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The rock partridge (*Alectoris graeca*): some studies on the Apennine Management Unit (MU)

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THE ROCK PARTRIDGE (*Alectoris graeca*): SOME STUDIES ON THE APENNINE MANAGEMENT UNIT

ABSTRACT

Alectoris graeca Meisner (Galliform: Phasianidae) is included in the Annex I of the EU Birds Directive and classified as Near Threatened (NT) in the IUCN Red List but the continuing decline in the area of occupancy suggests a next leap in the vulnerable (VU) category.

In mainland Europe, three different subspecies of rock partridge (*Alectoris graeca*) were described: 1) *A. g. graeca* presents in Greece, Albania and south-western Balkans, 2) *A. g. saxatilis*, presents in Alps, Dinaric Alps and north-western Balkans and 3) *A. g. orlandoi* limited to the central–southern Italian Apennines. In the island of Sicily the endemic *A. g. whitakeri* sub-species is present. Recent genetic investigations do not confirm the sub-specific differentiation of the Apennine rock partridge and suggest that *A. g. orlandoi* should be merged with the Balkan one (*A. g. graeca*). Despite this, the Apennine rock partridge population must be considered a distinct Management Unit (MU) because of its isolation and independence from other populations.

According to the national (Italy) and the regional (Lazio region) action plans for the conservations of the species, the study of the available literature highlighted important knowledge gaps that need to be filled for allowing the conservation of the species through the conservation of its “metapopulations”.

These gaps concern demographic assessment, morphology, sexual size dimorphism, sex determination methods, genetic pollution risks, autecology, *ex situ* conservation and effect of captivity on the animal’s ability to survive into the wild after release. These gaps are particularly large on the Apennine MU.

With the aim of contributing to gain new knowledge on some *key aspects* concerning the rock partridge of the Apennine MU, during the period 2013 – 2016 we performed four studies. Two of these studies have been published and two were submitted for publication. Furthermore, we built the bases for further research currently in progress.

First study. The first study was aimed to upgrade the status of the rock partridge (*Alectoris graeca*) in some suitable areas of the Rieti and Frosinone provinces (Lazio region) using data of presence collected during a six-year monitoring project. In Rieti province, the

estimated pre-reproductive density of territorial males varied between 0.12 and 0.62 per km^2 of suitable protected areas and between 0.12 and 0.45 of suitable hunting areas. In Frosinone province, the estimated pre-reproductive density of territorial males varied between 0.58 to 0.73 per km^2 of suitable areas in Private Hunting Farms bordering Abruzzo Lazio and Molise National Park and between 0.25 and 0.30 in suitable hunting areas. Overall, we found pre-reproductive density lower than that recorded in others areas of the Lazio region and of the Alps, confirming a bed conservation *status* of the Apennine MU that suggests also a negative correlation with the hunting permission of the area.

Second study. The aim of the second study was to ascertain the presence of the non-native red-legged partridge (*Alectoris rufa*) in the north of Lazio region as a first step towards updating the its distribution in central Italy and assessing the risks of contact with the native rock partridge (*Alectoris graeca*) and of consequent genetic pollution. Interviews (forestry agents, keepers, hunters and farmers) allowed collocating the species into 190 Territorial Units (TUs; 1 km x 1 km). On field surveys, performed in a random sample of cells (28.9% of the cells with presence), confirmed the presence of the species in 31 TUs almost exclusively bordering Tuscany region. Nowadays, the non-native red-legged partridge seems distributed away from possible contact zones with the native rock partridge. However, further researches are necessary to appreciate if the non-native partridge can potentially threat the genetic integrity of the native one trough dispersion in the medium-long term.

Third study. In captivity, behavioural, physiological and morphological changes need attentions because they make animals unable to survive in a natural environment. The main aim of this study was to assess at which stage of growth intensive rearing affects the external functional morphology of rock partridge (*Alectoris graeca*) offspring (G1). The second aim was to investigate this effect on the sexual size dimorphism (SSD) magnitude and on its occurrence. At these purposes comparison with a control group (G2) of related offspring housed in a complex wild-similar environment were performed. Significant ($p = 0.01$) morphological changes were detected in the G1 at 84 and 98 days post hatching when birds in the control semi-natural group (G2) showed longer head, deeper and wider tarsi probably as result of the physical and mental exercise they did in the complex environment. Our results suggest that the intensive rearing does not determine substantial non-functional morphological changes at least up to 70 days post hatching. Birds for restocking or reintroduction should be translocate before this time in pre-release complex semi-natural environment where they can exercise and compensate muscle, tendons and neuronal development.

Fourth study. Early sexing of animals is a fundamental factor in research, management and conservation and it becomes essential for the management of a sexually monomorphic species. Partridges (genus *Alectoris*) are sexually monomorphic in plumage and do not show obvious sexual dimorphism. Plastic morphological adaptations to contingent environmental factors are known and they could make a morphological sexing model not exportable to a context different from that in which it was developed. This study was aimed to identify exportable morphological criteria and methods for an early sex determination in *Alectoris graeca* regardless the growing conditions they are subjected to. At this purpose, we tested several morphological traits recorded on related *A. graeca* chicks grown under intensive or semi-natural rearing system for the immunity to the rearing system's effect (robustness) and for the discriminant capacity regardless the rearing system (DRRS). With this approach, three early and exportable morphological criteria for sex determination were identified. The best exportable method for sex determination was developed at 42 days post hatching when Discriminant Analysis (DA) retained only tarsus length (TL) as discriminant variable. This model was high discriminatory (canonical correlation = 0.89, Wilk's = 0.21) and 100% accurate regardless the rearing system.

Key words: *Alectoris graeca*, Apennine, *status*, threats, conservation, morphology, sexual size dimorphism, sex determination, rearing system, captive morphological changes.

RIASSUNTO

La coturnice (*Alectoris graeca*) è inclusa nell'Allegato I della Direttiva Uccelli (2009/147/CE) del Parlamento Europeo ed è classificata Near threatened (NT) nella lista rossa della IUCN, ma il declino continuo nell'intero areale distributivo suggerisce un prossimo aggiornamento nella categoria Vulnerabile (VU).

Nell'Europa continentale, sono state descritte tre diverse sottospecie di coturnice (*Alectoris graeca*): 1) *A. g. graeca* presente in Grecia, Albania e a sud ovest dei Balcani, 2) *A. g. saxatilis* presente nelle Alpi, nelle Alpi Dinariche e a nord-est dei Balcani, 3) *A. g. orlandoi* la cui distribuzione è limitata all'Appennino centro meridionale. In Sicilia è presente la sottospecie endemica *A. g. whitakeri*. Recenti indagini genetiche non confermano tuttavia la differenziazione sotto specifica della coturnice Appenninica e suggeriscono che *A. g. orlandoi* è affine alla sottospecie nominale dei Balcani (*A. g. graeca*). Nonostante questo, la popolazione Appenninica deve essere considerata una Unità di Gestione (MU) ben distinta per il prolungato isolamento che l'ha resa indipendente da tutte le altre.

In accordo con i Piani D'Azione per la conservazione della specie in Italia ed in regione Lazio, lo studio della letteratura disponibile ha evidenziato importanti vuoti conoscitivi che è necessario colmare per consentire di individuare strategie integrate di conservazione delle sue metapopolazioni. Questi vuoti riguardano valutazioni demografiche complete, la morfologia, il dimorfismo sessuale, metodi di sessaggio precoce, rischi di inquinamento genetico, l'autoecologia, la conservazione *ex situ* e l'allevamento con i suoi effetti sulla morfologia funzionale, l'etologia e la fisiologia degli animali in cattività. Questi aspetti risultano particolarmente lacunosi riguardo l'unità di gestione (MU) Appenninica.

Con l'intento di fornire nuove conoscenze riguardanti questi aspetti chiave, nel triennio 2013 – 2016, abbiamo eseguito 4 studi, di cui due sono stati pubblicati e due sottomessi per la pubblicazione. Inoltre, le aumentate conoscenze, ci hanno permesso di costruire le basi per ulteriori ricerche attualmente in corso d'opera.

Primo studio. Il primo studio era finalizzato ad aggiornare lo *status* della coturnice (*Alectoris graeca*) in alcune aree idonee delle province di Rieti e Frosinone (regione Lazio) utilizzando i dati raccolti in sei anni di monitoraggio in campo. In provincia di Rieti, le

densità pre-riproduttive, in aree protette, sono variate tra 0,12 e 0,62 maschi/100 ha di superficie idonea e tra 0,12 e 0,45 in aree in cui la caccia è consentita. In provincia di Frosinone le densità di maschi territoriali sono variate tra 0,58 e 0,73 nelle Aziende faunistico Venatorie (AFV) contigue al Parco Nazionale D'Abruzzo Lazio e Molise (PNALM) e tra 0,25 e 0,30 in aree in cui la caccia è consentita. Durante questo studio sono state rilevate densità pre-riproduttive addirittura più basse rispetto a quelle registrate in altre aree idonee dell'Appennino Laziale e delle Alpi, confermando una stato di conservazione assai precario dell'unità di gestione Appenninica e un effetto negativo legato al disturbo indiretto esercitato dall'attività venatoria.

Secondo studio. La finalità del secondo studio era quella di accertare la presenza della pernice rossa (*Alectoris rufa*) nel nord della regione Lazio, dove questa è alloctona, come primo passo verso l'aggiornamento del suo status distributivo in Italia centrale e la valutazione del rischio di contatto con la coturnice (*Alectoris graeca*) e del conseguente rischio di inquinamento genetico. Le interviste a fonti attendibili (agenti del corpo forestale dello stato, gurdiacaccia, cacciatori e allevatori) hanno permesso di collocare la specie in 190 Unità Territoriali (TUs; 1 km x 1 km). Indagini sul campo, eseguite su un campione casuale di celle (28,9% delle celle con presenza segnalata), hanno confermato la presenza della specie in 31 TUs quasi esclusivamente localizzate lungo il confine con la regione Toscana. Attualmente la presenza della pernice rossa all'esterno del suo areale distributivo originario non rappresenta una minaccia immediata in quanto sembra distribuita lontano da possibili aree idonee alla coturnice. Tuttavia, ulteriori indagini sono necessarie per comprendere se eventuali fenomeni dispersivi di questa pernice alloctona nel Lazio, possano, sul medio-lungo termine, minacciare l'integrità genetica della coturnice autoctona.

Terzo studio. I cambiamenti comportamentali, morfologici e fisiologici in animali mantenuti in stretta cattività richiedono attenzioni poiché possono determinare la loro incapacità di adattamento in natura, e quindi elevati tassi di mortalità post rilascio. Lo scopo principale di questo lavoro era quello di valutare a quale stadio di accrescimento l'allevamento intensivo determina cambiamenti nella morfologia funzionale esterna nella coturnice (*Alectoris graeca*). Il secondo obiettivo era quello di valutare l'effetto del sistema di allevamento sul dimorfismo sessuale. Per questi propositi un gruppo di pulcini allevato in modo intensivo (G1) è stato confrontato con un gruppo di controllo (G2) composto da fratelli accasati in un ambiente complesso semi-naturale. Cambiamenti morfologici significativi (p = 0,01) sono stati rilevati a 84 e 98 giorni successivi la schiusa, quando gli uccelli nel G2 mostravano teste più lunghe e tarsi più profondi e larghi probabilmente come risultato dell'esercizio fisico e mentale svolto in un ambiente complesso. I nostri risultati suggeriscono che l'allevamento intensivo non determina modificazioni morfologiche sostanziali almeno sino a 70 giorni dopo la schiusa. Uccelli destinati a rinforzare popolazioni selvatiche tramite ripopolamenti e reintroduzioni dovrebbero quindi essere traslocati, prima di questo limite temporale, in ambienti semi-naturali complessi dove possano fare esercizio e compensare i deficit di sviluppo fisico e mentale.

Quarto studio. Il sessaggio precoce degli animali è un elemento cruciale della ricerca e della gestione tesa alla conservazione delle specie, con particolare riferimento a quelle caratterizzate da assente o non evidente dimorfismo sessuale. La morfologia può adattarsi in modo plastico ai fattori ecologici cogenti potendo quindi determinare la non esportabilità di un metodo di sessaggio morfologico ad un contesto molto diverso rispetto a quello in cui è stato sviluppato. L'obiettivo di questo studio era quello di sviluppare metodi di sessaggio precoci ed esportabili, utilizzando come variabili discriminanti i tratti della morfologia esterna della coturnice non sensibili al sistema di allevamento (robusti) e precocemente discriminanti il sesso a prescindere dal sistema di allevamento (DRRS). A questo proposito alcuni tratti morfologici sono stati misurati su due gruppi di fratelli allevati rispettivamente in condizioni intensive e semi-naturali e sono stati effettuati confronti tra gruppi entro sesso e tra sessi in ogni combinazione dei gruppi. Con questo approccio tre tratti morfologici esportabili, in quanto robusti e contemporaneamente DRRS, sono stati identificati. Il miglior modello di sessaggio è stato sviluppato a 42 giorni dopo la schiusa, quando l'Analisi Discriminante (DA) ha trattenuto solo la lunghezza del tarso come variabile discriminante. Questo modello è risultato avere un elevato potere discriminante (correlazione canonica = 0,89, Wilk's = 0,21) ed ha classificato correttamente il 100% dei casi in ciascun gruppo.

