; Marie et al., 2010). Specifically, several authors have pointed out that restocking with domestic Japanese quails and hybrids can pose serious threats to the genetic integrity and survival of common quails (Guyomarc'h, 2003; Barilani et al., 2005; Chazara et al., 2006, 2010; Puigcerver et al., 2007). Their interbreeding can lead to introgression of maladaptive domestic Japanese quail alleles into the common quail population, potentially leading to alterations or loss of migratory behavior, and a decline in fitness in native quails (Guyomarc'h, 2003). A decrease in average fitness means a lower population growth rate, which could even become negative. However, even in the case that fitness of admixed individuals was not diminished, the massive releases of farm-reared quails could lead to a counter-intuitive reduction in the effective population size through Ryman-Laikre effects: a large portion of the breeding population could potentially derive from a small number of breeders from farms, resulting in a reduction of the genetic diversity and evolutionary potential (Ryman and Laikre, 1991). As a result of these concerns, the EU Quail Action plan has emphasized the need to preserve common quail genetic integrity (Perennou, 2009).

Several lines of evidence support the existence of introgressive hybridization from farm quails into wild common quails. Japanese and admixed quails have vocalizations that differ from those of common quails, with first generation hybrid male crows being intermediate between domestic Japanese quail and common quail (Derégnaucourt et al., 2001), and this has often been used to characterize the identity of free-ranging quail males (Guyomarc'h and Guyomarc'h, 1996; Collins and Goldsmith, 1998; Barilani et al., 2005; Puigcerver et al., 2007). Using this approach, hybrid quails were first detected in Catalonia by members of our research group in 1990 in an area close to the study site (Rodríguez-Teijeiro et al., 1993). Since then, and following the popularization of farm quails for restocking, hybrid individuals have been detected, using genetic methods, in common quail breeding areas in multiple European countries including Portugal, France, Italy, and Spain (Rodríguez-Teijeiro et al., 1993; Guyomarc'h, 2003; Barilani et al., 2005). Experiments in captivity have shown that common and Japanese quails present only partial mate discrimination (Derégnaucourt and Guyomarc'h, 2003), and that the two species interbreed easily and hybrids do not have reduced survival (Derégnaucourt et al., 2002). These observations suggest that Japanese quail alleles may have been introgressing into the European common quail population since the early 1990s.

However, the occurrence and frequency of interbreeding under natural conditions between native and released quails is not known. Furthermore, while hybrids do not show reduced survival in captivity, selection against hybrids in the wild could potentially lead to strongly reduced rates of introgression. For this reason, it has been suggested that individuals identified in nature as hybrids could correspond to farm released animals, without requiring any hybridization in the wild at all (Puigcerver et al., 2007, 2012). This is in part supported by the radio-monitoring of female farm quails, which showed an increased mortality and predation risk for their clutches (Puigcerver et al., 2014). However, this same study also revealed their success at attracting wild males and reproducing in the wild. Consequently, it remains unclear to what degree these massive releases are affecting European common quail populations.

Here we studied the genetic composition of wild quails sampled in a single locality in Northeast Spain across a 15-year period (about 15 generations), to evaluate to what extent the releases of farm quails are affecting the genetic composition of the local population. We combined genetic analyses with simulations to test the power of our markers to detect introgression of farm alleles. We also carried out simulations assuming different levels of fitness for the admixed individuals to investigate which values are compatible with the observed data. We then used these values in further simulations to forecast changes in the genetic composition of the local population in the near future under different scenarios.

MATERIALS AND METHODS

SAMPLES AND DATASETS

The field survey was carried out in an area of about 1 km^2 in Northeast Spain (Mas Esplugues, 41° 25′ N, 1° 23′ E, 628 m above sea level), a short, narrow valley with winter cereal crops (mainly barley and wheat) which constitute suitable breeding habitat for quails. The breeding population has been continuously monitored for over 25 years in the framework of a long-term project to study the biology and mating behavior of common quails (José D. Rodríguez-Teijeiro. and Manel Puigcerver). It is an open population and individuals continuously arrive during the breeding season, spend some time, and leave in search of suitable habitat patches and mates (Puigcerver et al., 1989; Rodríguez-Teijeiro et al., 2006, 2009). This is a game area, and the hunting season starts in mid-August, after the end of the breeding season (from about mid-April to early July). The great majority of quails breeding in the area were estimated to be 1 year old, with very few breeders being aged two or more (Puigcerver et al., 1992). For this reason, we considered generation time to be about 1 year.

A feather or a blood sample was obtained from 468 wild quails, captured during several breeding seasons (i.e., prior to the majority of farm releases), following protocols approved by an authorized Ethics Committee (for sampling methods see Puigcerver et al., 2007). Our sample represents a very large proportion of the quails breeding in the study area every season, but is very difficult to have precise count of the number of breeders. These wild quails were genotyped at 9–13 autosomal microsatellite loci. Genotypes were generated as three different datasets (**Table 1**) produced by different researchers, in different labs and using different instruments. Dataset I included quail samples collected during the breeding seasons of 1996 and 1997, genotyped by Ines Sanchez-Donoso. A subset of these genotypes was included in a previous study (Sanchez-Donoso et al., 2012). Dataset II included quail samples collected during the breeding seasons of 1999, 2002 and 2005, genotyped by Jisca Huisman as part of her MSc thesis at the University of Uppsala, Sweden (Huisman, 2006). Dataset III included quail samples obtained during the breeding seasons from the period 2007–2010, genotyped by Ines Sanchez-Donoso for this study.

Quail samples from Spanish game farms were analyzed as reference for the three wild quail datasets: 52 individuals from 5 farms (farm set A, sampled in 2009 and 2010, included in Sanchez-Donoso et al., 2012) were used as a reference in analysis with datasets I and III, while 29 individuals from 3 game farms (farm set B, sampled in 2006) were used with Dataset II (see **Table 1**). All farm individuals were intended for restocking. In a previous study, no genetic differentiation was observed among individuals from the different farms (Sanchez-Donoso et al., 2012, including some of the samples studied here), suggesting that all of them derive from the same stock and/or that the exchange between them is extensive. For this reason, and considering the large size of the overall population in farms and the lack of precise information about the origin and number of breeders, in the simulations we assumed that the allele frequencies in the farm population remained constant throughout the study period (see below).

Joining the three datasets offered the opportunity to compile a time series spanning 15 years. They could not be fully integrated, as the sets of genetic markers used were not identical (see below) and genotype scoring was done by different researchers. Since many DNA samples derived from small feathers, each providing only a small amount of DNA, it was not possible to re-analyze them under a unique set of conditions. Datasets I and III could be merged when restricting analyses to the markers used in both cases after ensuring the consistency of the allele call by genotyping a panel of reference samples.

LABORATORY PROCEDURES

For some individuals, DNA was extracted from blood samples (100 μl, stored at −20°C in 95% ethanol until analysis) using DNeasy Blood and Tissue Kit (Qiagen) following manufacturer's protocols, or following standard phenol-chloroform extraction procedures. For the rest of individuals, DNA was extracted from feathers (stored at −20°C in 95% ethanol) using a Chelex resin protocol (Walsh et al., 1991).

To characterize the maternal ancestry of quails and to determine directionality of hybrid matings, a 453 base pair (bp) fragment of the 5′ of the mitochondrial DNA (mtDNA) control region was sequenced for 26 wild quails from Dataset II (7 from 1999, 7 from 2002 and 12 from 2005) and the 29 farm quails from farm set B. The wild quails were selected to include diverse phenotypes and, potentially, admixture. Having admixed individuals of farm and wild origin could allow inferring if both sets of admixed birds had the same maternal origin. DNA samples were amplified via polymerase chain reaction (PCR) using primers PHDL and PH-H521 (Barilani et al., 2005). Sequencing reactions were carried out using the same forward and reverse primers, and were run on a MegaBACE 1000™ instrument (Amersham Biosciences) following manufacturer's protocols. From the two sequences obtained, a consensus was built using SEQUENCHER v. 5.1 (Gene Codes Corporation, Ann Arbor, MI, USA).

Individuals were genotyped for autosomal microsatellite loci originally developed for Japanese quail (Kayang et al., 2000, 2002) (genotype data sets in Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.kv733). Some loci (marked below with an asterisk) were analyzed for datasets I and III using primers modified to facilitate multiplexing (see Supplementary Material Table S1). The number and combination of loci typed varied between datasets. Dataset I was genotyped for 9 unlinked markers (each marker was located on a different chromosome): GUJ01, GUJ17*, GUJ39, GUJ44, GUJ57*, GUJ65*, GUJ85, GUJ93, and GUJ97*. Dataset II was typed for these same markers, excluding GUJ85, plus five additional markers: GUJ14, GUJ28, GUJ33, GUJ62, and GUJ68 (13 loci in total). Although some loci used for Dataset II are on the same chromosome (Kayang et al., 2004), previous analyses on this dataset showed they are not in linkage disequilibrium (Huisman, 2006). Dataset III was genotyped for the same 9 loci as Dataset I, plus GUJ28 and GUJ74 (11 loci in total, located on different chromosomes). Since datasets I and III were scored by the same researcher, and using a panel of reference samples to ensure that all allele calls were consistent, they could be analyzed together for the 9 shared markers.

Some markers were PCR-amplified in a multiplex, while others were amplified separately. Detailed protocols are available upon request. PCR products from dataset I and III, and from their reference farm set, were electrophoresed on an ABI 3730 sequencer (Applied Biosystems) following manufacturer's protocols, and alleles were sized and scored using the software GeneMapper v3.5 (Applied Biosystems). Electrophoresis of Dataset II PCR products, and of their reference farm set, was performed on a MegaBace 1000 capillary instrument (GE Healthcare) following manufacturer's protocols. The associated software, Genetic Profiler 2.2, was used for the allele calling.

DATA ANALYSIS

Mitochondrial DNA sequences were aligned with Muscle in MEGA v5.1 (Tamura et al., 2011). The same program was

Table 1 | Genetic diversity (averaged across loci, ± standard deviation) of wild and farm quails at autosomal microsatellites.

Dataset	Year	N	N loci	Alleles per locus (average)	A	PIC	H_e	H_O
I	1996	42	9	17.2 ± 6.2	15.3 ± 4.9	0.87 ± 0.07	0.90 ± 0.02	0.88 ± 0.02
	1997	51	9	18.0 ± 5.3	15.5 ± 4.1	0.88 ± 0.06	0.90 ± 0.02	0.87 ± 0.02
II	1999	31	13	13.5 ± 4.5	12.4 ± 4.1	0.81 ± 0.13	0.84 ± 0.03	0.74 ± 0.02
	2002	30	13	14.0 ± 5.5	12.7 ± 4.6	0.81 ± 0.15	0.84 ± 0.04	0.75 ± 0.02
	2005	31	13	13.6 ± 4.2	12.4 ± 3.7	0.83 ± 0.10	0.86 ± 0.03	0.75 ± 0.02
III	2007	50	11	19.6 ± 6.6	15.9 ± 4.6	0.89 ± 0.05	0.90 ± 0.01	0.84 ± 0.02
	2008	33	11	16.6 ± 4.8	15.3 ± 4.0	0.87 ± 0.07	0.89 ± 0.02	0.84 ± 0.02
	2009	97	11	21.7 ± 6.6	15.3 ± 4.1	0.88 ± 0.05	0.90 ± 0.01	0.84 ± 0.01
	2010	103	11	23.6 ± 8.2	15.9 ± 4.4	0.89 ± 0.06	0.90 ± 0.01	0.85 ± 0.01
Farm set A		52	9	14.6 ± 4.6	12.0 ± 3.6	0.80 ± 0.07	0.83 ± 0.02	0.80 ± 0.02
Farm set A		52	11	14.0 ± 4.3	11.4 ± 3.3	0.77 ± 0.11	0.82 ± 0.02	0.80 ± 0.02
Farm set B		29	13	11.4 ± 4.3	10.4 ± 3.6	0.80 ± 0.08	0.81 ± 0.03	0.73 ± 0.02

The entire sample is composed of three datasets genotyped independently and using partially different sets of markers (see text). Reference farm samples are the same for datasets I and III (farm set A), but with different number of loci. Farm set B is constituted by reference farm samples used with Dataset II. N, number of samples genotyped; N loci, number of loci genotyped; A, allelic richness; PIC, Polymorphic Information Content; H_e, expected heterozygosity; H_o, observed heterozygosity.

used to compare sequences and for phylogenetic reconstructions using maximum likelihood (ML) with the Tamura-Nei model of sequence evolution and a gamma distribution rate of variation across loci, and neighbor-joining (NJ) based on the maximum composite likelihood model. Support for nodes was assessed with 500 bootstrap replicates.

Average number of alleles per locus and allelic richness (mean number of alleles per locus corrected for the minimum sample size; Leberg, 2002) were calculated using FSTAT version 2.9.3.2 (Goudet, 1995). In order to measure marker informativeness we calculated the Polymorphic Information Content (PIC; Botstein et al., 1980) which takes into account the number of alleles per locus and the frequency of these alleles, using EXCEL MICROSATELLITE TOOLKIT 3.1.1 (Park, 2001). We used the same software to calculate observed (H_o) and expected (gene diversity, H_e) heterozygosities (Nei, 1987). Overall differentiation between wild and farm samples (for each sample set) was estimated using Weir and Cockerham's (1984) estimate of the fixation index F_{st} in GENETIX (Belkhir et al., 1996–2004), Hedrick's (2005) standardized measure of genetic differentiation (G'_{ST}) and Jost'D estimator of actual differentiation (D_{est}, Jost, 2008) with SMOGD (Crawford, 2010).

Genetic composition of wild and farm quails was analyzed using the Bayesian clustering procedure implemented in STRUCTURE 2.3.4 (Pritchard et al., 2000). Other likelihood approaches could have been used to infer population structure, but we chose the approach implemented in STRUCTURE because its use is widespread for hybridization studies, and because it is easy to scale up to analyze thousands of simulated datasets across multiple computer processors (see below). Since our goal was to track the introgression of farm alleles into the wild population we chose to always use the same analytical approach and we avoided using approaches aimed at identifying individual hybrids and backcrosses (for example NEWHYBRIDS, Anderson and Thompson, 2002).

For a given number (K) of genetically distinct clusters, STRUCTURE provides an estimate of the fraction of the genome (q_i) that belongs to each one of the clusters. Analyses were performed under the admixture model, as each individual may have ancestry in more than one parental population, and were run with correlated allele frequencies and without population or sampling location information (USEPOPINFO and LOCPRIOR set to 0). Analyses were done for $K = 2$ to separate farm and wild clusters, since previous analyses showed that this is the highest level of population structure (Sanchez-Donoso et al., 2012). Analyses were run for 100,000 steps following a burn-in period of 100,000 steps, and were replicated five times. Results did not vary significantly with longer runs and likelihood values were observed to converge during the runs. STRUCTURE proportion of membership to each cluster (q_i) and 90% posterior probability intervals were estimated for each individual. For consistency across real and simulated datasets, STRUCTURE was always run using these parameters.

All STRUCTURE analyses included wild-caught quails together with quails from farms used as reference to allow the identification of two clusters that would facilitate tracking farm and wild origins. The value of $q_1 = 0.90$ was used as an arbitrary threshold above which individuals were assigned to cluster i as pure (see Barilani et al., 2005). Individuals that showed lower values ($q_i < 0.9$ for both clusters) were considered as potentially admixed.

To study if there was an increase through time in the proportion of farm alleles in the wild population, we focused on the estimated yearly proportion of wild-caught individuals classified as admixed ($0.1 < q_{\text{farm}} < 0.9$) in the population, rather than the degree of admixture for each individual. The classification as being admixed or not for each individual was the response variable (binary) and was analyzed using Generalized Linear Models (GLM). Sampling year was the continuous explanatory variable, since the interest was to test whether there was a trend in the ratio of admixed individuals over time. A binomial error structure and logit link function was used, and the models were fitted in R (version 2.15.2; R Core Team, 2012) using the glm() function. The factor "dataset" was included to account for differences due to investigator and marker set when analyzing the three different data sets. All models were tested for overdispersion to avoid false positives and for collinearity between "year" and "dataset." With these models we investigated if the probability of being classified as admixed was different between years and, if so, if it was increasing over time.

SIMULATION OF FARM ALLELE INTROGRESSION

Initial analyses of the observed data showed low levels of admixture between wild and farm quails. To test if these analyses had enough statistical power to detect an increase of admixture over time, we performed STRUCTURE analyses on data simulated with a series of scripts (available at http://www.consevol.org). These simulations were intended to represent the population at the breeding season (when field surveys took place). The scenarios that we investigated involved varying the proportion of farm individuals in the breeding population every generation, and different fitness values for admixed individuals born in the wild.

We first simulated genotypes for a wild population of quails where every generation a certain proportion of the potential breeders originated from farm releases. The simulations thus portrayed the composition of our study population during the breeding season. Because wild quails in the area show high mobility (Rodríguez-Teijeiro et al., 1992) and are part of a much bigger population (millions of individuals), we simulated a large population with a constant size of 10,000 breeding individuals to minimize the effects of random genetic drift. Genotypes at generation 0 were generated by randomly drawing two alleles for each locus according to their observed frequency. Starting allele frequencies were based on Dataset III, after excluding individuals whose genetic composition was clearly dominated by farm alleles ($q_{\text{farm}} \geq 0.40$ according to STRUCTURE analyses of that dataset alone; this threshold was selected to exclude individuals born in farms—q_{farm} close to 1—and first generation admixed individuals—q_{farm} close to 0.5). Similarly, 10,000 genotypes were generated for farm quails by randomly drawing pairs of alleles for each locus based on the observed allele frequencies from farm set A. Since we had selected unlinked markers (see above), alleles at one locus did not influence allele selection at other loci (markers are assumed to recombine freely). Also, since our simulations only cover a limited time span (a few decades at the most), we did not consider the effect of mutations. Although Datasets III and farm set A may have an allelic composition different to the allelic composition of the populations before any admixture had taken place in the wild, we consider that these datasets may be an appropriate overall representation of the degree of differentiation between wild and farm quails and can offer useful insight on how introgression can be expected to proceed.