Parole chiave: *Alectoris graeca*, Appennino, *status*, minacce, conservazione, morfologia, dimorfismo sessuale, sessaggio, allevamento, cambiamenti morfologici in cattività.

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Chapter 1

GENERAL INTRODUCTION

Systematic

The genus *Alectoris* (Galliform: Phasianidae) is distributed in the Palearctic, China, Himalayas and southern Arabia (Randi 1996; Randi & Lucchini 1998) including seven ground-dwelling, non-migratory, monogamous and monomorphic in plumage galliform species known with the common name of partridges. These species share many anatomical and morphological characters (Parkin & Knox, 2010) and to distinguish among species and to identify eventual crossbreeding some plumage colour variations were described (Johnsgard 1988, Bernard-Laurent 1984). In the Italian mainland this genus is present with two native species: the rock partridge (*Alectoris graeca*) and the red legged partridge (*Alectoris rufa*) (Brichetti and Fracasso 2004). These two species are normally separated from an ecological point of view because the rock partridge occupies the phytoclimatic sector of cold *fagetum* and shows preference for cacuminal grasslands (Alps and Apennines), low scrub or scattered conifers (Griffin 2011) while the red-legged one prefers agricultural landscape with presence of shrubs and abandoned fields at lower altitude (Bulgarini 2011). However, in some particular areas of the Alps, these two species coexist and the case of natural hybridization is well-documented (Bernard-Laurent 1984, 1987, 1990; Priolo & Bocca 1992; Randi and Bernard-Laurent 1999). Spanò (2010) suggested to prevent the introduction of red-legged partridge below a line connecting the provinces of Grosseto and Ascoli because out of the original distribution range of the species. However, red-legged partridges are still massively released in Tuscany for hunting purposes and there are anecdotal evidences that the species is going to colonize the north of Lazio Region. The eventual expansion to the south of its distribution range as well as the illegal introduction of the non-native oriental chukar partridge (*Alectoris chukar*) could threat the genetic integrity of the Apennine rock partridge. The genetic introgression of *Alectoris chukar* genes into native Mediterranean partridges was widespread detected (Baratti *et al.* 2005; Barilani *et al.* 2007; Barbanera *et al.* 2007, 2009).

In Europe, three different subspecies of rock partridge (*Alectoris graeca*) were described (Cramp & Simmons, 1980; Madge & McGowan 2002; Randi *et al.* 2003):

1) Nominal subspecies (*A. g. graeca*) presents in Greece, Albania and south-western Balkans,
2) Alpine subspecies (*A. g. saxatilis*), presents in Alps, Dinaric Alps and north-western Balkans,
3) Sicilian subspecies (*A. g. whitakeri*) endemic to the island of Sicily.

Priolo (1984) described a fourth Apennine subspecies (*A. g. orlandoi*) on the base of plumage colour variations and time of feather changes. In this regard, Randi et al. (2003) reported that plumage colour and time of feathers change evolve rapidly, shaped by natural selection and/or non-genetic ecological factors. At present, genetic investigations do not confirm the sub-specific differentiation and suggest that the Apennine rock partridge (*A. g. orlandoi*) should probably be merged with the Balkan one (*A. graeca graeca*) (Randi et al. 2003), as previously suggested by Vaurie (1959). Randi et al. (2003) suggested that partridges in the Apennines and Albania-Greece might have been interconnected until the beginning of the Holocene (10,000 years ago) by the Adriatic land-bridge (Fig. 1.1 - double black line). Despite this, the Apennine rock partridge population must be considered a distinct management unit (MU; Moritz 1994) because of its isolation and independence from other populations (Randi et al. 2003).

Figure 1.1. Distribution of the three subspecies described in mainland Europe: *Alectoris graeca graeca* (blue), *Alectoris graeca saxatilis* (grey) and *Alectoris graeca orlandoi* (violet) (Randi 2003).

Status of the species

In Europe, the reproductive population of rock partridge (*Alectoris graeca*) is estimated in 41,000 – 54,000 breeding pairs of which 10,000 – 20,000 in Italy that is the nation with the most conspicuous population (Trocchi *et al*. 2016).

Actually, the species, including all subspecies, is included in the Annexes I and II/A of the EU Birds Directive (2009/147/CE) and classified as Near Threatened (NT) in the IUCN Red List but the continuing decline in the area of occupancy (BirdLife International 2015) suggests a next leap in the vulnerable (VU) category. Bernard-Laurent and Boev (1997) declared the conservation status of *Alectoris graeca* vulnerable (VU) in Europe already at the end of the twentieth century. The species is listed also in the annex III of the Berna Convention and it is classified SPEC 2 (BirdLife International, 2004).

At the national scale, Peronace *et al.* (2012) and Rondinini *et al.* (2013) declared the species VU also in Italy on the base of a severe demographic decline in all the sector of presence of this sedentary species (Trocchi *et al.* 2016).

Status of the Apennine MU

For the Apennine management unit (MU) presence data recorded on a long period with an interregional approach and standardized methods are not available.

Petretti *et al.* (1985) estimated, in the Lazio region, 2,975 - 9,640 breeding pairs. In the same Apennine sector, only 10 years later, Arcà *et al.* (1995) estimated 50 – 200 breeding pairs. Although the results reported by Petretti *et al.* (1985) could be overestimated, as commented by Trocchi *et al.* (2016), a severe population decline in the Apennine's sector of Lazio

Region is accredited and can be given as an example of the critical conservation status of the entire Apennine population. Furthermore, the results of a recent study conducted by Sorace *et al.* (2011), suggest that the rock partridge is locally extinct in the pre-Apennine and anti-Apennine areas (Lucretili, Lepini and Aurunci mountains) where the species presence was previously documented (Boano et al., 1995). Sorace *et al.,* (2011) found also that the protection state of the area is positively correlated with the conservation status of the species and estimated in the Lazio region a pre-reproductive population of 171-342 breeding pairs allowing to describe a stable situation in the period between 1995 (Arcà *et al.*) and 2011 (Sorace *et al.*).

Threats and limiting factors

The threats to the conservation of the species are many, varied and well studied. For a full discussion of the limiting factors, refer to the action plans for the conservation of the rock partridge drawn up for Italy (Trocchi *et al.* 2016), Lazio Region (Sorace *et al.* 2011) and the Province of Rieti (Amici *et al.* 2007) that reviewed exhaustively the scientific literature on the specific field.

Among the many factors of threat described in the available literature, three in particular require the most attention at every spatial scale (international, national, regional and local):

1. environmental changes due to the abandonment of the traditional agro-pastoral activities that are responsible of the increase in shrubs and woodlands, and consequently of the loss of open montane grasslands suitable for the species (Bernard-Laurent & De Franceschi 1994; Rippa, *et al.* 2011) and of invertebrates

richness (Kirby 1992; Vickery *et al.* 2001) fundamental for the offspring feeding during the first weeks of life;

2. hybridization with non-native species of the genus *Alectoris,* and in particular with red-legged partridge (*Alectoris rufa)* and chukar (*Alectoris chukar)* that are frequently introduced out of their original distribution range for hunting purpose by unaware hunters, determine genetic pollution and outbreeding depression (Randi *et al.* 1998; Baratti *et al.* 2005; Barilani *et al.* 2007; Barbanera *et al.* 2005, 2009; Negri *et al.* 2013);
3. distribution gaps, due to the discontinuities of suitable environments, determine the fragmentation of the meta-populations in isolated small populations. In absence of genetic flow, low-density populations risk a dangerous increase of the *inbreeding* rate and a consequent significant *fitness* loss that may trigger an "*extinction vortex*" (Sorace *et al.* 2011; Trocchi *et al.* 2016) due to any stochastic variation of the biotic or abiotic environment (Primack 2000).

Gaps of knowledge

According to the objectives of the regional (Sorace *et al.* 2011) and the national (Trocchi *et al.* 2016) action plans for the conservation of the species, the study of the available literature highlighted the necessity to increase knowledge on different incomplete *key aspects*:

1. **genetic aspects**:

a) degree of differentiation among Apennine and Balkan populations,
b) genetic flow and richness,
c) purity and hybridization,

d) actual distribution of non-native partridges and assessment of the risk of contact with the native rock partridge;

2. morphological aspects:

a) morphology,
b) morphometry,
c) sexual size dimorphism,
d) sex determination methods;

3. demographical aspects:

a) demographic assessments (effective population sizes, breeding success, mortality rate, trends, dynamics and populations viability analysis),
b) identification of distribution gaps,
c) localization of small isolated nuclei;

4. ecological aspects:

a) habitat suitability with particular regard to the arduous wintering period,
b) juvenile dispersion, movements, habitat and space use,
c) predation,
d) diseases;

5. *ex situ* conservation and breeding:

a) hand rearing techniques,
b) effect of intensive rearing and tight captivity on the ability of the birds to survive into the wild (restocking or reintroduction purposes),
c) tools and strategies allowing for the mitigation of negative effects of tight captivity.

Overview

Starting from the lack of knowledge listed above, my PhD focused on the rock partridge (*Alectoris graeca*) of the Apennine MU with the aim of contributing to gain new knowledge on incomplete *key aspects* with a view to submit them separately for publication as stand-alone manuscripts.

As such, there are some overlaps in the introduction and methods of chapters four and five.

In the present PhD thesis, I present four studies (Chapters 2 – 5) concerning *key aspects* 1d; 2a, 2b, 2c, 2d, 3a, 5a, 5b and 5c.

Works in progress and planned reaches concerning *key aspects* 1a, 1b, 1c and planned research on *key aspects* 4a and 4b are mentioned after the general conclusions in chapter 7 "*perspectives*".

All applicable international, national, and/or institutional guidelines for the care and use of animals were adopted.

For genetic analysis aimed to identify the haplotype of the studied birds (Chapters 4 and 5), additional expertise was required. Dr. Federica Gabbianelli, of the Department for Innovation in Biological, Agro-food and Forest systems (DIBAF, Unitus), worked on the genetic.

Titles and content of the manuscripts not yet published are potentially subject to partial changes before the final acceptance.

Specific objectives

First study (chapter 2)

The first study was aimed to upgrade the *status* of the rock partridge (*Alectoris graeca*) in some suitable areas of the Lazio region using data of presence collected during a six-year monitoring project in the Provinces of Rieti and Frosinone.

Second study (chapter 3)

The principal aim of this study was to ascertain the colonization of the north of Lazio region by the non-native red-legged partridge (*Alectoris rufa*), with a view to updating the status of its distribution in central Italy, and assessing the risk of contact with the native rock partridge (*Alectoris graeca*) and the consequent risk of genetic pollution.

Third study (chapter 4)

In captivity, behavioural, physiological and morphological changes need attentions because they make animals unable to survive in a natural environment. The main aim of this study was to assess at which stage of growth intensive rearing affects the external functional morphology of rock partridge (*Alectoris graeca*) offspring (G1). The second aim was to investigate the effect of intensive rearing conditions on the sexual size dimorphism (SSD) and on its occurrence. The third aim was to assess the feasibility of sex determination in intensive reared birds when the intensive rearing was not yet effective on the animal morphology.

Fourth study (chapter 5)

Early sexing of animals is a fundamental factor in research, management and conservation and it becomes essential for the management of a sexually monomorphic species. Partridges (genus *Alectoris*) are sexually monomorphic in plumage and do not show obvious sexual dimorphism. Plastically morphological adaptations to the contingent environmental factors are known in both mammals and birds. This study was aimed to identify exportable morphological criteria and methods for an early sex determination in *Alectoris graeca* regardless the growing conditions they are subjected to.

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Chapter 2

Status of the rock partridge (*Alectoris graeca*) in Lazio Region, Central Italian Apennine: six years of monitoring

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Abstract

Data on the presence of the rock partridge were collected in suitable areas of Frosinone and Rieti provinces. Suitable areas were investigated with a pre-reproductive (playback) and post-reproductive (with the aid of pointing dogs) census. To avoid under or double counting, different teams of censors performed counts in adjacent areas simultaneously. Using the technique of playback in the province of Rieti, 153 territorial males were detected. Between 2005 and 2011, the number of territorial males intercepted varied between 4 and 33 per year. The estimated density in protected areas varied between 0.12 and 0.62 territorial males per km^{-2} of area suitable for the species. The estimated density in the hunting areas ranged between 0.12 and 0.45 territorial males per km^{-2} of area suitable for the species. In the province of Frosinone, 67 territorial males were counted. The estimated density on Private Hunting Farms bordering Abruzzo Lazio and Molise National Park varied from 0.58 to 0.73 territorial males per km^{-2} of area suitable for the species. The estimated density in the hunting areas varied between 0.25 and 0.30 territorial males per km^{-2} of area suitable for the species. The density of rock partridge in the region was not quite as high. In the early survey years (2005/2007), we highlighted the limiting factors for the presence of rock partridge. This information allowed for the drafting of a specific action plan for the conservation of the species.

Introduction

In Europe, where the estimated abundance of rock partridge ranges from 40,000 to 78,000 nesting pairs, the species was recently classified as near threatened because it is likely to undergo a moderately rapid population reduction despite its relatively large population and range (IUCN 2012). In Italy, *Alectoris graeca* is present with disjoint populations in the Apennines, Alps and Sicily. Priolo (1984) separated an Apennine subspecies (*Alectoris graeca orlandoi*) based on morphological characteristics. At present, the sub-specific differentiation is not confirmed (Randi *et al.* 2003, Randi 2006). However, in view of the Apennine population's reproductive isolation, it should be considered as a distinct management unit (Moritz 1995, Randi *et al.* 2003). The Directive 2009/147/EC (on the conservation of birds) identifies the need to protect and conserve habitats in which species mentioned in Annex I (including the rock partridge) live through the establishment of Special Protection Areas. Additionally, the rock partridge is listed in the National Red List (Peronace *et al.* 2012), but it is included in the list of hunting species both in Law 157/92 as well as Law 17/95 of Lazio Region. Previously, the rock partridge had a wider distribution range and higher population density compared to the present time (Spanò *et al.* 1985, Brichetti & Massa 1998). Today, the Italian population represents approximately 30% of the world patridge population (Brichetti & Fracasso 2004). Land cover data indicate that during the last century, the Apennines habitat for the species has been progressively reduced and fragmented (Pelorosso *et al.* 2007a, 2007b, Rippa *et al.* 2011). The species is composed of small and locally isolated nuclei (Petretti 1985, Spanò *et al.* 1985, Amici *et al.* 2011, Sorace *et al.* 2011). Although several surveys have been carried in the last decade (Brunelli *et al.* 2011, Sorace *et al.* 2011, P. Viola pers. com.), many study results have not been shared with the scientific community. This reduces the scientific value of the acquired data and does not contribute to the depiction of a complete picture of the status and dynamics of the species.

The rock partridge has a marked selectivity in the habitat requirements for both the winter and spring-summer (breeding season) seasons (Bernard-Laurent & Laurent 1984, Memoli 2003). This has allowed the development of habitat suitability models (Amici *et al.* 2004) that are both generic for the species (Bernard-Laurent & Leonard 2000, Boitani *et al.* 2002, Amici *et al.* 2011) and for the nesting period (Amici *et al.* 2009, in press).

In this paper, we report an upgrade of the status of the species in the Lazio region using information that was obtained through a six-year monitoring project in the Provinces of Rieti and Frosinone. To ensure the comparability of the results obtained in different areas of the region, density was referred to the suitable habitat for the species (Amici *et al.* 2004).

Methods

In Rieti Province the monitoring program was performed in Reatini Mountains group, Duchessa and Cicolano Mountains. In Frosinone province monitoring was performed on the Ernici Mountains (ATC FR1) and in Private hunting farms adjacent to the National Park of Abruzzo, Lazio and Molise. The investigated areas were chosen on the basis of a deterministic habitat suitability model (Amici *et al.* 2011). This model includes both the generic model for the species (Amici *et al.* 2004) and the spring-summer habitat (suitability of the site for nesting, proposed by Amici *et al.* 2004; 2011). For Rieti Province this procedure has shown a suitable area for the species of about 229 km^2 and a suitable area for nesting of 79 km^2; the same figures for Frosinone province are about 95 km^2 and 26.2 km^2.

In both provinces, protected (Natural reserves) and unprotected areas (free hunting areas, private hunting farms) were investigated.

The count of the territorial males in nesting period was performed using the technique of the playback (Bernard-Laurent and Laurent (1984). Family groups were looked for with the aid of pointing dogs, during the late summer/autumn (post-reproductive) according to the available literature.

Playback technique has been applied in accordance with the guidelines described by Bernard-Laurent and Laurent (1984) with the modifications introduced by Serrani *et al.* (2005b). This technique is based on the emission of calls of the male along transects. These were identified in suitable areas for the species. The transects were plotted in two different altitudinal belts. A series of transects at higher altitudes, a second set at lower levels (in areas with lower suitability for nesting) (Amici *et al.* 2007b, 2009).

A digital recorder and an emitter with a digital loudspeaker (50 W power) were the instruments used for the vocal emission. The vocalisations were emitted along 200 m long transects, and at each change of mountainside (Bernard Laurent and Laurent 1984). In each location emissions were broadcasted toward the four cardinal points. Each emission lasted 20 sec for each of the cardinal points, followed by 10 minutes of listening. Each site of emission was georeferenced with a GPS. The position of the reactive males was identified with the polar coordinates (0°-360°) and in case of sighting measuring the distance with a rangefinder.

Playback data were collected between April 20 and June 15 of each year. Each identified transect was run at least twice within 20 days. According to literature, emissions were made in early morning (05:00 - 12:00), with favourable climatic conditions. The data were collected by a variable number of teams depending on the complexity of the area to be investigated (minimum 2 teams). Each team consisted of two operators. In 2010, as a result of unfavourable climatic conditions, and then in 2011 three teams of detectors were engaged.

In the study area, 38 transects were georeferenced and plotted (Table 2.1).

Table 2.1. Covered kilometres (km) for breeding census of rock partridge in each investigated area. Legal status of each area.

Area	N° transect	mileage (km)	Protected area
Province of Rieti			
Pozzoni Mountain	2	5.5	No
Reatini Mountains*	6	24.9	Yes/No
Giano Mountain	2	6.4	No
Cicolano Mountain	6	25.9	No
Duchessa Mountains	6	26.5	Yes
Total	**22**	**89.2**	
Province of Frosinone			
Ernici Mountain	3	11.3	No
Private hunting farm bordering ALMNP**	7	33.2	No
Total	**10**	**44.5**	

*Including “Terminillo Oasis”, ** Abruzzo, Lazio and Molise National Park

Some sites of presence of rock partridge are separated by short distances, but they are rather distant to reach by following any ordinary road (e.g. Colle Alto, Serra, Nuria, Nurietta and Monte Giano). Bernard-Laurent (1988) has shown, with the use of radiotelemetry, that a population of hybrids (*A. graeca x A. rufa*) within the French Maritime Alps, moved within a range of a few hundred meters to 6.3 km. These findings suggest that mobility of the species among these areas is possible. In order to limit the possibility of missed or double male counting emission was simultaneously performed on both mountain sides.

The onset of the territorial phase of males is dependent by the climatic conditions (Sorace *et al.* 2009, Amici *et al.* 2004), and the period of maximum reactivity vary in the years (Cramp e Simmons 1980, Petretti 1985, Amici *et al.* 2004). For these reasons, some sample areas have been chosen (Amici et al, 2007b, 2009) to detect the presence of reactive males and to start the monitoring plan on a wide scale.

In the post-reproductive period, rock partridge was recorded performing transects in areas with good/high suitability for the species with the aid of pointing dogs. The investigated areas included summer and winter areas for the species. The first are found at altitudes above 1,600 m a.s.l. (Petretti 1985, Brichetti and Massa 1998) the second between 1,000 m a.s.l. and 1,600 m a.s.l. (Cattadori *et al.* 2003).

Compared to other standardized methods, the application of this technique favours a considerable saving of time (Warren 2003). The dogs are carefully chosen among those available (adults, well connected to the conductor and with certification of Ente Nazionale per la Cinofilia d'Italia). The task of active searching of rock partridge is entrusted to them (Smith *et al.* 2001, Calladine *et al.* 2002) ranging within a buffer of 50 m transect from the predetermined path covered by surveyor and conductors.

The transects were chosen according to the bibliography i.e., those at higher altitudes were run before those at lower altitudes. The execution took place in early morning (06:00 - 09:00; Smith *et al.* 2001), only on days with mostly clear sky and wind of restrained intensity (Thirgood *et al.* 1995).

Results

In Rieti province, with the technique of playback, 153 territorial males were heard/observed (Tab. 2.2). In different years, between 2005 and 2011, the number of territorial males intercepted varies between 4 and 33. The density of territorial males were expressed as number of territorial males Km^{-2} of suitable habitat (Amici *et al.* 2004).

Table 2.2. Number of males of rock partridge recorded with playback and pre-reproductive density (expressed as number of territorial males Km^{-2} of suitable habitat) of the species in each study area.

Area	Number of males						Pre-reproductive density					
Province of Rieti	**2005**	**2006**	**2007**	**2009**	**2010**	**2011**	**2005**	**2006**	**2007**	**2009**	**2010**	**2011**
Pozzoni Mountain	3	3	2	3	3	2	0.21	0.22	0.18	***	0.24	0.18
Reatini Mountains*	16	21	17		18	17	0.48	0.62	0.55		0.57	0.55
Giano Mountain	3	2	4	1	3	4	0.12	0.08	0.16	***	0.12	0.16
Cicolano Mountain	3	3	3		5	5	0.31	0.35	0.35		0.45	0.45
Duchessa Mountains	2	4	6				0.12	0.24	0.36			
Province of Frosinone	**2005**	**2006**	**2007**	**2009**	**2010**	**2011**	**2005**	**2006**	**2007**	**2009**	**2010**	**2011**
Ernici Mountain			3	2	3	3			0.25	0.30	0.30	0.30
Private hunting farm bordering ALMNP**			12	15	14	15			0.73	0.58	0.69	0.58

*Including Terminillo protected Oasis, ** Abruzzo, Lazio and Molise National Park; ***Inconsistent data

The estimated density in protected areas varies between 0.12 and 0.62 males km^{-2} of suitable areas. The estimated density in the hunting areas ranges between 0.12 and 0.45 territorial males km^{-2} of suitable habitat.

In Frosinone province monitoring was yearly conducted between 2007 and 2011. During the survey 67 territorial males were observed (Tab. 2.2.). Surveys conducted in Private Hunting Farm bordering Abruzzo Lazio and Molise National Park showed the presence of 12 - 15 territorial males, and an estimated density ranging from 0.58 to 0.73 males km^{-2} of suitable areas. In hunting areas of Ernici Mountains density varied between 0.25 and 0.30 males km^{-2} of suitable habitat.

The census with pointing dogs showed poor reliability. This probably also depends on low densities of the species. Therefore, the results of post reproductive census are summarised.

In the Reatini Mountains a total of 22 brigades were observed, composed by 3.6 individuals on average.

Discussion

The comparison between the densities of rock partridge found in protected vs. hunting areas indicates that the status of the species is positively correlated to the level of protection of the area (Sorace *et al.* 2011). However the level of protection is not the only important factor.

The values obtained in this study are comparable to those recorded by Bernard-Laurent and Leonard (2000), but they are lower than the maximum found in the Alps by the same authors. However, the numerical comparison is complicated by the heterogeneity in the identification of areas of investigation (Tab. 2.3) (De Franceschi and Odasso 1998, De Filippo *et al.* 1999, Renzini *et al.* 2001).

Table 2.3. Density values reported in literature. *Alps

Density	Units	Author
1.85	Territorial males km^{-2}	Renzini *et al.* 2001
0.3-4.7	males/ km^{-2}	Bernard-Laurent and Leonard 2000*
1.4-1.7	pairs km^{-2}	Spanò *et al.* 1985
5.4-8.0	males/ km^{-2}	Bocca 1990*

Standardisation is needed for the census method (e.g. frequency of the emission, listening time, number of operating units with respect to suitable surface, etc.).

Despite the cited advantages of using pointing dogs in post reproductive census (reducing the detection time, the direct involvement of locals in management actions etc..) the technique has shown some limitations. The efficiency of the dogs is closely related to the climate and the degree of training. This made it difficult to have teams (drivers/dogs) homogeneous and characterised by ability to standardised and constant investigation. The usefulness of the technique to determine reproductive success of species (number of individuals / brigade) should be stressed.

The low densities of rock partridge registered in this study may be attributable in general to the habitat suitability.

The preference of sward with low xeric grassland interrupted by rocky outcrops, rocks, shrubs and dwarf (Petretti, 1985) puts the species in relation to the areas of pasture, where the higher is food availability. The reforestation of the high pastures in the province of Rieti started after World War II (Pelorosso *et al.* 2007a). The expansion of forests (Van Gossum *et al.* 2010) with the consequent expansion of wild ungulates (Carnevali *et al.* 2009) has reduced the habitat available for other species (Memoli 2003, Pelorosso *et al.* 2007a) as the rock partridge.

According to forecasts of medium-long term, this situation should deteriorate further due to the progression of forest in grasslands (Pelorosso *et al.*, 2007b, Sorace *et al.* 2011). Variation produced by global warming (Bolli *et al.* 2007, Peñuelas *et al.* 2007) should be also stressed.

This phenomenon, reducing the food availability for wild ruminants (deer, chamois) and domestic ones (cattle and horses in the pasture) will impact negatively bird populations associated with altitude meadows (Laiolo *et al.* 2004, Tellini-Florenzano 2004).

The negative effects of fox, hooded crow and dogs (Sorace *et al.* 2011) found in investigated areas might be very important. Recently it has been highlighted the limiting effect due to the presence of the wild boar (Amici *et al.* 2007a).

The monitoring program conducted in the study area allowed to highlight the major factors limiting the occurrence of the species: progressive reduction of the use of upland areas by livestock, poaching and predators (Amici *et al.* 2007a, 2011, Serrani *et al.* 2005b, Sorace *et al.* 2011). All phenomena that are not easily counterbalanced (Serrani *et al.* 2005b). The investigations have led to the first draft of the management statement for the conservation of rock partridge in Central Apennines (Amici *et al.* 2007a). This document represent the milestone to set future management decisions that, in addition to the hunting ban, should increase habitat suitability for the species. Finally the lack of coordination between the various working groups that operate simultaneously in the same area should be avoided because it favours a waste of energy and resources, and unnecessary duplication of the same investigation.

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Chapter 3

Is the red-legged partridge *Alectoris rufa* naturally colonising the north of Lazio region, Italy?