Successive generations (up to 20 generations to parallel the time period since the first hybrids were detected in the study area) of wild quails involved 10,000 new individuals per generation. To simulate the continuous restocking of quails, each of these new individuals had a certain probability of originating from farm releases (between 1 and 5%, according to the genetic analyses of our field samples; see below) and, in this case, was directly drawn from the file of farm genotypes. The genotypes for the rest of the individuals were generated by drawing alleles from two parental genotypes randomly selected from the previous generation. The simulation was run for 20 generations. We assumed non-overlapping generations and a generation time of 1 year. We did not assign sex to the genotypes and any pair of genotypes could act as parents for a genotype in the following generation. A schematic representation of these simulations is portrayed in **Figure 1**.

Initial simulations assumed that all individuals had the same chances of producing offspring in the following generation (fitness = 1) independently of their origin (farm, wild, or admixed). However, it is likely that farm individuals contribute maladaptive traits (Lynch and O'Hely, 2001; Frankham, 2008; Baskett and Waples, 2013), and that they and their offspring have a lower fitness. To introduce this factor into a second set of simulations, we tracked for each individual the proportion of the genome expected to come from the farm population (1 for farm individuals, 0 for pure common quails, 0.5 for first generation admixed individuals). Each time that a new genotype was generated and one or both of the parents had a proportion of farm ancestry of 1 or 0.5, its probability of survival to the breeding season was determined by a predefined fitness value varying between 1 and 0 (1 when the probability of contributing to the next generation for admixed individuals was the same as for wild common quails, 0 when they never contributed; in the simulations we used the following fitness values: 1, 0.8, 0.6, 0.4, or 0.2). To simplify, we assumed that the fitness value was the same independently of the individual's proportion of farm ancestry. If the genotype did not survive, it was not included among the genotypes for a given generation (this would only include those that could participate as potential breeders), and a completely new genotype was generated to replace it and to keep the total population size constant. We assumed that lower fitness affected the chances of survival for the first two generations of admixed individuals, which are the ones that are most likely to suffer loss of extrinsic or intrinsic adaptation (i.e., presence of maladaptive alleles or breakup of co-adapted gene complexes, Laikre et al., 2010). After two generations genetic incompatibilities may be less likely and those individuals were considered as if they were pure wild quails for subsequent generations (proportion of farm ancestry: 0).

ANALYSIS OF SIMULATED DATA AND POWER TESTS

For a visual examination of the level of introgression in the simulated population, we randomly sampled individuals from multiple generations (50 individuals per generation for a few predefined generations) and analyzed them with STRUCTURE ($K = 2$) together with a simulated farm dataset (50 farm

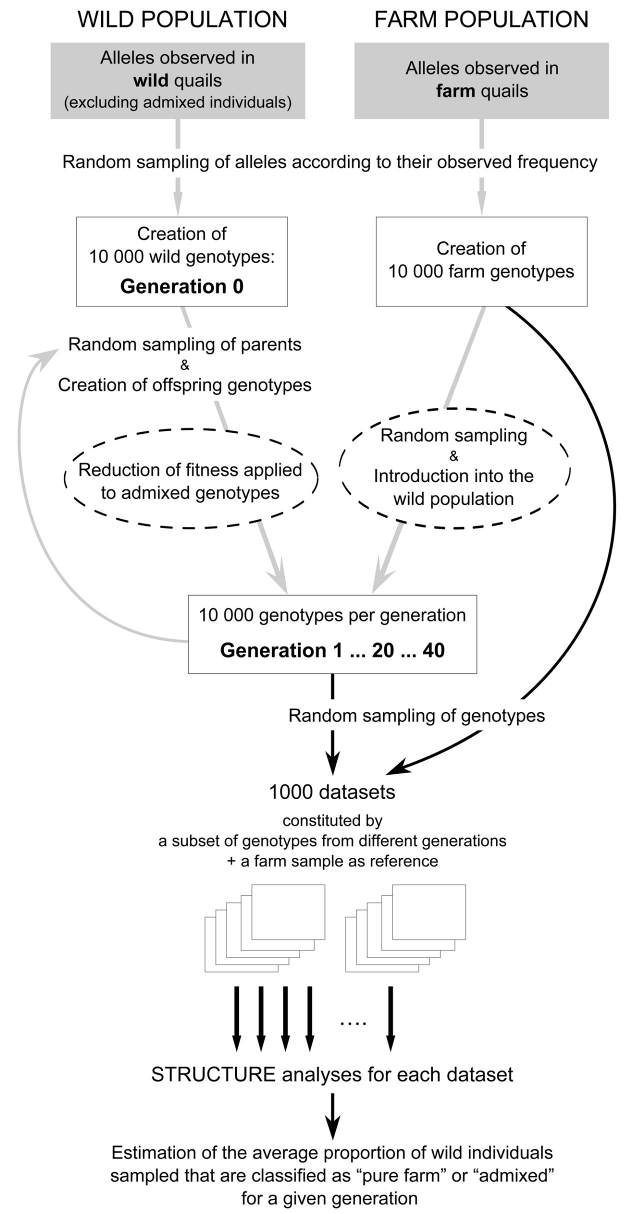


FIGURE 1 | Schematic representation of the population simulations (gray arrows) and analysis of simulated data (black arrows). Boxes represent simulated populations or datasets. Simulations were used to assess the effect over time (across different generations) of the proportion of potential breeders of farm origin and varying fitness of admixed individuals (variables affecting the population simulations, marked with discontinuous circles). The first set of simulations was carried out assuming that all individuals, independently of having farm ancestry or not, had the same probability of contributing to the next generation (fitness = 1). For details, see text.

individuals). The same analysis was also carried out breaking the temporal series into three sets of samples, covering a shorter time span each, mirroring the three datasets resulting from the field work (**Table 1**). In addition, in order to compare the results from the simulated data with the results obtained for the field datasets and to assess the power to detect introgression with the markers used, we prepared a program to replicate our field sampling pattern. Since the first hybrid individual in a neighboring area was detected by its vocalizations in 1990 (Rodríguez-Teijeiro et al., 1993), several years before the first of the wild-captured quails analyzed in this study, individuals from 1996 were assumed similar to the 5th simulated generation, 1997 to the 6th, and so on. The program produced 1000 sets of simulated data extracting genotypes from the simulated population of 10,000 individuals per generation and with the same number of individuals per year as in real data (see **Table 1**). Each one of these simulated datasets was analyzed with STRUCTURE as described above (**Figure 1**).

For each STRUCTURE run we calculated the proportion of simulated genotypes that were not classified as pure wild ($q_{\text{farm}} > 0.10$; admixed or pure farm). The 95% confidence interval for this proportion, for a given combination of potential breeders coming from farms and fitness value, was based on the 2.5th and 97.5th percentiles over each set of 1000 STRUCTURE runs. The proportion observed for the real data was then compared with these confidence intervals to assess which simulated scenarios were compatible with the real data (analysis of field samples).

RESULTS

To clarify analyses and results, a flow chart of the analyses and conclusions reached with them is provided as Supplementary Material (Figure S1).

MITOCHONDRIAL DNA

Since mtDNA is maternally inherited and common quail and Japanese quail have well differentiated sequences (Barilani et al., 2005), this marker is useful for establishing directionality in hybrid matings. ML and NJ phylogenetic trees showed the existence of two clearly differentiated clades (**Figure 2**). The control region sequences of 29 farm quails revealed 8 haplotypes and all of them were located within the same clade, with pairwise differences ranging between 1 and 10 substitutions (prefix F in **Figure 2**). One of the haplotypes (F1W1 in **Figure 2**) was observed in 22 individuals, while the remaining 7 were observed in one individual each. In contrast, the diversity observed in wild quails was much higher, with 13 haplotypes in 26 quails, mostly located within a second clade (prefix W plus F1W1 in **Figure 2**). Five of the wild haplotypes were observed in more than one individual. One of these (F1W1), present in five wild individuals, was the only one shared with farm quails and corresponded to the most frequent farm haplotype. A GenBank BLAST search showed that F1W1 was identical to sequences previously identified in Japanese quails, supporting the hypothesis that farm quails derive from captive domestic Japanese quail females used as breeders, as suggested by Sanchez-Donoso et al. (2012). These sequences constitute the Japanese quail clade in **Figure 2**. Importantly, all analyzed farm individuals carried mtDNA sequences from this clade.

Two of the haplotypes found in wild quails were either identical to (F1W1, in five individuals) or clustered with (W6, in one individual) farm haplotypes within the Japanese quail clade. The remaining sequences made up the second clade that did not include any farm sample (common quail clade), with differences

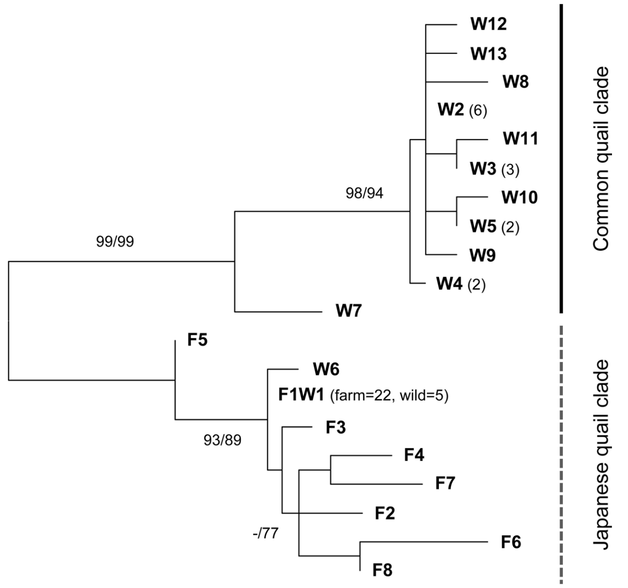


FIGURE 2 | Unrooted maximum likelihood (ML) phylogenetic tree of mitochondrial control region haplotypes identified in wild (indicated with the prefix W) and farm (F) quails. Only one haplotype was shared between wild and farm quails (F1W1) (it was found in 22 farm quails and 5 wild quails). Number of quails sequenced with each haplotype is indicated in parenthesis when larger than 1. Bootstrap support for ML and NJ (in this order) is indicated at the nodes when higher than 70%. Two clades are found, separating common quail (*Coturnix coturnix*) from Japanese quail (*C. japonica*) sequences with high support.

between haplotypes also ranging between 1 and 10 substitutions. Despite the short size of the fragment studied here, sequences from the two clades were clearly differentiated by 17 to 24 substitutions. The existence of mitochondrial sequences from the Japanese clade in some wild quails confirms the presence of genetic material from quails of farm origin among the wild-sampled quails (all of these individuals were confirmed as being of farm origin or admixed in the microsatellite analyses, see below).

IDENTIFICATION OF ADMIXED INDIVIDUALS

All studied microsatellite loci were highly variable in both farm and wild quails. Despite the high polymorphism, previous paternity studies (not shown) revealed that the markers and protocols used did not result in false alleles. All individuals were successfully genotyped for at least 70% of the markers. The number of alleles per locus was high for all datasets and markers, with an average number of alleles per locus and allelic richness higher than 10 for all years and datasets (**Table 1**). As expected, the average number of alleles per locus was closely associated with sample size, but allelic richness remained practically constant from year to year within datasets. PIC values were high in all cases (with average PIC over 0.81 in wild quails and over 0.77 in farm quails), showing that the allelic variation was potentially very informative of population structure. Expected heterozygosity (H_e) was above 0.8 in all cases. This high heterozygosity results from the high number of alleles and similar values have been observed in other Galliform species with high allele diversity (He et al., 2008; Hassen et al., 2009; Bei et al., 2012). Observed heterozygosity (H_o) was slightly lower than H_e in all cases, which could be due to the presence of sets of alleles of different origin (farm and wild) that had not reached Hardy-Weinberg equilibrium. The values for H_e and H_o were almost constant within datasets, even when comparing datasets I and III (typed by the same person and equipment), which supports our initial assumption of negligible genetic drift.

Game farm quails showed lower average number of alleles per locus and allelic richness than wild quails analyzed with the same markers (**Table 1**). There was significant genetic differentiation between the wild (including possibly admixed individuals) and farm populations, and F_{st} values between them were very similar across datasets: F_{st} for Dataset I = 0.05, for Dataset II = 0.06, for Dataset III = 0.05 ($p < 0.05$ in all cases). Due to the high heterozygosity of our loci and the upper limit this imposes on F_{st} [1–(average within population H_e); Hedrick, 2005], these relatively low values actually represent a pronounced differentiation: Hedrick's standardized G'_{ST} values were between 0.35 and 0.41 (ca. 40% of the maximum differentiation possible), and D_{est} between 0.25 and 0.31.

We used the software STRUCTURE to divide each dataset (together with the corresponding farm set) in two clusters ($K = 2$) in order to identify the contribution of the two quail lineages (wild or farm) to the genome of each individual. In each analysis (all five replicates produced practically identical output), a farm cluster and a wild cluster appeared clearly separated, and several individuals were identified as admixed (**Figure 3**). Using $q_{farm} \geq 0.90$ as a threshold for the proportion of farm ancestry, between 79 and 90% of farm animals in each analysis were classified as pure farm, and none appeared as pure wild (**Figure 4A** and Supplementary Material Table S2A). Admixed individuals in the farm (between 10 and 21% of the farm animals) may represent descendants of crosses between farm quails and common quails (males) that are regularly introduced into farms as breeders (Sanchez-Donoso et al., 2012). Of the quails sampled in the wild, between 81 and 94% were identified as pure wild ($q_{farm} \leq 0.10$). Some wild individuals (up to 10%) had very high q_{farm}, compatible with pure farm origin, and likely represent quails from restocking events. Overall, between 3 and 16% of wild-caught birds were classified as admixed ($0.10 < q_{farm} < 0.90$).

Since we identified some individuals with admixed nuclear genotypes among farm quails, the presence of admixed individuals in the wild is not, *per se*, indicative of matings between restocked farm quails and native wild quails; these individuals could represent admixed animals released from farms. However, there are two lines of evidence that point to interbreeding in the wild. First, the ratio between admixed and pure farm, as classified by STRUCTURE, was much higher in the wild sample than in the farm sample (**Figure 4A**, Supplementary Material Table S2A; Yates Chi-square = 67.21, $df = 1$, $p < 0.0001$). We would expect the same ratio in farms and in the wild if no interbreeding occurred in natural conditions; the observed admixed birds in the wild would just have resulted from their release from farms. Therefore, the inflated proportion of admixed quails in the wild compared to the proportion of freely roaming pure farm quails points to possible crosses in the wild between restocked and wild quails. However, the observed pattern would also emerge

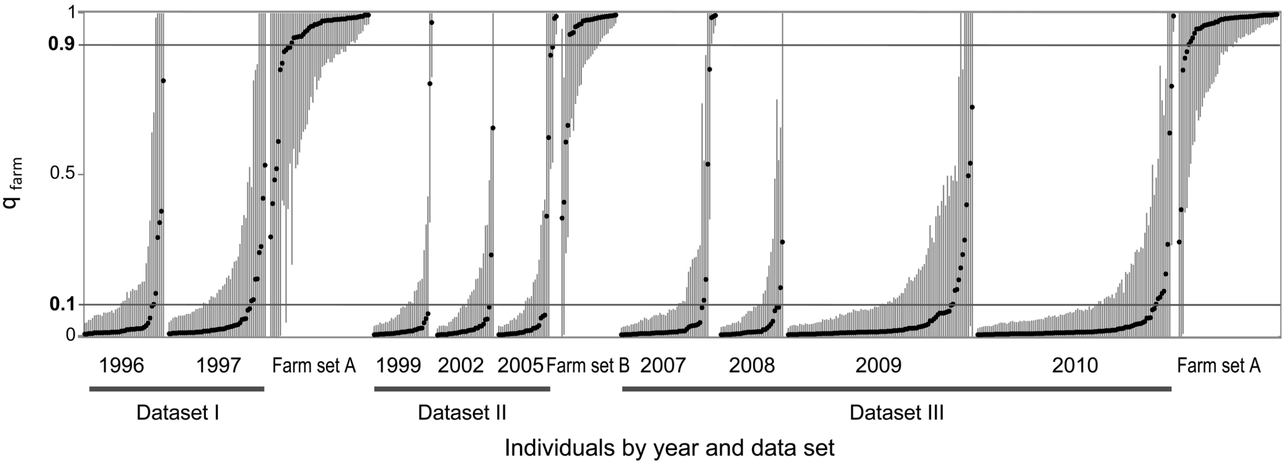


FIGURE 3 | STRUCTURE analyses for $K = 2$ for wild and farm quails for three datasets from different time periods. Dots represent individual proportion of membership to farm cluster (q_{farm}) and vertical lines are 90% highest posterior density intervals. Individuals are sorted by dataset, year, and q_{farm} value. Horizontal lines at $q_{\text{farm}} = 0.10$ and 0.90 indicate threshold values used to separate pure ($q_{\text{farm}} \leq 0.10$ for pure common quails, $q_{\text{farm}} \geq 0.90$ for pure farm quails) and admixed individuals ($0.10 < q_{\text{farm}} < 0.90$; see text).