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Abstract

The present study was aimed to update the distribution of the red-legged partridge in Central Italy, with particular reference to the northern Lazio Region. Spanò (2010) suggested to prevent the introduction of red-legged partridge below a line connecting the provinces of Grosseto and Ascoli because certainly out of the original distribution range of the species.

In Tuscany, the species was successfully reintroduced in the Provinces of Grosseto and Siena and it is still massively released for hunting purposes.

Several reports on the species' presence and reproduction were received from the northern Viterbo province, suggesting the necessity of a survey. Local people were interviewed (forestry agents, keepers, hunters and farmers), and a field survey was performed utilizing detection signs of presence and playback count. All data were registered in a geodatabase. Signs were georeferenced in a grid of 3,864 territorial units (TUs; 1 km x 1 km). Red-legged partridges were found in 190 TUs (19,000 ha): brigades, composed by 6 individuals on average, within 31 TUs; breeding pairs within 31 TUs; single birds within 128 TUs. To confirm the presence of breeding pairs, a playback survey schedule was performed throughout the spring in a random sample of cells (28.9% of the cells in which the presence of the species had been reported) along line transect of 118 km length in total. Playback

survey confirmed the presence of 25 breeding pairs and 6 single individuals within 31 TUs. Because the province of Viterbo did not perform restocking of the species, the detected distribution appears to be caused by the natural expansion of populations from Toscana and Umbria regions. Further studies are currently in progress to better appreciate the population parameters.

Introduction

Declines in the distribution and abundance of many wildlife species associated with agricultural ecosystems have been acknowledged across Europe since the 1960s (Tucker & Heath 1994). The major causes of this reduction is found to be the abandonment of traditional agriculture in favour of mechanized and intensive agricultural practices (Murphy 1989), as well as excessive hunting pressure (Tapper 1999). However, the widespread introduction of set-aside throughout Europe from the mid-1980s and the reforestation of marginal agricultural areas were considered as possible mechanisms to reduce the adverse effects of agricultural impacts (Sotherton 1998). This has produced benefits for farmland wildlife, facilitating the colonization of species related to transitional and forested habitats (Amici *et al.* 2011).

Although the conservation status of the grey partridge *Perdix perdix* in Italy is also critical after many years of massive reintroduction (Simonetta & Dessì Fulgheri 1998), the red-legged partridge *Alectoris rufa*, a species more adapted to dry hilly land with small bushes, has extended its areal distribution without management intervention, exploiting the abandoned marginal landscape and the transition habitat (Spanò 2010).

The Italian Red List 2011 currently classifies the red-legged partridge as Data Deficient (DD), because there was not enough data to analyse the degree of genetic pollution and the dependence of the sub-populations on restocking for hunting purposes (Peronace *et al.* 2012). The species is listed in Annexes 2 and 3 of 2009/147/CE Directive, in Annex 3 of the Berne Convention, as it is considered to be at low risk (Least Concern) by the International Union for Conservation of Nature (IUCN 2011) and category 2 of SPEC (species with unfavourable conservation status in Europe).

Italian and regional laws include the red-legged partridge on the list of hunting species. The Lazio region hunting regulation, however, did not include the species in the list of hunting species (Lazio Region 2011).

Widespread evidence of species presence and nesting in the northern region of the Viterbo province suggested the necessity of a monitoring plan. The aim of this study was to ascertain the stable presence of red-legged partridge in the north of Lazio region as a first step towards updating the status of its distribution in central Italy and assessing the risk of contact with the native rock partridge (*Alectoris graeca*) and the consequent risk of genetic pollution.

Materials and methods

STUDY AREA

The study area was located in the Viterbo Province, central Italy, in the zone managed by the ATC VT1 (ATC: Hunting Territorial Zone) (187,496 ha) (Fig.3.1).

The elevation ranges from 0 m above sea level (a.s.l.) to 1,053 m.a.s.l. Based on the climatic averages of the last 6 years (January 2005–December 2011), the average temperature of the coldest month, January, is 5.9 °C, while the average of the hottest month, July, is 23.8 °C. On average, there are 33 frost days per year and 50 days per year with a maximum temperature equal to or above 30 °C. In the period examined, the extremes of temperature were +38.6 °C in July 2005 and −5.8 °C in March 2005. The annual rainfall was 848 mm, distributed over 203 days on average, with the minimum in summer and the peak in autumn (Tuscia University weather station, 301 m a.s.l., 42°25′31.86″ N, 12°04′ 43.47″ E).

The landscape is highly fragmented, with cultivated fields that are interspersed with woodland and scrubland as well as with a gradient that increases from the coast towards the interior hills. Along the coast, the fields are planted with vegetables; in the inland, irrigable plain summer crops such as maize and sunflowers are the predominant cultivations, and winter cereals and forages are very often sowed after autumn. At the higher altitudes, orchards (vineyards, olive groves, chestnut and hazelnut) and woodlands dominated the hills. Most of the forests are coppiced, while timbers are present more frequently into the protected areas.

The survey plan was performed using bibliographic research, interviews and field investigations. Scientific journals and conference proceedings were the principal source of the bibliographic data. By means of the major scientific search engines available on the Web, a search was performed using “red-legged partridge”, “*Alectoris rufa*”, “Italy”, and “distribution” as keywords. Subsequently, literature reviews were performed using the same descriptors (subjects), and a further literature search was conducted on general Web search engines.

With particular attention to pre-reproductive (March) and post-reproductive (August and September) periods, a two years plan for direct acquisition of information by interviewing reliable sources was designed. Direct or telephone consultations were made to agents of the State Forestry and Province Police Corps, hunters, volunteers from the Observatory for the Study and Management of Wildlife Resources at the University of Tuscia, and to others (hikers, keepers, and farmers). In particular, the information collected focused on direct and indirect (signs of presence) sightings within the ATC VT1. On the basis of received reports, a geodatabase was built and upgraded using the ArcMAP™ 10.0 software (ESRI®). Each reference was inserted in a record containing the location, the type of sighting (individuals, couples, brigades, etc.), and all other information useful for the study (Amici *et al.* 2008). All reports were ordered and connected, through the same software, to a grid layer for subsequent analysis. In particular, a square grid with 3,864 cells (1-km side), was superimposed on aerial photographs and on the Technical Map 1:25,000 and 1:10,000 scale. Each cell was regarded as a territorial unit (TU). The reference system used was the European Datum (ED) 50, Zone 33 Northern Hemisphere. Each TU was assigned a value based on information of the presence/absence of the species, according to the types of sighting (single individual, couple, and brigade) (Fig. 3.1). Using the geo-referenced reports, a casual network of sample line transects was identified. These started from the 1-km cells that fall within a buffer of 25 km from the borders of Tuscany and Umbria. A random sample of 163 TU (28.9% of the total positive, 16,331.00 ha) was extracted, and transects were identified considering existing road layouts, altitude, and exposure, among other factors. These line transects were investigated using the technique of playback census (Bernard-Laurent & Laurent 1984, Serrani *et al.* 2005) in the period between March and April. This technique involves the selection of transects where the males emit calls, within the territorial period, respond and often become visible. The calls were emitted by a transmitter with a digital speaker (50 W power) every 500 m for 20 sec at each cardinal point, followed by 5 minutes of listening. The position of the male was identified through the terrestrial coordinates (with a GPS), and in cases of sighting, the distance was measured with a rangefinder.

Results

BIBLIOGRAPHIC SURVEY

The red-legged partridge subspecies *rufa* is a native species of Italy (Snow and Perrins 1998, Andreotti *et al.* 2001); it is sedentary and nests on the northern Apennines as well as on the Toscana Archipelago (Elba, Giglio, Pianosa and Capraia). Additionally, the species was introduced and partially acclimatized in the Lazio, Umbria and Molise regions, without success elsewhere (Alps, Friuli, Sardinia, etc.). Populations at the southern limits of the Apennines distribution (Tuscany) are regularly restocked. In historical times, the subspecies was believed to be widely spread in the north-central Apennines (Piedmont-Marche) and the Archipelago Toscano (Elba and Monte Cristo), but almost everywhere, there has been a considerable decrease in the population (Brichetti & Fracasso 2004). In the Capraia and Pianosa islands, it became extinct at the end of the nineteenth century, but was successfully reintroduced in recent years (Tellini Florenzano *et al.* 1997).

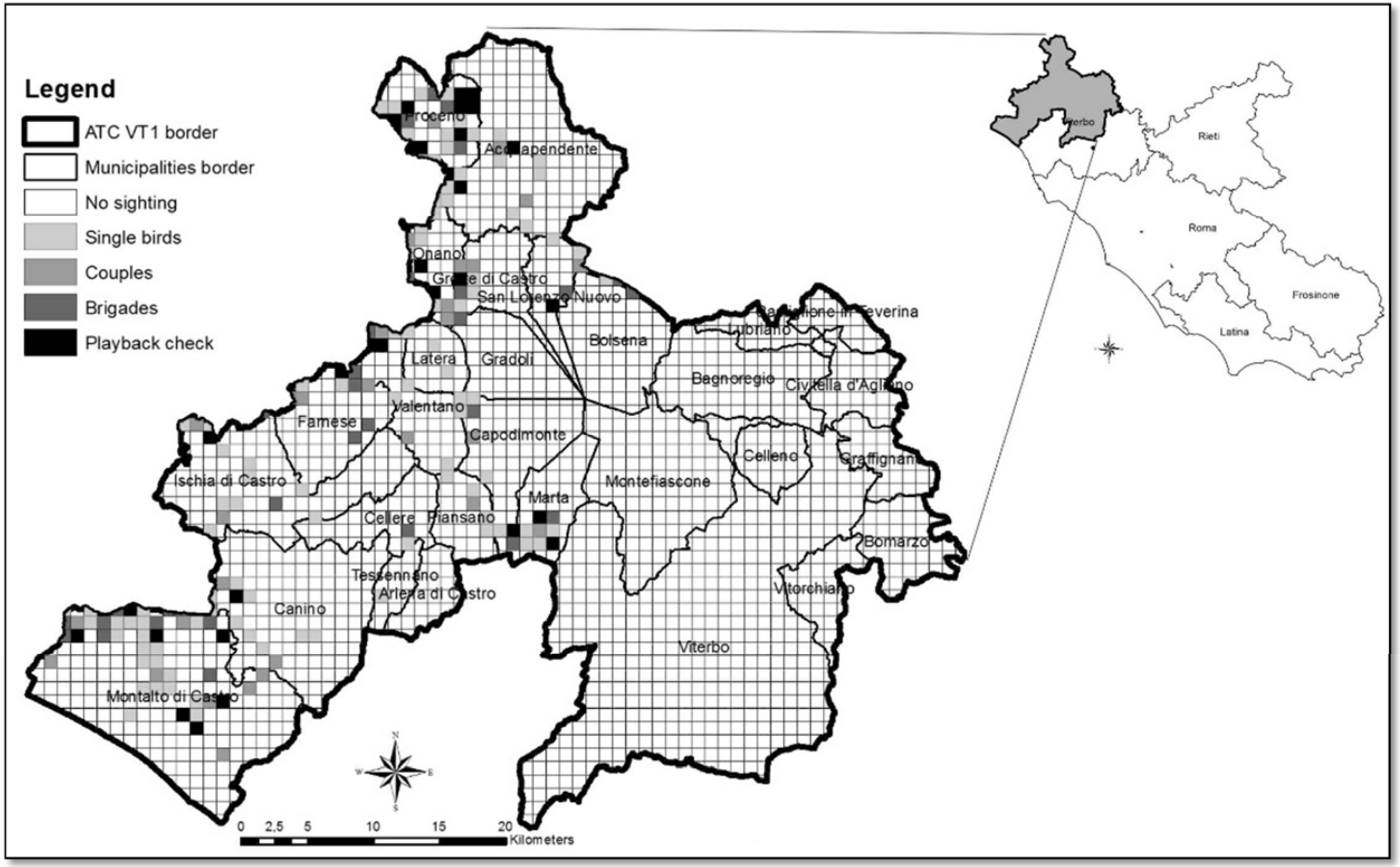
In Toscana, a region of particular interest for this study because it borders the northern region of the ATC VT1, the red-legged partridge underwent a general and sharp decline in the last decades, but recently, it has been successfully reintroduced after restocking (Lucchini *et al.* 1999, Meriggi *et al.* 2007, Spanò 2010). Densities observed between the years 1995 and 2002 in the reintroduction areas of the province of Siena (Tuscany region) totalled approximately 5.7 heads km^2 and 1.4 broods km^2 (Brangi *et al.* 2003). In Umbria, the red-legged partridge was introduced and now is partially acclimatized (Brichetti & Fracasso 2004, Spanò 2010). Its "patchy" distribution is closely associated with the recovery actions of hunters exclusively for hunting purposes. These actions may lead to the formation of autonomous small populations, especially in wildlife areas and other regions off-limits to hunting (Renzini 1997). In the Lazio region, the species was introduced in the Lucretili (Angelici 1995) and Lepini Mountains (province of Rome, Frosinone and Latina) (Boano *et al.* 1995, Brichetti & Fracasso 2004), but its presence in these areas was not confirmed during recent data collection (Bulgarini 2011). The same author (Bulgarini 2011) reports observations of the species in the municipality of Blera (province of Viterbo) and inside the Natural Reserve of Marturanum (province of Rome) and suggests a possible consequence of restocking for hunting purposes. These two reports were registered in shrubby agricultural areas located around 300 m a.s.l.