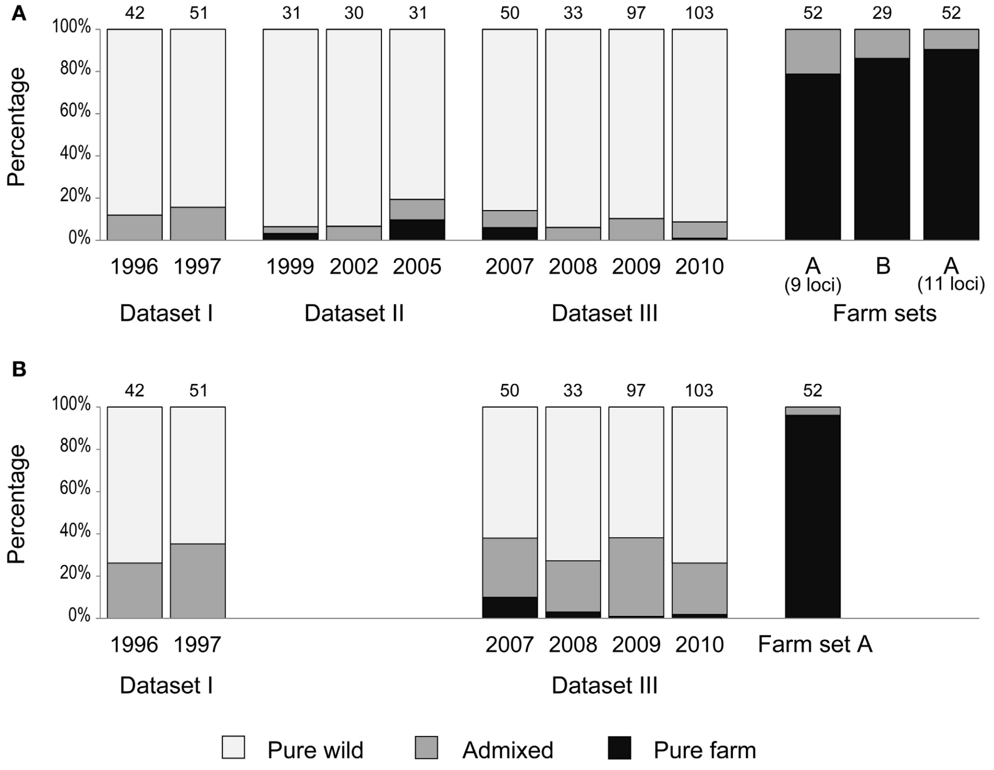


FIGURE 4 | Percentage of individuals classified as pure wild (pale gray), pure farm (black) and admixed (dark gray) according to q_{farm} values obtained from STRUCTURE ($K = 2$). Number of samples per year is indicated above each bar. **(A)** Analysis carried out separately for each dataset (as in **Figure 3** and Supplementary Material Table S2A). **(B)** Datasets I and III analyzed together (as in **Figure 6** and Supplementary Material Table S2B).

without crosses in the wild if restocked pure farm quails had lower survival in the wild than admixed quails, thus leading to an over-representation of the admixed individuals in the field sample.

The second line of evidence derives from the mtDNA analysis. For the 26 wild samples from Dataset II used for mtDNA analyses, 6 were identified as admixed by their nuclear DNA and out of these, 4 had common quail mtDNA (3 had haplotype W2, and 1 had W3; **Table 2**, **Figure 2**). The other 2 had Japanese quail mtDNA, as had the 4 wild-caught individuals assigned as farm quails by their autosomal genotypes. These last individuals probably represent animals released from farms. Since none of the farm individuals had common quail mtDNA (0 out of 29) and female common quails are known to reproduce very poorly in captivity (Caballero de la Calle and Peña Montañés, 1997), finding four admixed individuals in the wild population with common quail mtDNA by chance is highly unlikely (Fisher exact test: $p = 0.008$) if they just represented animals released from farms. Our findings therefore strongly indicate successful mating in the wild between restocked quails (males) and native wild quails (females).

CHANGES THROUGH TIME IN THE LEVEL OF INTROGRESSION

During the 15 years encompassed by our study, there was no clear increase in the proportion of admixed individuals in the wild (**Figures 3**, **4A**). GLM analyses using the year of sampling as independent variable and controlling for dataset failed to show a significant trend in the proportion of admixed individuals through time ($\beta = 0.122$, $SE = 0.131$, $p = 0.352$). Results did not change when we did not control for dataset ($\beta = -0.028$, $SE = 0.030$, $p = 0.347$).

To assess our power to detect an increase of the proportion of admixture in the wild, we simulated 15 generations of a population where about 1.5% of the potential breeders every generation were quails restocked from farms. This proportion is below the estimated average from our field data (2.2%; see **Figure 4A** and Supplementary Material Table S2A) and our simulations could thus exemplify the changes that are expected to occur in the population with a low-moderate contribution of farm alleles every generation. We randomly selected 50 simulated genotypes from different generations and 50 simulated farm genotypes. The proportion of admixed genotypes significantly increased over time ($\beta = 0.133$, $SE = 0.035$, $p < 0.001$; **Figure 5A**) when the simulated dataset spanning 15 generations was analyzed as a whole. Ten additional sets of simulated genotypes were generated and all of them showed a significant increase in the proportion of admixed genotypes ($p < 0.001$ in all cases). However, when the same data was analyzed subdivided into three datasets, mirroring the analysis of real data (**Figure 3**), the degree of introgression was underestimated. A smaller proportion of the simulated wild population appeared as admixed in later generations, and this proportion did not show changes over time ($\beta = -0.031$, $SE = 0.039$, $p = 0.432$; **Figure 5B**). The comparison of the q_{farm} values for the same simulated genotypes from the last generations (12 to 15) showed that fragmenting the data into shorter time periods led to an underestimation of q_{farm} in wild quails in later generations, and of the proportion of admixed individuals in the population (**Figure 5D**). The same comparison for the first simulated generations (1 and 2) did not show the same differences in q_{farm} values (**Figure 5C**). Thus, limiting the time span analyzed greatly reduced our ability to detect the progressive introgression of alleles from the farm population.

Table 2 | Classification of 26 wild quails from Dataset II, based on autosomal microsatellites (as suggested by STRUCTURE: pure wild: $q_{\text{farm}} \leq 0.10$; admixed: $0.10 < q_{\text{farm}} < 0.90$; pure farm: $q_{\text{farm}} \geq 0.90$), and mtDNA (common quail or Japanese quail mtDNA, Figure 2).

Autosomal genotype	mtDNA haplotypes	
	Common quail	Japanese quail
Pure wild	16	0
Admixed	4	2
Pure farm	0	4

Similarly, the field dataset that spanned the longest time period (Dataset II, 6 years, **Table 1**) did not show a change in the proportion of admixed individuals over time ($\beta = 0.184$, $SE = 0.183$, $p = 0.314$); the same result was obtained for a different wild population located over 100 km north of our study site monitored during the same 6 years (data not shown, see Supplementary Material Text S1). We used simulations based on Dataset II to investigate the power to detect an increase of admixed individuals over a period of 6 years using the same number of markers and samples. We found that increased introgression of farm alleles would not be consistently detectable (in at least 90% of the cases) unless the proportion of potential breeders coming from farms was above 3% (see Supplementary Material Text S2), higher than the proportion observed in our study area.

Since the simulations showed that the ability to detect introgression of farm alleles into the wild population (with the sample size available) depends on the length of the time series considered, we joined datasets I and III for a STRUCTURE analysis covering a longer time span (although reducing the loci used to the nine shared by both datasets). This resulted in wider confidence intervals for the estimates of q_{farm} (**Figure 6A**). In agreement with the simulations (**Figure 5A** compared to **Figure 5B**), joining datasets I and III indicated larger admixture (**Figure 6B**). The proportion of individuals classified as pure wild decreased compared to previous analyses (**Figure 4B** and Supplementary Material Table S2B). Again, no noticeable change was observed in the proportion of admixed individuals through time ($\beta = -0.007$ $SE = 0.021$, $p = 0.737$): about 31% for the first two years of the series (years 1996 and 1997) and 28% for the last four years (years 2007–2010).

In conclusion, the genetic analysis of the field data did not show an increase in the proportion of admixed individuals in the population, even after joining datasets I and III to overcome limitations associated with a short time series. However, these analyses suggested that perhaps the level of admixture had been underestimated by considering only short time periods in each analysis.

INTROGRESSION AS A FUNCTION OF THE PROPORTION OF FARM BREEDERS AND FITNESS OF ADMIXED INDIVIDUALS

One limitation of our simulations was that we considered a fixed proportion of the potential breeders to originate from farms (1.5%) while in real data this proportion was variable between years. It was not clear if a lower proportion would also produce a detectable signal of introgression, nor what level of introgression could be expected if the fitness of admixed individuals was lower than that of the native common quail population. To assess the

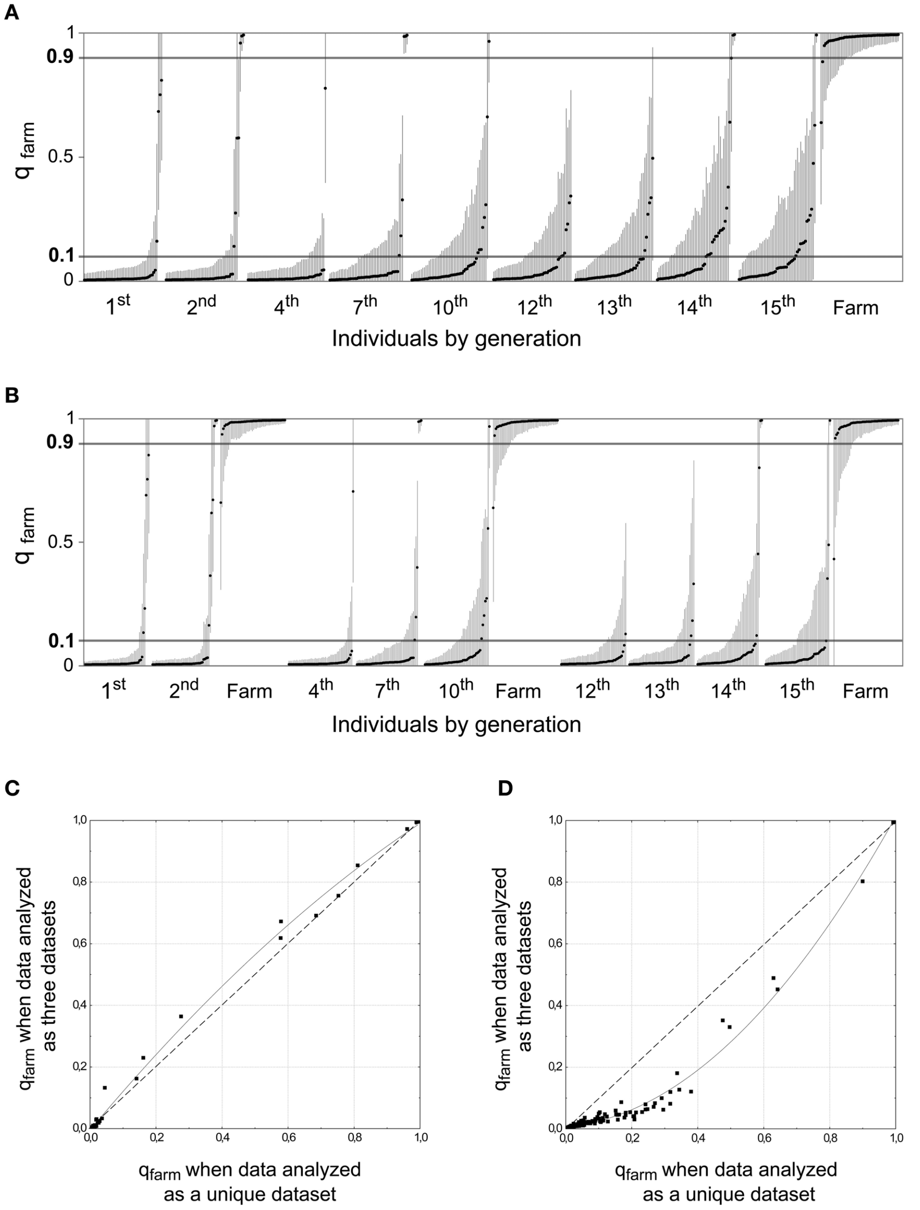


FIGURE 5 | STRUCTURE analyses ($K = 2$) for simulated data assuming that about 1.5% of the potential breeders are of farm origin. Fifty genotypes were sampled from each generation and 50 simulated farm genotypes were included in the analyses. **(A)** All data included in the analysis as a unique dataset. **(B)** Same simulated data as above, but analyzed in three datasets separately, analogous to those in **Figure 3** (same 50 simulated farm genotypes included in all analyses). Generations 12 to 15 do not show the degree of introgression that was inferred above. **(C)** Scatterplot comparing q_{farm} values for individuals from generations 1 and 2 obtained in (a) (X axis) B and (b) (Y axis). The curve represents the polynomial curve that best fits the data: q_{farm} values were almost identical for the two axes. **(D)** Similar scatterplot for individuals from generations 12, 13, 14, and 15. In this case, intermediate values of q_{farm} are underestimated when analyzed using short time series and more individuals appear as pure wild.

likely fraction of restocked farm individuals among the potential breeders that could produce the observed patterns of low admixture, we simulated 20 generations with fractions of 1, 1.5, 2, 2.5, 3, 4, or 5% per generation. For each case we generated 1000 datasets of simulated genotypes analogous to the field sampling (same number of samples per year as in datasets I + III) and we analyzed them with STRUCTURE. Since the proportion of individuals with $q_{\text{farm}} > 0.10$ in Dataset III was 32.4% (when combining datasets I and III; **Figure 4B** and Supplementary Material Table S2B), we investigated in which cases this value was inside the 95% confidence interval obtained from the simulations. The observed value was compatible with the simulations only when the proportion of potential breeders coming from farms was 1% (**Figure 7**, not shown for values higher than 3%). However, this value was almost a third of the observed average in the field sample (2.7%; **Figure 4B** and Supplementary Material Table S2B).

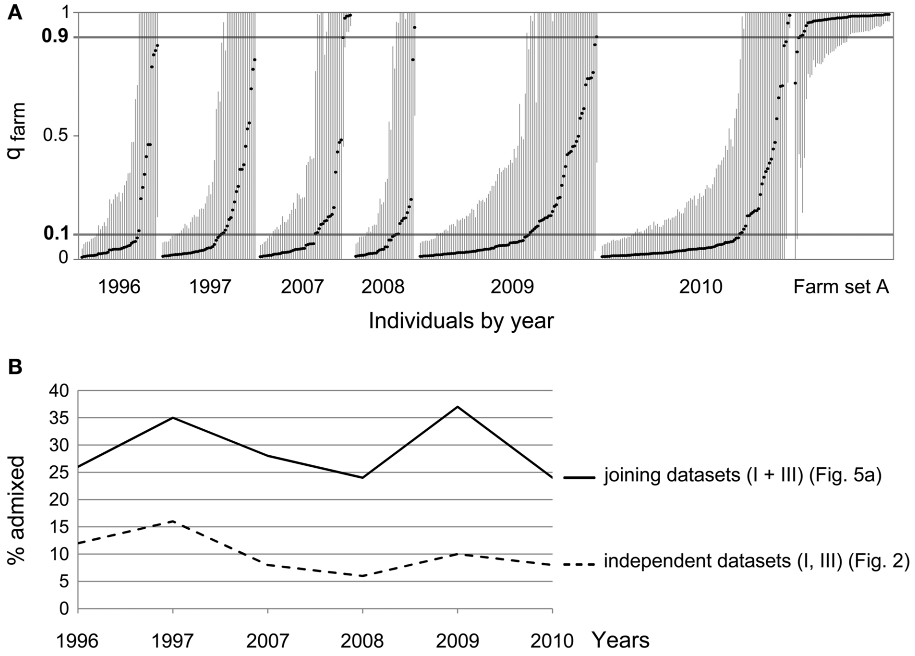


FIGURE 6 | (A) Joint STRUCTURE analysis ($K = 2$) for wild quails from datasets I and III. **(B)** Proportion of admixed individuals ($0.10 < q_{\text{farm}} < 0.90$) per year. Continuous line: datasets I and III analyzed together (as above). Dashed line, datasets I and III analyzed independently (as in **Figure 3**). No increase in the proportion of admixed individuals is observed.

We suggest two possible explanations for this mismatch: first, the percentage of farm individuals potentially contributing to the breeding population may have been overestimated. Alternatively, farm and admixed individuals could exhibit lower fitness in the wild than pure common quails, slowing down the spread of farm alleles in the wild population. In simulations incorporating a variety of fitness values (1, 0.8, 0.6, 0.4, and 0.2) and assuming that between 2–3% of the potential breeders come from farms, the observed proportion of admixed (and pure farm) quails in the field was compatible with relative fitness values as low as 0.6 (**Table 3**). If the proportion of farm individuals among potential breeders was larger than 3%, the data would be compatible with even lower fitness values for admixed birds. These results indicate that admixed individuals may have lower chances of contributing offspring to the next generation than common quails. Interestingly, our data was not compatible with the simulated scenarios in which fitness values of admixed individuals was 0.2. Introgression would only be completely negligible if the results were compatible with fitness = 0. This implies that some introgression must be taking place.