INTERVIEWS

Each of the 156 respondents indicated the municipality and location of sightings, the date and whether the partridges were grouped (brigade), in pairs or appeared as single individuals. A subsequent screening of the responses made it possible to exclude double counting.

The number of TUs with positive signals was 190: brigades were detected in 31 TUs and were composed of 6 individuals on average; couples and single individuals were detected in 31 and 128 TUs, respectively (Fig.3.1, Tab. 3.1).

Figure 3.1. Map of TUs with sightings of single birds, pairs and brigades, and playback positive response.



ON-FIELD SURVEY

The on-field survey confirmed the presence of a minimum of 25 territorial males, to which 6 random encounters with single birds were added for a total of 31 sightings (one for each TUs; Tab. 3.1, Fig. 3.1). The Index of Kilometric Abundance (IKA) relating only to "breeding pairs", calculated on the 118 km of covered transects, was equal to 0.21 pairs/km[1].

Table 3.1 Number of territorial units (TU) investigated and number of TU where sightings (brigades, couples and individuals) were reported for each of the two used methods.

	TUs	Positive TUs with brigades	Positive TUs with couple	Positive TUs with single birds
Interviews	3,864	31	31	128
On field survey	163	-	25	6

Discussion

The results of this study show a homogeneous distribution along the ATC border with the provinces of Grosseto and Siena where the red-legged partridge was actively managed for decades. The presence of the species on the border with the province of Terni was scarce. A descending presence has been detected from the border with Toscana (municipalities of Montalto di Castro, Canino, Ischia di Castro, Farnese, Valentano, Onano, Proceno, and Acquapendente) and Umbria (municipalities of San Lorenzo Nuovo and Bolsena) to the most internal municipalities such as Grotte di Castro and Latera. This situation, together with the absence of significant distribution gaps, suggests that the presence of the species is the result of natural expansions of the populations from the south of Tuscany and Umbria. This fact is also hypothesized based on the literature.

The distribution, although still patchy, does not present large voids. Among the probable factors of the disturbance due to natural evolution, we can list the illegal killing and the widespread presence of opportunistic predators (foxes, corvids) typical of unmanaged habitats. In the study area, the authors were able to observe the direct predation of nests and nestlings of pheasants in specific wildlife areas (repopulation and capture areas). Therefore, predation pressure might represent a limiting factor that is also effective for the natural expansion of red-legged partridge, as also confirmed by Vargas *et al.* (2006).

A monitoring program must be planned in the near future to increase the knowledge regarding the population parameters and to predict the dynamic expansion of the species as well as promote the most suitable management actions.

In this regard, we can exclude any immediate risk of hybridization with Apennine rock partridge because there is no suitable habitat within the ATC VT1 territories, but further researches are necessary to appreciate if the non-native partridge can potentially threat the genetic integrity of the native one trough dispersion.

Additionally, the risk of intraspecific competition with the grey partridge, restocked in the years 2006-2007 (Amici *et al.* 2007), must be carefully assessed.

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Chapter 4

Effect of the intensive rearing on the functional morphology and the sexual size dimorphism in Apennine rock partridges (*Alectoris graeca graeca*)

Submitted to *British Poultry Science*

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Abstract

In captivity, behavioural, physiological and morphological changes need attentions because they make animals unable to survive in a natural environment. The main aim of this study was to assess at which stage of growth intensive rearing affects the external functional morphology, the sexual size dimorphism (SSD) and its occurrence, in rock partridge (*Alectoris graeca*) offspring. At this purpose, six exterior morphological traits recorded on birds subjected to typical intensive rearing conditions (G1) were compared with the respective recorded on related offspring housed in a complex wild-similar environment (G2). Significant ($p = 0.01$) morphological divergences were recorded in the G1 at 84 and 98 days post hatching when birds in the control semi-natural group showed longer head, deeper and wider tarsi probably as result of the physical and mental exercise they did in the complex environment. Our results suggest that the intensive rearing does not determine substantial non-functional morphological changes at least up to 70 days post hatching. Birds for restocking or reintroduction should be translocate within this time limit, and optimally before 56 days post hatching, in pre-release complex semi-natural environments where they can do exercise and compensate muscle, tendons and neuronal development. Starting from 42 days

post hatching sex determination is also possible with high accuracy allowing for the translocation of sex balanced groups of birds from tight captivity to acclimatization sites.

Introduction

The conservation status of *Alectoris graeca* Meisner (Galliform: Phasianidae) was declared vulnerable (VU) in Europe by Bernard-Laurent and Boev (1997) as well as at the National scale (Italy) by Peronace *et al.* (2012) and Rondinini *et al.* (2013).

Actually, the species is included in the Annex I of the EU Birds Directive and classified as Near Threatened (NT) in the IUCN Red List (BirdLife International 2015) but the continuing decline in the area of occupancy (BirdLife International 2015) suggests a next leap in the vulnerable (VU) category.

To reinforce wild populations and to restore the metapopulation functionality, captures of wild birds for translocations are ill-timed (Ellis, Dobrott & Goodwin 1978) because the critical state of its little and isolated populations (Cattadori *et al.* 2003). Therefore, *ex situ* conservation programs and production systems of captive rock partridges suited to the wild are necessary for insurance, reintroduction and restocking targets (Armstrong & Seddon 2008).

In *ex situ* conservation programs efforts are commonly concentrated on preserving the genetic variability of the captive populations (Frankham *et al.*, 1986).

This is clearly a principal objective but also behavioural, physiological and morphological changes need attentions because they make animals unable to survive in a natural environment (Liukkonen – Anttila *et al.*, 1999, 2000; Millàn *et al.*, 2003; Price 2002; Alonso *et al.* 2005; O'Regan & Kitchener 2005). A reduction of the sexual dimorphism, that is crucial for many sexual selection strategies and essential for developing expeditious sex determination methods, was also detected in captive animals.

Such changes were commonly detected in multigenerational captive animals and often attributed to the '*passive selection for animals that are behaviourally more suited to captivity'* as O'Regan & Kitchener (2005) commented, with possible severe consequences on the ability of the successive generations to survive and to reproduce into the wild. It is also reported that morphology and behaviour can early respond and plastically adapt to early environmental stimuli (Witheside *et al.*, 2016).

Natural or semi-natural rearing systems can mitigate the negative effects of typical intensive rearing allowing for better survival rate of hand-reared animals after release (Buner and Aebischer 2008, Pérez *et al.* 2015).

However, there is anecdotal evidence that breeders of galliforms rarely exploit this kind of complex and integrated rearing strategy for logistic and commercial reasons. At present, the typical intensive rearing continues to play a dominant role especially for the restocking of harvested populations.

The effect of typical hand-rearing systems on behaviour, physiology, soft tissues (gut, heart, liver, gizzard) and muscles (pectoral, thigh) development, have been relatively well studied in galliform species (Bergero *et al.*, 1995; Putaala & Hissa, 1995; Liukkonen-Anttila *et al.*, 2000; Tobalske *et al.*, 2000; Pérez *et al.*, 2002; Millan *et al.*, 2003; Hess *et al.*, 2005; Gaudioso *et al.*, 2011a). Some possible mitigation measures have also been proposed: environmental enrichment protocols (Whiteside *et al.*, 2016), antipredator training (Gaudioso *et al.*, 2011b) and complex diets (Whiteside *et al.*, 2015).

Differently, exterior morphological changes need more attentions and insights to understand: 1) if, 2) how early and 3) how strongly, the effect of tight captivity could affect the functional morphology of hand-reared galliformes.

The main aim of this study was to assess at which stage of growth intensive rearing affects the external functional morphology of Apennine rock partridge (*A. g. graeca*) offspring (G1). The second aim was to investigate the effect of intensive rearing conditions on the sexual size dimorphism (SSD) and on its occurrence. The third aim was to assess the feasibility and the accuracy of sex determination when the intensive rearing was not yet effective.

Material and methods

BASIC ASSUMPTIONS

We based our study on four basic assumptions:

1. wild phenotype, including the morphotype, it's the most functional into the wild;
2. the genotype of offspring of wild parents is not corrupted by conscious or inadvertent artificially selection;
3. offspring of wild parents subjected to "wild similar stimuli" can adequately represent the wild phenotype including the morphotype;
4. morphological changes recorded on offspring of wild parents living under typical intensive rearing conditions, are the result of plastically adaptations to contingent non-natural stimuli and they must be interpreted as a loss of functionality into the wild.

ANIMALS

Founders and incubation

In 2013, a wild *A. graeca* breeding pair from the Sirente Velino Mountains group (AQ – Abruzzo Region), was transferred from the rehabilitation centre of the Vico Lake Regional protected area to "la Starniana" fosterage located in the municipality of Onano (Viterbo Province, Italy). Genomic DNA was extracted from feathers as described by Barbanera *et al.* (2005) and quantified using a DTX Multimode Detector 880 (Beckman) with PicoGreen method (Quant-iT, Invitrogen) according to the manufacturer's instructions. The entire D-loop was amplified using the primers PHDL (5'-AGG ACT ACG GCT TGA AAA GC -3') and H1321 (5'-TAG YAA GGT TAG GAC TRA GTC TT-3'), following Barbanera *et al.* (2005). Each sample was amplified, purified by ExoSap-IT (USB Corporation) and sequenced. Sequencing was outsourced to Macrogen (www.macrogen.com). They were aligned with sequences obtained by GenBank belonging to different *A. graeca* haplotypes (AM492997, AM084673, AJ222731, DQ834524, AJ222730), *A. chuckar* (HQ735256, D66890) and *A. rufa* (HQ674699). The wild *A. graeca* breeding pair proved to belong to *A. g. graeca* haplotype 23 (H23) (Randi *et al.* 2003).

The wild breeding pair was located in a dedicated rehabilitation aviary of 50 m^2 and 3 m high, where scattered rocks and composite vegetative cover were available. Different seeds and a specific food for breeders (21% CP and 2750 MJ/Kg ME) were provided *ad libitum* with a feeder hopper protected by a hut as well as water.

The animals were maintained for two successive reproductive seasons in standard environmental, food and climatic conditions. These wild birds mated and the female laid 19 eggs in June 2013, and 20 between mid-May and mid-June 2014.

Aiming to obtain two successive groups of contemporary birds, eggs were collected daily, stored at 12 – 14 °C and 70 - 80% RH, 45° turned every 12 hours, and artificially incubated all together on June 28 and June 19 respectively in year 2013 and 2014. In both the years, eggs hatched after approximately 23 days of artificial incubation.

Animals in the experiment

The broods were composed respectively of sixteen and fourteen birds. At sexual maturity, 1:1 sex ratio (8 male and 8 females) was verified in the first brood (2013) while in the second one (2014) the male class was more numerous than the female one (8 males and 6 females).

EXPERIMENTAL DESIGN

The brood of the first year (2013) was reared under typical intensive conditions and it was assigned to the treatment group (G1). After hatching, chicks were located into a heated room in a cage at a density of 16 chicks/m^2 up to 14 days post hatching (DPH). Heating process was conducted as described in Günlü *et al.* (2007). From 15 DPH to 35 DPH, the available indoor area was enlarged to allow a density of 2 chicks /m^2. From hatching to 35 DPH chicks were indoor on a concrete floor covered with straw. Starting from 36 DPH the "*indoor - outdoor phase*" began using an adjacent aviary of 25 m^2. Hence, 2 m^2 per bird were overall available up to the end of the trial. The group was fed ad libitum with a starter diet (28% CP and 2850 MJ/kg ME) during the first three weeks, and subsequently with a second period diet (22% CP and 2850 MJ/kg ME). Water was provided ad libitum and during the first day after hatching, it was enriched with sugar and mineral salts.

The brood of the second year (2014) has grown in a natural complex environment under the guidance of the natural parents and was assigned to the control group (G2). After hatching, chicks were immediately relocated under the hen that meantime continued to incubate some surrogate eggs. The parental couple accepted chicks without reticence and the “brigade” stayed into a 50 m^2 aviary in a complex semi-natural environment (alternation of composite vegetative cover, draining ground, stones, rocks and ramps) up to the end of the trial. A consistent presence of ants, attracted positioning upturned plant pot as suggested by Buner and Aebischer (2008), was verified into the aviary. Different edible seeds and green foods were constantly available, but during the first three weeks, chicks ate almost exclusively arthropods (pupae and eggs) naturally present in the aviary and moths artificially provided.

DATA RECORDING

Chicks were individually tagged on the right wing *patagium* with non-toxic marker. Starting from 14 DPH and up to 98 DPH birds were fortnightly weighted and measured for eight exterior morphological traits (MTs) by the same operator. MTs recorded were: live weight (LW), tarsus length (TL), tarsus depth (TD), tarsus width (TW), head width (HW), head length (HL), wing length (WL) and beak length (BL). TL was the distance between the proximal and the distal ends of the tarsus-metatarsus (Pis, 2012). TD and TW were respectively the depth and the width of the tarsus-metatarsus at the distal end of the spur scale (Çağlayan *et al.*, 2011). HW was the distance between the two orbital faces of the frontal bone and HL between the midpoint of the occipital and the nasal face of the frontal bone (Çağlayan *et al.*, 2011). WL was the distances between the wrist joint (radio-carpal) and the longest primary (Bernard-Laurent *et al.* 2003) and BL the length of the straight line along the upper margin of the beak (Pis, 2012). Partridges were weighed with an electronic balance (±0.01 g accuracy). All the linear measures were taken to the nearest 0.01 mm with a digital calliper except for WL that was measured by a ruler (1 mm accuracy). All the bilateral measurements were taken on the left side of the bird.