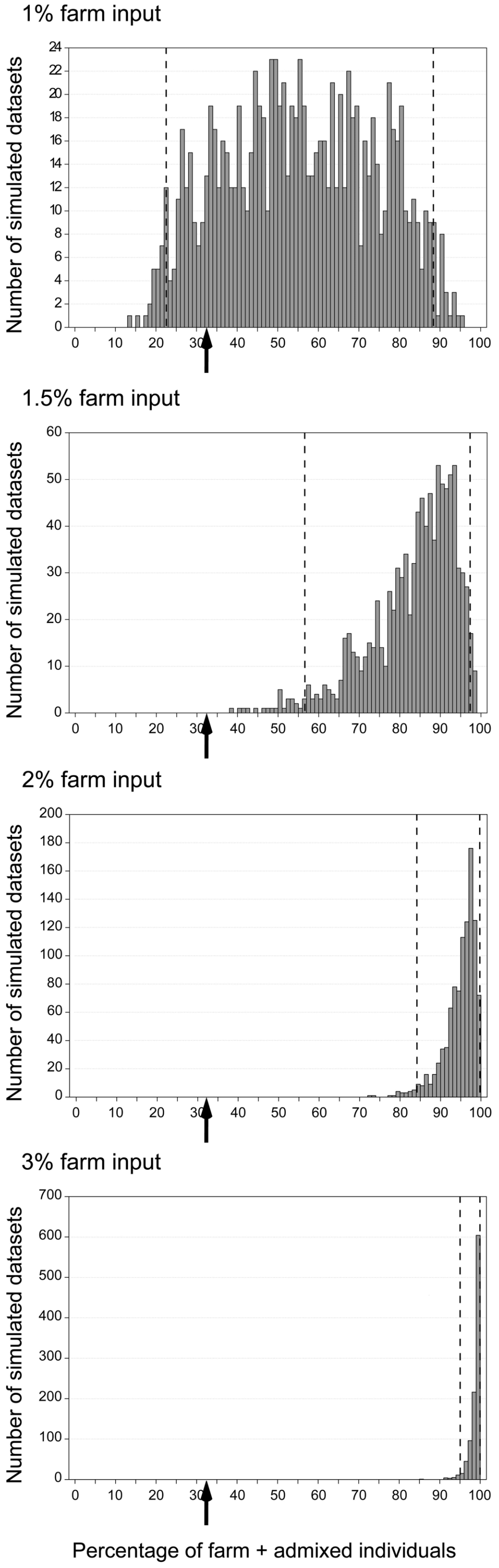
LONG-TERM EFFECTS

Our simulations showed that even though we did not observe a clear increase over time in the proportion of admixed individuals, the genetic analysis of field samples was compatible with slow but accumulating introgression (**Table 3**). In order to investigate the effects of such introgression on wild quail populations in the near future, we simulated populations evolving under two scenarios compatible with field data: (i) 1% of potential breeders deriving from restocked quails and hybrids without diminished fitness, (ii) 2% of the potential breeders coming from farms and a fitness of 0.6 for hybrids (**Figures 8A,B**, respectively). In both cases, running the simulations for 20 more generations (40 generations in total) translated into extensive introgression of farm alleles and more than 20% of the wild individuals reached values of q_{farm} indicative of admixture ($0.10 < q_{\text{farm}} < 0.90$; **Figure 8C**).

DISCUSSION

Studies on the population-wide impact of introgression have a long history in areas like fisheries research and aquaculture, where massive restocking practices are common (see, e.g., Hedgecock and Coykendall, 2007; Waples et al., 2007; Waples and Drake, 2008). On the other hand, only little is known about the genetic effects of large scale releases for supportive breeding in other wildlife groups (Laikre et al., 2010). Studies on birds and mammals often focus on the individual identification of hybrids and the estimation of their proportion in the population. From this information, however, it is not straightforward to infer the rate of introgression and its potential impact on the population. In this study we integrated three separate datasets to cover a large time period of 15 years (ca. 15 generations), which could allow separating hybridization and introgression, and thus monitor the advance of introduced alleles into the focal population and assess the fitness of hybrids.

In the study region (Catalonia, NE Spain), the number of farm quails restocked every year was 3–11 times larger than the estimated wild population (from Rodríguez-Teijeiro et al., 2004 and Hunting order plans of the Regional Government of Catalonia). However, only a small proportion of the released farm quails become potential breeders: those that escape hunting



1% farm input

1.5% farm input

2% farm input

3% farm input

FIGURE 7 | Distribution of the proportion of non pure wild individuals (farm or admixed, $q_{\text{farm}} > 0.10$) in 1000 simulated datasets representing a sampling pattern analogous to that of Dataset III (2007-2010) *(Continued)*

FIGURE 7 | Continued
assuming various proportions of potential breeders coming from farms: 1, 1.5, 2, and 3%. Dashed lines show 2.5th and 97.5th percentiles. Arrows indicate proportion observed in the real data: 32.4% (**Figure 4B** and Supplementary Material Table S2B).

Table 3 | Ninety five percentage confidence intervals of the proportion of not pure wild (admixed and pure farm individuals, $q_{\text{farm}} > 0.10$) for the simulated data corresponding to Dataset III.

Farm %	Fitness				
	1	**0.8**	**0.6**	**0.4**	**0.2**
1	22.6–88.3	11.0–49.5	6.7–20.2	4.6–12.4	2.1–7.8
1.5	56.5–97.2	19.8–79.9	10.2–31.1	5.7–15.2	3.2–9.2
2	84.1–99.7	50.2–96.1	17.3–66.4	7.4–18.4	3.9–10.6
2.5	91.9–100.0	71.4–98.2	17.3–63.6	9.9–24.4	5.3–12.4
3	95.1–100.0	84.1–99.3	30.7–87.6	13.1–33.6	5.7–13.4
4	98.9–100.0	94.7–100.0	60.4–95.4	18.7–50.2	8.5–17.3
5	99.3–100.0	97.9–100.0	83.4–99.3	28.6–69.6	11.3–21.6

Simulations consisted of 1000 STRUCTURE runs, varying (i) the levels of fitness for admixed individuals, and (ii) the proportion of potential breeders that come from farms (restocked) each generation (35,000 STRUCTURE runs in total). Simulated data sets were built replicating the sampling pattern from the field data for datasets I and III (same number of samples per year). Gray cells represent the parameter combinations compatible with our field data, i.e., simulations where confidence intervals include the proportion of individuals with $q_{\text{farm}} > 0.10$ observed in our sample (32.4%; Supplementary Material Table S2B).

and predation and those that also survive the winter, with a probably depressed immune system response (due to relaxed selection in captivity) and in breeding grounds with limited food resources, or that successfully migrate to wintering latitudes and return the next breeding season (which seems unlikely due to the reduced migratory impulse they may have inherited from domestic Japanese quails; Derégnaucourt, 2000; Derégnaucourt et al., 2005). Since our results indicate that only 2–3% of the potential breeders in the study area were of farm origin, approximately only one out of every 100–500 farm quails released can potentially contribute to the wild breeding population. However, our results show that those individuals could have an important impact on the wild population.

Based on the proportion of quails with vocalizations indicating hybrid origin, Puigcerver et al. (2007) suggested that the proportion of admixed birds in the population had remained relatively constant, and proposed that those individuals could be animals of farm origin that survived in the field for some time but failed to reproduce. However, the proportion of admixed relative to pure farm in the wild is much larger than in captivity and this, together with the capture of admixed birds with common quail mtDNA, indicating that the mother was a wild quail, confirm that interbreeding has happened in the wild. Field experiments carried out by Puigcerver et al. (2014) have shown that farm and wild individuals can mate in the wild, but our study provides the first evidence that this in occurring outside the experimental conditions.

The detection of offspring from wild × farm quail crosses does not necessarily imply a progressive build-up of Japanese quail

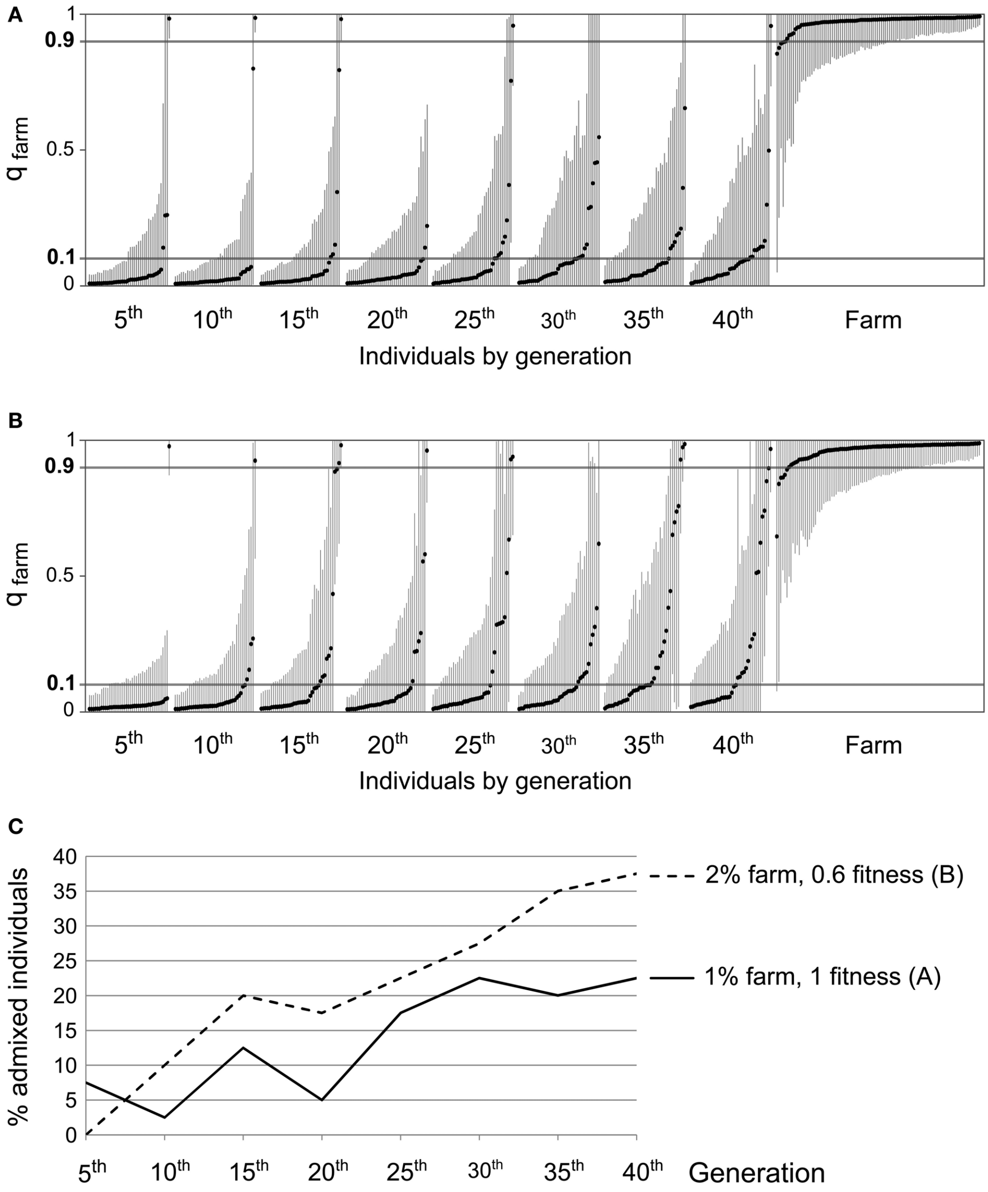


FIGURE 8 | Examples of results from an analysis using STRUCTURE ($K = 2$) on data simulated over 40 generations, with 50 individuals randomly sampled every 5 generations, along with 100 simulated farm individuals. (A) 1% of potential breeders derived from farms, fitness of 1 for all individuals. **(B)** 2% derived from farms, fitness of 0.6 for first and second generation admixed individuals. **(C)** Proportion of admixed individuals ($0.10 < q_{\text{farm}} < 0.90$) over time under scenarios **(A)** (continuous line) and **(B)** (dashed line). In both cases, a clear increase in the introgression of farm alleles into the wild population is observed.

alleles in the common quail population. Continued backcrossing of the crossbred individuals with common quails is required to produce an increase in the proportion of admixed individuals and introgression of alleles. An analysis of three separated studies of field samples did not show such increase (**Figure 3**). However, not seeing such a trend could either be due to its non-existence or to the lack of power to detect it, showing that the observed data is inconclusive. Our simulations pointed to the latter, as they revealed that analyses covering short time frames (as those generated when analyzing the three datasets separately) lack the power to detect an increase in introgression, especially when the proportion of potential breeders coming from farms is relatively small. In these cases, STRUCTURE analyses underestimated the degree of admixture (**Figure 5D**), possibly due to an inaccurate estimation of the ancestral allele frequencies (before introgression). After a few generations of admixture, some introgressed alleles may have spread in the native population, complicating the identification of their alien origin. This is an especially important outcome of our simulations since studies of hybridization and introgression are generally based on samples collected over a short time period, and this could result in similar biases in the estimation of the rate of admixture. It is possible that increasing sample sizes during the different time periods or genotyping a larger number of genetic markers would also increase the power to detect introgression. However, this is often not possible or too expensive. Overall, our results illustrate how power tests are essential in determining if a study can robustly test the hypotheses presented.

To increase our power to detect changes through time, we jointly analyzed the first and last datasets, spanning 15 years. This implied reducing the power to individually classify samples as pure or admixed and increased confidence intervals for the proportion of farm ancestry q_{farm} (see **Figures 3**, **6A**). This cannot be just result of reducing the number of genetic markers included in the analysis since it remains the same for Dataset I (9 loci) but may be indicating important admixture.

Simulations replicating the sampling pattern of datasets I and III indicated that the power of the markers and samples analyzed should be sufficient to detect increased introgression in the case that as little as just 1.5% of the potential breeders came from farms. However, the real data did not show an increase in the proportion of admixed individuals even though the average proportion of farm animals detected in the study site every generation was about twice as much. This suggests that either the proportion of farm animals contributing to the reproduction is well below 1.5%, or that farm and admixed individuals have a lower fitness and, thus, have a lower contribution to the following generations than pure common quails. This would be possible if most of the admixed individuals were not involved in the reproduction. However, this does not seem likely because field experiments have shown that female quails of farm origin are more successful than wild common quails in attracting and mating wild common quail males (Puigcerver et al., 1999, 2013, 2014). The limited introgression of farm alleles into the wild population may be due to the lower fitness of the released birds and their descendants, which is also supported by experimental observations in the wild that point to a decreased survival and increased nest predation for females of farm origin (Puigcerver et al., 2014). Our simulations indicate that their overall fitness could easily be 40% lower than the fitness of wild common quails.

Common and Japanese quails have evolved under different selective forces and have experienced different demographic changes. Consequently, it is highly possible that alleles of Japanese quail origin present in the farm population are maladaptive and contribute to a lower fitness in common quail populations. However, probably more important than this is the change in selective forces imposed first by the domestication process in Japanese quails, and later by the captive breeding with common quails for restocking. These further promote the fixation of maladaptive alleles and traits (Lynch and O'Hely, 2001; Frankham, 2008; Baskett and Waples, 2013) that could, for example, contribute to reduce anti-predatory behavior, flight restlessness or ability to explore alternative food sources. As a result, restocking practices often have very low success rates (Gortázar et al., 2000; Guyomarc'h, 2003). This could contribute to the reduced fitness of admixed birds.

Interestingly, real data was not compatible with simulated scenarios where the fitness of hybrids was very low (0.2). When we simulated data with this very low fitness for admixed individuals, fewer individuals from the simulated datasets were identified as admixed than in the real data, suggesting that farm alleles may be slowly, but progressively, integrating into the native common quail gene pool. Our simulations were simple and relied on several assumptions (large effective population size, no population structure, generation time of 1 year, non-overlapping generations, etc); the simulations were also based on the assuming that farm quail releases were initiated at the time our group first detected them (around 1990) and took place at a constant rate (constant proportion of farm individuals added every generation), but this may not be the case. Also, fitness effects could be introduced into the simulations in a number of ways (for example, varying fitness depending on the proportion of the genome originating in the farm, or reduced survival could be modeled at different stages in the life cycle). Consequently, the fitness estimations obtained can only be taken as rough approximations, but we believe that they reflect population trends and indicate that although admixed individuals have reduced fitness some introgression is taking place every generation.

Our results highlight the possible consequences of continuing the current restocking practices. While we do not detect an increase in the proportion of admixed individuals over a 15 year time period, our simulations show that this does not guarantee that introgression is not taking place. Two simulated scenarios, compatible with estimates from real data, predict extensive introgression of farm alleles in the near future (**Figure 8**). Of course, these simulations are based on estimates obtained from a very small area, and the proportion of potential breeders coming from farms is likely to vary from place to place and from year to year. However, considering the high mobility of common quails and how widespread restocking practices are, we do not have reasons to expect very different patterns in other areas within the western distribution of the common quail.

CONCLUSIONS

Extensive restocking with farm-reared quails does not yet appear to have had large impacts on the genetic integrity of common quails in Northeast Spain. However, we here provide evidence that interbreeding has already happened in the wild and that overall admixture levels may be slowly increasing over time. Although some degree of selection against introgressed alleles appears to exist, our simulations predict a marked risk of genetic swamping of the common quail population, if release practices are maintained. Our results therefore support initiatives currently being taken to control quail restocking practices across Europe.

More generally, our results highlight that the impacts of introgression on wild populations can be difficult to detect, especially when hybridization occurs over long time periods and at low frequency. In such cases, data collected over a short time period may be insufficient and lead to an underestimation of the degree of admixture. Our study therefore underlines the need for long time series to reliably assess the consequences of hybridization in natural populations and the need for power tests.

DATA ACCESSIBILITY

Newly obtained mitochondrial DNA sequences have been submitted to GenBank (accession numbers: KF410830-KF410849). Microsatellite data files are deposited in Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.kv733. Scripts were set to run in a computer with a Linux operative system (Ubuntu 12.10) and are available at http://www.consevol.org.

AUTHOR CONTRIBUTIONS

This study is part of a long-term study on quail populations led by Manel Puigcerver and José D. Rodríguez-Teijeiro. It is part of the doctoral thesis of Ines Sanchez-Donoso (at Barcelona University, Spain), supervised by José D. Rodríguez-Teijeiro, Manel Puigcerver and Carles Vilà, and the master thesis of Jisca Huisman (at Uppsala University, Sweden), supervised by Carles Vilà and Frank Hailer, Carles Vilà and Ines Sanchez-Donoso conceived the genetic study. Ines Sanchez-Donoso, Manel Puigcerver and José D. Rodríguez-Teijeiro did field work. Ines Sanchez-Donoso, Jisca Huisman, Frank Hailer, and Jorge Echegaray performed laboratory work. Ines Sanchez-Donoso and Carles Vilà did genetic and statistical analyses and simulations, with input from Jisca Huisman and Frank Hailer. All authors contributed to the text.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fevo.2014.00015/abstract

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