STATISTICAL ANALYSIS

Statistical analysis was performed with SPSS statistical software (IBM Corp. 2013). Normality of data distribution was tested in each group using Normal Q-Q plot and Shapiro-Wilk test.

All the measures of the investigated MTs were reported as means ± standard deviations.

With the aim to assess, by age, morphological changes and the occurrence of SSD, the Fisher test (GLM F-test) was used to compare groups and sexes at different DPH. Group and sex were the main factor in the analysis. The effects of the group and of its interaction with the sex (group x sex) were investigated by age in all the MTs. The Fisher's least significant difference (LSD) post hoc test was used to determine the significance in pairwise comparisons. The homogeneity of the variance (DPH) of each MT was preliminary verified by Levene – test for all the morphological traits.

The Sexual Size Dimorphism Index (SSDI) proposed by Calabuig et al. (2011) was used to assess the magnitude of the sexual dimorphism in size:

$$\text{SSDI} = \frac{mean\ size\ of\ the\ males - mean\ size\ of\ the\ females}{mean\ size\ of\ the\ females} \text{ x } 100.$$

With the aim to evaluate the feasibility of early sex determination aimed to translocate, at the appropriate time, sex balanced group of young birds from tight captivity to semi-natural and complex acclimatization sites, we processed discriminant variable (MTs showing significant differences between the sexes in the G1) by Discriminant analysis (DA). The equality of the covariance matrices was preliminary checked using Box's M test. The forward stepwise method was used. In the stepwise approach, the Wilk's Lamba method was used to select the discriminant variables. The values of F-enter and F-remove for the selection of the discriminant variables were leave as default, respectively 3.84 and 2.71.

The discriminatory power of the functions was measured considering canonical correlation (r) and Wilk's Lamba (Wilk's). Their accuracy was tested a) on the original sample and b) by the bootstrap method with 1.000 iterations, fixing equal a priory probability (0.5).

For all the statistics, the 1% significance level ($p = 0.01$) was accepted. Significance level at $p > 0.01$, although conventionally relevant, in this case could be misleading because the limited number of samples in the present study or other factors could generate the occurrence of transitory differences.

Results

Already at 14 DPH beaks and tips of outer primaries resulted respectively slightly wounded and ticked in the treatment group (G1). This fact prompted us to consider the lengths of the beak and of the wing not accurate measures of the respective MTs and consequently not reliable enough for the comparisons between groups. WL and BL were then excluded from the measurement protocol.

MORPHOLOGICAL COMPARISONS BETWEEN GROUPS

The homogeneity of the variance was confirmed by the Levene – test for all the MTs.

The group x sex interactions were never significant. Differently the effect of the group was responsible of some significant morphological changes (Fig. 4.1, Table 4.1).

Figure 4.1. Comparisons (GLM *F-test* at p = 0.01) between the treatment group (G1) subjected to typical intensive rearing conditions (grey bars) and the control one (G2) housed in a semi-natural complex environment (white bars). Compared morphological traits were live weight (a), tarsus length (b), tarsus depth (c), tarsus width (d), head width (e) and head length (f). Comparisons were performed by age at 14, 28, 42, 56, 70, 84 and 98 days post hatching (DPH). Vertical bars denote standard deviations (SD). ** = $p < 0.01$; *** = $p < 0.001$.

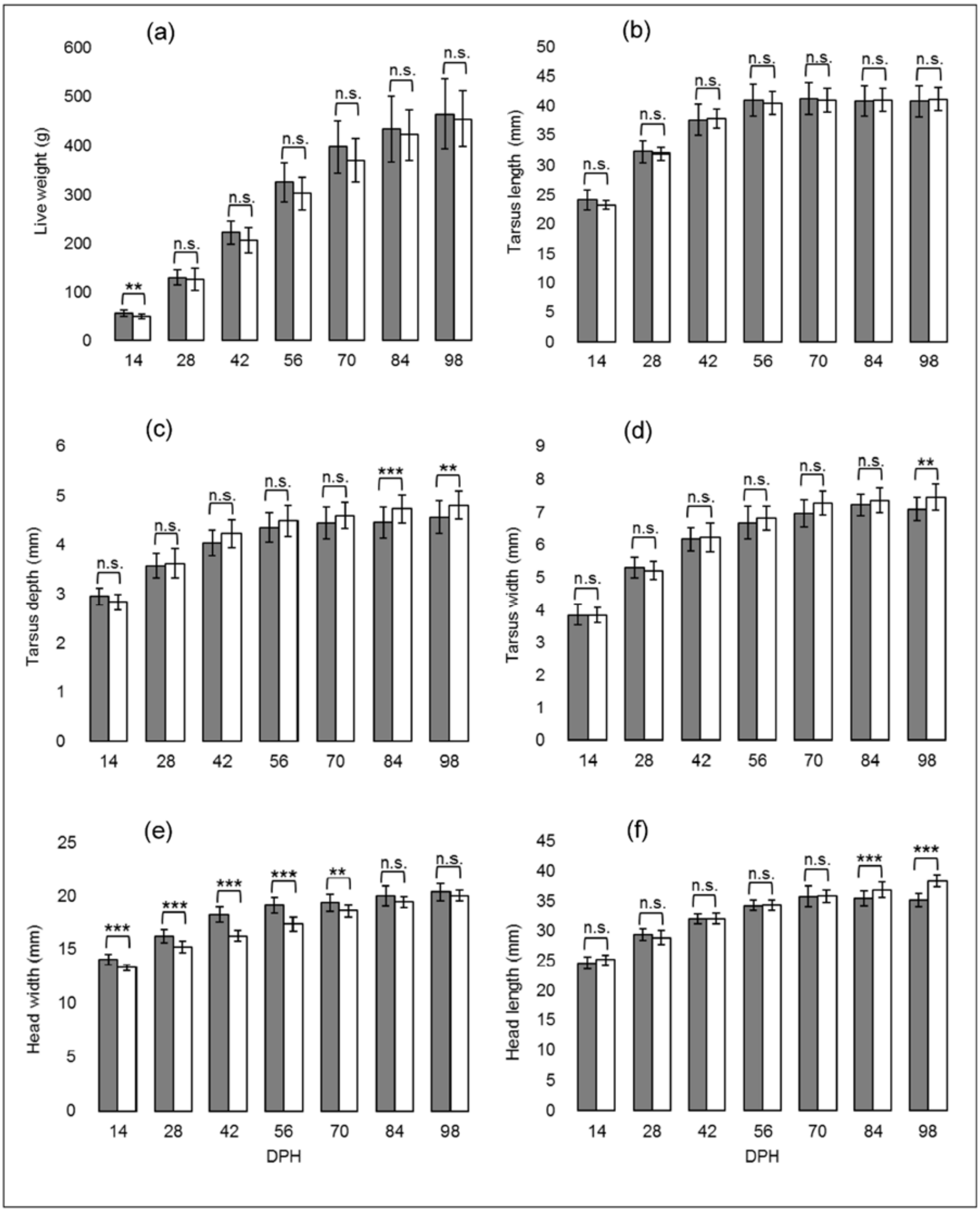

Table 4.1. F and *p-values* of the comparisons (GLM *F-test* at p = 0.01) performed at 14, 28, 42, 56, 70, 84 and 98 days post hatching (DPH) between the treatment group (G1) subjected to typical intensive rearing conditions (grey bars) and the control one (G2) housed in a semi-natural complex environment. Compared morphological traits were live weight (LW), tarsus length (TL), tarsus depth (TD), tarsus width (TW), head width (HW) and head length (HL). The group x sex effect was never significant; therefore, the effect of the group only is reported.

	LW		**TL**		**TD**		**TW**		**HW**		**HL**	
DPH	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
14	11.720	**0.002**	4.390	0.061	4.136	0.069	0.039	0.922	23.780	**0.000**	2.800	0.098
28	1.898	0.370	2.020	0.295	0.224	0.535	1.460	0.317	26.060	**0.000**	2.160	0.188
42	4.247	0.054	0.300	0.514	3.449	0.055	0.030	0.693	97.390	**0.000**	0.010	0.903
56	9.061	0.015	2.060	0.333	1.680	0.109	0.499	0.337	61.140	**0.000**	0.040	0.845
70	6.067	0.047	1.080	0.564	2.730	0.051	5.790	0.011	10.480	**0.004**	0.030	0.970
84	1.104	0.530	0.040	0.724	9.800	**0.000**	0.800	0.161	5.220	0.046	10.500	**0.000**
98	0.869	0.617	0.000	0.540	6.290	**0.004**	8.710	**0.001**	3.290	0.143	121.390	**0.000**

Live weight (LW) was affected by the intensive rearing at 14 DPH, when birds in the G1 were significantly heavier than birds in the G2 (F = 11.72, p = 0.002). After 28 DPH, birds in the G1 were constantly heavier than birds in the G2 but not significantly. TL was not sensitive to the intensive rearing; in fact, the average values of the two groups approximately constantly overlapped during the entire observation period. TD and TW showed the first significant differences between groups respectively at 84 DPH (F = 9.800, p = 0.000) and 98 DPH (F = 8.710, p = 0.001). These measures were higher in the G1 at an early stage of growth but respectively at 28 and 42 DPH, the average values registered in the G2 exceeded that registered in the G1 (Fig. 4.1). Birds in the G1 had wider head (HW) than birds in the G2 but this difference, maximum at 42 DPH (F = 97.390, p = 0.000), narrowed progressively and maintained the significance until 70 DPH but not after. HL was not sensitive to the intensive rearing until 84 DPH; in fact in the period between DPH 14 and DPH 70, the average values of two groups constantly overlapped. After DPH 70 HL stopped growing in the G1 and grew up yet in the G2. Differences in HL between two groups became significant at 84 DPH (F = 10.500, p = 0.000).

SEXUAL SIZE DIMORPHISM

In both G1 and G2, all the MTs often showed significant SSD in favour of the male class (Tables 4.2 and 4.3) except for head that was always larger in males but never significantly ($p \leq 0.01$).

Table 4.2. Descriptive statistics (mean ± SD) of the morphological traits (MTs) and comparisons (GLM *F – test* at p = 0.01) between males (n = 8) and females (n = 8) *Alectoris graeca graeca* living under typical intensive rearing conditions (G1), at different days post hatching (DPH). The Sexual Size Dimorphism Index is SSDI = (mean size of male – mean size of female) x 100 / mean size of female. LW: live weight (g), TL: tarsus length (mm), TD: tarsus depth (mm), TW: tarsus width (mm), HW: head width (mm), HL: head length (mm).

		Days post hatching (DPH)						
		14	**28**	**42**	**56**	**70**	**84**	**98**
LW	♂	57.68±6.7	141.91±8.4	237.57±21.2	355.12±20.3	430.30± 37.5	469.11±64.4	507.11±64.4
	♀	53.65±6.4	118.32±11.2	206.83±12.4	295.65±29.8	365.15± 46.8	403.61±54.1	427.42±57.1
	F	1.52	22.70	12.65	21.77	9.45	4.59	6.45
	p	0.238	**0.000**	**0.003**	**0.000**	**0.008**	0.051	0.024
	SSDI	7.52	19.94	14.86	20.12	17.84	16.23	18.65
TL	♂	24.76±1.6	33.70±0.7	39.72±1.0	43.14±1.6	43.52±1.2	42.98±1.9	43.03±1.7
	♀	23.55±1.3	30.83±1.3	35.12±1.4	38.83±1.6	39.06±1.7	38.94±1.5	38.80±1.6
	F	2.197	28.48	50.96	28.15	36.13	22.04	24.80
	p	0.160	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
	SSDI	5.16	9.30	13.10	11.11	11.42	10.38	10.90
TD	♂	2.98±0.2	3.75±0.1	4.20±0.2	4.56±0.3	4.66±0.3	4.71±0.3	4.81±0.3
	♀	2.91±0.2	3.39±0.2	3.87±0.1	4.14±0.1	4.22±0.1	4.23±0.1	4.35±0.1
	F	0.77	17.31	7.89	14.63	14.25	20.80	14.24
	p	0.394	**0.000**	0.014	**0.001**	**0.002**	**0.000**	**0.002**
	SSDI	2.58	10.35	8.53	10.09	10.52	11.38	10.77
TW	♂	3.89±0.3	5.49±0.2	6.40±0.3	6.85±0.5	7.18±0.4	7.45±0.3	7.36±0.2
	♀	3.83±0.3	5.13±0.3	5.94±0.3	6.51±0.4	6.76± 0.4	7.04±0.2	6.88±0.3
	F	0.14	6.99	9.38	1.87	5.16	8.72	12.64
	p	0.708	0.019	**0.009**	0.190	0.039	0.011	**0.003**
	SSDI	1.60	7.05	7.68	5.22	6.21	5.80	7.05
HW	♂	14.18±0.6	16.57±0.6	18.62±0.4	19.42±0.6	19.65±0.8	20.40±1.0	20.81±0.9
	♀	13.87± 0.4	15.82±0.4	17.90± 0.8	18.82± 0.7	19.08±0.8	19.60±0.7	19.96±0.5
	F	1.71	8.73	5.22	3.26	2.00	3.29	5.15
	P	0.212	0.011	0.039	0.092	0.178	0.092	0.040
	SSDI	2.23	4.72	4.05	3.21	2.97	4.08	4.25
HL	♂	24.74±0.9	29.87±0.6	32.56±0.5	34.86±0.5	36.74±1.2	36.14±0.5	36.08±0.7
	♀	24.28±1.0	28.69±0.9	31.36±0.5	33.58±0.7	34.78±1.6	34.82±1.4	34.33±0.7
	F	0.92	9.17	22.97	18.89	7.48	5.55	24.23
	p	0.354	**0.009**	**0.000**	**0.000**	0.016	0.034	**0.000**
	SSDI	1.92	4.11	3.83	3.81	5.62	3.80	5.10

Table 4.3. Descriptive statistics (mean ± SD) of the morphological traits (MTs) and comparisons (GLM *F – test* at p = 0.01) between males (n = 8) and females (n = 6) *Alectoris graeca graeca* living in a complex semi-natural environment (G2), at different days post hatching (DPH). Sexual Size Dimorphism Index (SSDI) = (mean size of male – mean size of female) x 100 / mean size of female. LW: live weight (g), TL: tarsus length (mm), TD: tarsus depth (mm), TW: tarsus width (mm), HW: head width (mm), HL: head length (mm).

		Days post hatching (DPH)						
		14	**28**	**42**	**56**	**70**	**84**	**98**
	♂	51.14±4.2	139.43±15.1	215.58±17.1	324.41±16.4	401.68±17.7	455.41±32.2	489.02±45.1
	♀	46.54±2.9	107.43±16.5	194.62±31.6	273.82±26.1	329.25±33.5	378.86±37.9	409.31±31.1
LW	*F*	5.328	13.38	2.56	19.05	27.67	16.70	13.33
	p	0.039	**0.003**	0.135	**0.000**	**0.000**	**0.001**	**0.003**
	SSDI	9.87	19.80	10.77	18.48	22.00	20.21	19.47
	♂	23.66±0.5	32.63±0.7	39.01±0.6	41.88±1.2	42.39±1.4	42.19±1.6	42.32±1.7
	♀	22.69±0.7	30.96±0.4	36.26±0.9	38.61±0.7	39.14±0.9	39.50±1.1	39.54±1.1
TL	*F*	9.08	24.12	44.67	32.71	23.21	12.12	12.65
	p	0.011	**0.000**	**0.000**	**0.000**	**0.000**	**0.004**	**0.004**
	SSDI	4.28	5.37	7.60	8.46	8.29	6.79	7.05
	♂	2.89±0.1	3.70±0.3	4.36±0.2	4.67±0.2	4.77±0.2	4.92±0.2	5.00±0.2
	♀	2.76±0.2	3.52±0.2	4.05±0.3	4.25±0.3	4.37±0.1	4.48±0.1	4.55±0.1
TD	*F*	2.36	1.35	5.54	10.03	19.66	18.36	25.85
	p	0.150	0.267	0.036	**0.008**	**0.000**	**0.001**	**0.000**
	SSDI	4.47	5.08	7.64	9.88	9.31	9.89	10.05
	♂	3.91±0.2	5.32±0.2	6.48±0.2	7.06±0.2	7.51±0.3	7.61±0.3	7.70±0.2
	♀	3.77±0.2	5.06±0.3	5.90±0.4	6.51±0.3	7.01±0.2	7.05±0.2	7.12±0.3
TW	*F*	1.61	3.79	9.89	15.53	11.18	15.14	15.67
	p	0.228	0.075	**0.008**	**0.001**	**0.005**	**0.002**	**0.001**
	SSDI	3.75	5.27	9.83	8.50	7.16	7.91	8.10
	♂	13.30±0.3	15.24±0.6	16.46±0.5	17.57±0.3	18.83±0.6	19.60±0.5	20.27±0.4
	♀	13.33±0.2	15.17±0.5	15.96±0.3	17.02±0.8	18.27±0.2	19.21±0.4	19.68±0.5
HW	*F*	0.03	0.06	4.46	2.85	4.24	2.07	6.16
	P	0.854	0.807	0.056	0.116	0.061	0.175	0.028
	SSDI	-0.21	0.49	3.18	3.18	3.09	1.99	3.00
	♂	25.13±0.7	29.07±1.2	32.51±0.5	34.83±0.6	36.34±0.7	37.69±0.7	38.86±0.7
	♀	24.97±0.8	28.44±1.10	31.31±0.79	33.51±0.80	35.03±0.9	35.71±1.2	37.68±0.1
HL	*F*	0.14	1.034	12.32	12.94	8.52	15.96	7.42
	p	0.712	0.329	**0.004**	**0.003**	0.012	**0.001**	0.018
	SSDI	0.63	2.21	3.81	3.95	3.72	5.54	3.13

At 14 DPH, no significant differences in size were detected between sexes in both G1 and G2. At 28 DPH, in the G1, the males were significantly larger than the females in LW, TL, TD and HL. At the same time, in the G2, only LW and TL differed significantly between sexes. In this group, TD showed the first significant difference between sexes at 56 DPH (4 weeks later than in the G1) and HL at 42 DPH (2 weeks later than in the G1). Tarsus became significantly wider (TW) in males than in females at 42 DPH in both the groups.

SSDI allowed identifying when sexual dimorphism in size reached its maximum (Tables 2.4 and 3.4). In both G1 and G2 SSDIs in LW showed two isolated peak at 28 DPH ($SSDI_{G1}$ = 19.94, $SSDI_{G2}$ = 19.80) after that decreased before reaching the maximum values at 56 DPH in the G1 (SSDI = 20.12) and at 70 DPH in the G2 (SSDI = 22.00). SSDI in TL reached its maximum in the G1 and G2 respectively at 42 DPH ($SSDI_{G1}$ = 13.10) and at 56 DPH ($SSDI_{G2}$ = 8.46). In TD maximum SSDI occurred respectively at 84 DPH ($SSDI_{G1}$ = 11.38) and 98 DPH ($SSDI_{G2}$ = 10.05). The third measure of the tarsus (TW) reached its maximum dimorphism in size at 42 DPH in both the groups ($SSDI_{G1}$ =7.68, $SSDI_{G2}$ = 9.83). The maximum difference between sexes in HL occurred in the G1 and G2 respectively at 70 DPH ($SSDI_{G1}$ = 5.62) and 84 DPH ($SSDI_{G2}$ = 5.54). Overall, maximum SSD occurred with not very different magnitude (SSDI) in the two groups but always at least two weeks before in the treatment group (G1) than in the control one (G2).

SEX DETERMINATION IN THE TREATMENT GROUP (G1)

The equality of the covariance matrices was confirmed by Box's M test. In the G1, sex determination was possible with adequate accuracy (percentage of individuals of each sex correctly classified > 80%) starting from 42 DPH when DA retained TL (Wilk's = 0.203, p = 0.000) and HL (Wilk's = 0.145, p = 0.000) as the strongest predictors of the sex. The discriminant model developed at 42 DPH correctly classified 100% of the males and 87.5% of the females in both the original and the bootstrap sample (Table 4.4). The better discrimination (accuracy 100% in both the sample and bootstrap) occurred at 70 DPH when DA retained only TL (Wilk's = 0.28, p = 0.000) as variable in the discriminant model (Table 4.4).

Table 4.4. Discriminant functions developed, at different days post hatching (DPH), by Discriminant Analysis (DA) at p = 0.01. Percent of birds correctly sexed of the treatment sample (G1) and of the bootstrap sample (1.000 iterations). TL: tarsus length; HL: head length; Wilk's: Wilk's Lambda and r: canonical correlation. Values of D > 0 indicate males and values of D < 0 indicate females.

DPH	Discriminant function	r	Wilk's	p	% males correct		% females correct	
					Sample (n)	Bootstrap	Sample (n)	Bootstrap
28	D = 0.931 (TL) - 30.034	0.82	0.33	0.000	100.0 (8)	100.0	75.0 (8)	75.0
42	D = 0.673 (TL) + 1.138 (HL) – 61.661	0.93	0.14	0.000	100.0 (8)	100.0	87.5 (8)	87.5
56	D = 0.477 (TL) + 1.082 (HL) – 56.583	0.88	0.22	0.000	100.0 (8)	87.5	100.0 (8)	100.0
70	D = 0.674 (TL) – 27.824	0.85	0.28	0.000	100.0 (8)	100.0	100. 0 (8)	100.0

Discussion

EFFECT OF THE INTENSIVE REARING ON THE MORPHOLOGY

The slight wounds and cuts on the tip of the beaks and of the primary feathers recorded in the G1 during the "*indoor*" phase, are probably the consequences of aggressiveness and agonistic interactions between captive chicks housed at high densities without shelters or shields. In these conditions aggressive behaviour are well known (Duncan & Hawkins 2009; Whiteside *et al.* 2016). These slight injuries affect, at least at an early stage, the representativeness of BL and WL measures but do not determine a definitive loss of ability of the birds to survive into the wild. In fact, during the "*indoor – outdoor*" phase, the density reduction and the availability of vegetative covers and shields determined an evident reduction of the aggressiveness, according to the observations of Whiteside *et al.* (2016), allowing for the healing of the beaks and a regular advancement of the post-juvenile moulting.

At the beginning of the observation period, the birds in the G1 were overall larger than the birds in the G2. However, more or less rapidly according to the morphological trait considered, the birds in the G2 recovered the disadvantages and in some cases exceeded the birds in the G1. This evidence suggests that the intensive rearing system minimizes energy costs and provides more resources to growth at an early stage, but also that the life in a complex semi-natural habitat allows for a progressive higher development of muscles and tendons and probably for larger brain that allow for the mitigation of the overall differences in LW.

In the present study, the intensive rearing determined higher LW in the G1 even if the differences between groups were significant only at an early stage (14 DPH). Our result is in contrast with that of Whiteside *et al.* (2016) who reported that 7 weeks old pheasants (*Phasianus colchicus*) grown in an enriched environment were heavier ($p < 0.01$) than pheasants subjected to standard commercial rearing conditions. The authors explained this result highlighting that 1) when natural or artificial perches are available, young pheasants are incentivized flying to perches for roosting or to escape chases and that 2) a frequent take-off allows for a marked development of pectoral and thigh muscles.

Differently, in rock partridges the tendency to use perches is unknown in both nature and captivity. Birds in the control group (G2) moved inside the semi-natural aviary by foot, walking and running. In this group, the use of the wings was limited to occasional short flights and also the Wing Assisted Incline Running (WAIR) (Dial, 2003) was rarely observed, probably because this behaviour is shown only for running up very inclined obstacles (Bundle and Dial, 2003). In the present study, there is no evidence suggesting the occurrence of stimuli for a higher development of the big pectoral muscles in the control group (G2); otherwise, this group did exercise in a complex environment enriched with rocks, ramps and uneven ground allowing for a remarkable development of the muscles of the limbs. In fact, birds in the control group (G2) showed wider and deeper tarsi than that observed in the treatment group (G1) starting from 28 and 42 DPH respectively; differences became significant at 84 and 98 DPH respectively. In this case, our results are concordant with the evidences of Whiteside *et al.* (2016).

TL was approximately constantly overlapped between groups. Likewise, Bernard-Laurent *et al.* (2003) did not appreciate significant differences in TL between hand-reared and wild *A. g. saxatilis* males. Most probably, the constant absence of differences in TL between groups is consistent with its high heritability (Lerner 1939; Kondra and Shoffner 1955).

There is evidences that individuals of many animal species, exposed to enriched and complex environment, showed larger brain and cranial volume than conspecifics reared in spatially simple environment, as the result of a significant higher development of neurones connected to spatial learning and memory as reviewed by O'Regan and Kitchner (2005). A better cognitive ability was also verified in young pheasants grown in an enriched environment than in ones reared in intensive conditions by Whiteside *et al.* (2016). Similarly Sol *et al.* (2005, 2007) proved that larger brains allow for high cognitive skills and for high survival rate in nature. Møller (2010) proved that exterior dimensions of the theoretical head ellipsoid are strongly correlated with the brain mass of a passerine bird (*Hirundo rustica).* These evidences suggest that probably the head was longer in rock partridges living in a complex semi-natural environment (G2) than in conspecifics grew in a banal environment (G1) as the result of a higher neuronal development that reflects in a larger brain and consequently in an increase of head size. For the same reason, we expected also a narrower head in the G1 than in the G2, but the contrary occurred. This evidence is difficult to explain. Bagliacca *et al.* (2008) reported that in closed heated rooms, fermentation of the bedding may result in irritant concentrations of ammonia. Coherently, we believe that a plausible hypothesis for explaining the wider head recorded in the G1 until 70 DPH, could be the occurrence of swelling of eyelids or eyebrows due to a slight irritation of the respiratory mucosa affecting the HW measure. Evidently this irritation state was not detectable by eye and it emerged only at the data processing stage that also allowed us to detect a spontaneous and progressive reshaping during the "*indoor – outdoor*" phase.

EFFECT OF THE INTENSIVE REARING ON SEXUAL SIZE DIMORPHISM

Our result reveal a significant sexual dimorphism in size in both G1 and G2 in favour of the male class and with not notable differences in magnitude (SSDI) and time of occurrence between groups. However, the maximum distance between males and females occurred first in the G1 and at least 2 weeks later in the G2, suggesting a faster growth of the males subjected to intensive rearing. Anderson *et al.* (1993) proved that in sexually dimorphic bird's species the larger sex grows faster than the smaller one and that it requires more energy during the growth. Evidently, the intensive rearing and artificially heating, low exercise and *ad libitum* availability of industrial feed in particular, amplify the differences between sexes in growth rate at least at an early stage of growth. This was objectively favourable for the purpose of early sex determination.

SEX DETERMINATION IN INTENSIVE REARING CONDITIONS

We proved that sex determination in intensive-reared Apennine rock partridges (*A. g. graeca*) is possible with high accuracy starting from 42 DPH when DA retained TL and HL as discriminant variables. However, the best predictive accuracy (100%) was reached at 70 DPH when DA retained only TL as variable in the analysis. At this time TL reached a plateau in both the control (G2) and the treatment group (G1). Our results are concordant with that of Woodard *et al.* (1986) who reported that using TL as discriminant variable the sex of chukar partridges (*Alectoris chukar*) can be determined with the best accuracy at 70 days of age when this morphological trait stopped growing.

Conclusions

In the present paper we present evidence that the intensive rearing does not determine substantial non-functional morphological changes in rock partridges at least at an early stage of growth. Consequently, we believe that from a strictly morphological point of view, this rearing system could be successfully adopted for restocking or reintroduction purposes, as long as it is guaranteed the early removal of the birds from the tight captivity in the period between 42 and 70 DPH, when sex determination is possible with high accuracy and the intensive rearing is not yet effective. Even if the better determination of the sex occurs at 70 DPH, we are confident that an earlier translocation from tight captivity to pre-release acclimatization sites, where the birds can do exercise in a complex semi-natural environment, can allow for faster compensative muscle, tendon and neural (cognitive ability) development.

In conclusion, we suggest to adopt the intensive rearing with the only aim to produce a large number of birds in limited spaces, but also to translocate them from tight captivity to pre-release acclimatization sites before 56 DPH as also suggested respectively by Cocchi *et al.* (1998) for restocking pheasants and by Buner and Aebischer (2008) for reintroducing grey partridges.

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Ethical approval: "All applicable international, national, and/or institutional guidelines for the care and use of animals were followed".

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Chapter 5

Identifying morphological exportable criteria for sex determination: a case study with *Alectoris graeca graeca*

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Abstract

Partridges (genus *Alectoris*) are sexually monomorphic in plumage and do not show obvious sexual dimorphism at least at a juvenile stage. For reintroduction or restocking purposes, an early and accurate sex determination is crucial to ensure a pre-release acclimatization in wild-similar conditions of sex-balanced groups. A reliable sex determination method should be simply detectable, early discriminant and widely exportable to different rearing conditions potentially affecting animal morphology. With the aim to identify early and exportable criteria for sex determination in *A. graeca*, we tested several morphological traits for the immunity to the rearing system's effect (robustness) and for the discriminant capacity regardless the rearing system (DRRS). With our approach, three early and exportable morphological criteria for sex determination were identified. The best exportable method for sex determination was developed at 42 days after hatching considering only tarsus length (TL). This model assessed a discriminant function with high discriminatory power (canonical correlation = 0.89; Wilk's = 0.21) and accuracy (100%) regardless the rearing system. More broadly, we present evidence that a preliminary identification of robust and DRRS morphological traits makes possible the development of early and exportable sex determination methods.

Introduction

Alectoris graeca Meisner (Galliform: Phasianidae) is included in both the Annex I and II/A of the EU birds Directive (BirdLife international 2015) because it is interesting for both conservation and hunting purposes. In Italy, the conservation status of the species is considered Vulnerable (VU) (Peronace *et al.* 2012; Rondinini *et al.* 2013). The most serious threat to its conservation at every spatial scale (EU, national, regional, local), is represented by the fragmentation of the metapopulations in small isoleted populations as the result of the discontinuities of suitable habitat (Cattadori *et al.* 2003; Amici *et al.* 2011; Sorace *et al.,* 2011; Trocchi *et al.* 2016).

Therefore *in situ* conservation strategies should focus on: 1) improving the availability of suitable habitats; 2) restoring predator-prey balance; 3) restoring Minimal Viable Population (MVP) sizes and 4) recovering distribution gaps (Bernard-Laurent & De Franceschi 1994; Primack, 2000; Sorace *et al.* 2011; Holá *et al.* 2015; Trocchi *et al.* 2016).

Comprehensive guidelines for habitat restoration are available (Sorace et al. 2011; Trocchi et al. 2016). Otherwise, actions for reinforcing wild populations with captive birds require more insights. The translocations of wild birds (Rymešová et al. 2013, Kallioniemi et al. 2015) is dangerous (Ellis et al. 1978) because the critical conservation status of the source populations. Therefore, the start-up of *ex situ* conservation programs and the production of captive rock partridges suited to reintroduction or restocking, are both crucial to preserve the genetic heritage on one hand and to restore the wild populations on the other one (Armstrong and Seddon 2008; Conway 2011). These actions are also crucial with a view to ensure a balanced trade-off between insurance, restocking and reintroduction targets (Canessa *et al.* 2014).

There are widespread scientific evidences on morphological, physiological, behavioural and neuronal deficiencies affecting many animal species in captivity, with severe consequences on their ability to survive into the wild after release (Price 2002; O'Regan & Kitchener 2005; Gaudioso *et al.,* 2011a). In a previous paper (see chapter 4) we proved that offspring of rock partridge reared under typical intensive rearing conditions, showed significant morphological changes (shorter head, and lower tarsal thickness) respect related housed in a semi-natural complex environment, starting from 70 days post hatching. Therefore, translocations from tight captivity to complex semi-natural acclimatization sites, should be performed within 70 days post hatching and optimally earlier than 56 days as also suggested by Cocchi *et al.* (1998) for pheasants (*Phasianus colchicus*) and by Buner & Aebischer (2008) for grey partridges (*Perdix perdix*).

Rock partridge is monomorphic in plumage and do not shows obvious sexual size dimorphism at a juvenile stage. In this case, early expeditious sex determination methods are crucial to ensure the translocation of sex balanced groups of young birds.

The principal aim of this study was to develop expeditious tools for sex determination in *A. graeca graeca* earlier than 56 DPH. Furthermore, assuming that manipulation and translocation of chicks at a very early stage of growth (indoor, artificially heating) and moult, could determine excessive stress and high mortality rate, we started at 28 DPH.

Because morphology can plastically adapts to contingent ecological factors, making potentially a morphological sex determination method not exportable to different frameworks, we focused on the search of morphological criteria not affected by antipodal environmental stimuli and discriminant anyhow.

Materials and methods

We based our study on the belief that working on multigenerational captive birds would make no sense if the objective was to develop sexing methods for effective reintroduction and restocking, because captivity alters animal phenotype, including morphology (Price 2002; O'Regan & Kitchener 2005).

Therefore, we accepted to work with small samples as long as representative of an uncorrupted morphotype.

At this purpose, we recorded simply and accurately measurable morphological traits on two groups of related first generation offspring of a wild breeding pair grown under antipodal rearing conditions.

We first tested the hypothesis that a sex determination method is accurate within the framework in which it was developed but not under different conditions because the morphological changes occurring under different environmental stimuli (hypothesis of non-exportability).

After, we filtered the choice of the morphological criteria for sex determination on the base of their *robustness* (immunity to the rearing system) and of their discriminant capacity regardless the rearing system (DCRRS), and improvements in sex prediction accuracy in the antipodal sample were investigated.

Lastly, we organized robust and DRRS morphological data of both the groups in a pooled dataset to consider all the possible variance contained in the two samples.

ANIMALS, EXPERIMENTAL DESIGN AND DATA RECORDING

We used a wild rock partridge (*A. graeca*) breeding pair from the Sirente Velino Mountains group (AQ – Italy) to produce two experimental groups of related offspring (see Chapter 4).

Mitochondrial DNA analysis (see chapter 4) proved that both the founders belonged to the nominal subspecies (*A. g. graeca)*, haplotype 23 (H23), according to Randi *et al.* (2003).

Chicks were assigned to two experimental groups: intensive rearing (G1) and semi-natural rearing (G2).

The birds in the G1 were reared under typical intensive rearing conditions with a first period *indoor*, until 35 days post hatching (DPH), and a second period ("*indoor – outdoor*" phase), between 36 DPH and the end of the trial, during that the available surface was little expanded *outdoor*. For details about group's composition, managing/rearing strategies, experimental program and data recording refer to the previous work, see chapter 4.

The birds in the G2 were housed in a complex semi-natural environment under the guidance of their natural parents for the whole duration of the trial. For details about group's composition, managing and rearing strategies, experimental program and data recording refer to the previous work, see chapter 4.

DATA ANALYSIS

Statistical analysis was performed with STATISTICA 7 (StatSoft Inc. 2004).

According with the main aim of this study, only discrimination between sexes occurring starting from 28 DPH and earlier than 56 DPH were investigated. Therefore, for testing the hypothesis of non-exportability, we developed discriminant functions at 28 and 42 DPH with morphological data recorded in the G1, and we exported them to the G2.

Data were processed by Discriminant Analysis. The forward stepwise method with unchanged default settings (F – enter = 1.00; F – remove = 0.00) was used.

The discriminatory power of the discriminant functions was assessed on the bases of the canonical correlation (r) and Wilk's Lambda (Wilk's).

Each Discriminant function was first tested on the sample used for the analysis (G1) fixing equal a priori probability (0.5) (internal validation) and after on the external semi-natural sample (G2) (external validation).

In the second step, we tested the *robustness* and the DCRRS of each morphological trait.

A morphological trait was *robust* when not significant differences between groups by sex were detected and not obvious differential trends of growth were observed.

A morphological trait was DRRS when significant differences between sexes were verified in all the possible combinations of the groups.

Differences between groups were investigated by sex and differences between sexes were investigated in all the possible combinations of the groups, with the aim to exclude the eventual occurrence of masked differences by variation in sexual dimorphism between the two samples.

The t-test, independent by group was used to perform comparisons. For each morphological trait, the variance equality between compared groups was verified with the F-test.

In the third step, only data on *robust* and DRRS morphological traits recorded in the G1 were processed by Discriminant Analysis (DA) for the development of new discriminant functions. Accuracy and exportability were verified by internal and external validation.

In the last step, for considering all the possible variance contained in the two antipodal samples, data recorded on *robust* and DRRS morphological traits were collected in a pooled dataset.

The 70% of the cases of each sex in each group was randomly extracted by the random number generator of Microsoft® Excel 2013 and organized in a pooled dataset to be processed by DA. The remaining 30% of the cases was preserved for the final external validation of the sexing models and it was called test sample. Accuracy of the models was estimated a) on the sample used to develop the models fixing equal a priori probability (0.5); b) by external validation on the test sample.

For all the statistics, the 1% significance level ($p = 0.01$) was accepted. Significance level at $p > 0.01$, although conventionally relevant, in this case could be misleading. In fact, the limited number of samples in the present study or other factors could generate the occurrence of transitory differences.

Results

Wing and beak length were excluded because both wing and beak were compromised by usury and slight injuries.

HYPOTHESIS OF NON - EXPORTABILITY

Discriminant functions developed at 28 and 42 DPH with data recorded on birds in the G1 are reported in Table 5.1.

Table 5.1. Discriminant functions developed at 28 and 42 days post hatching (DPH), by Discriminant Analysis (DA), with data recorded on intensive reared birds (G1). Percent (%) of correctly sexed intensive (G1) and semi-natural (G2) for males and females are reported in the last four columns. TL: tarsus length; LW: live weight; HL: head length; Wilk's: Wilk's Lambda and r: canonical correlation. Values of D > 0 indicate female cases. (n): number of individuals in each sample.

DPH	Discriminant function	r	Wilk's	p	% males correct G1 (n)	% males correct G2 (n)	% females correct G1 (n)	% females correct G2 (n)
28	D = -0.629 (TL) - 0,056(LW) - 0.018 (HL) + 28.075	0.86	0.26	0.001	100.00 (8)	75.00 (8)	100.00 (8)	100.00 (6)
42	D = - 0.808 (TL) - 0.984 (HL) + 0.012 (LW) + 59.297	0.89	0.19	0.000	100.00 (8)	100.00 (8)	100.00 (8)	83.33 (6)

The 28 days model was 100% accurate for sex determination in the G1 but not accurate enough in the G2. In the G2, this model correctly classify 100% of the females but wrongly assigns two males to the female class. Otherwise, the 42 days model was accurate in both the G1 and the G2. Overall, this model correctly classify the 96.7% (29/30) of the cases. Only one female in the G2 was wrongly assigned to the male class.

This result denied the starting hypothesis of non-exportability.

ROBUSTNESS AND DCCRS

Differences between groups by sex and between sexes in all the combinations of the groups, tested at 28 and 42 DPH with the t-test at p = 0.01, are reported in Table 5.2. Mean values and standard deviations of each morphological trait were reported in Fig. 5.1.

At 28 and 42 DPH, LW, TL, TD, TW and HL were *robust* (Table 5.2). At both 28 and 42 DPH, TL and HL approximately overlapped between groups by sex (Fig. 5.1). Otherwise, some interesting different trends were observed between groups by sex in TD and TW. These measurements of the tarsus-metatarsus recorded in the G2, at one stage of growth exceeded the average values registered in the G1 (Fig. 5.1). The overtaking in TD occurred at 28 DPH as well as in TW at 42 DPH (Fig. 5.1). HW was constantly higher in the G1 than in the G2.

At 28 DPH, only LW and TL were DRRS as well as at 42 DPH TL and HL.

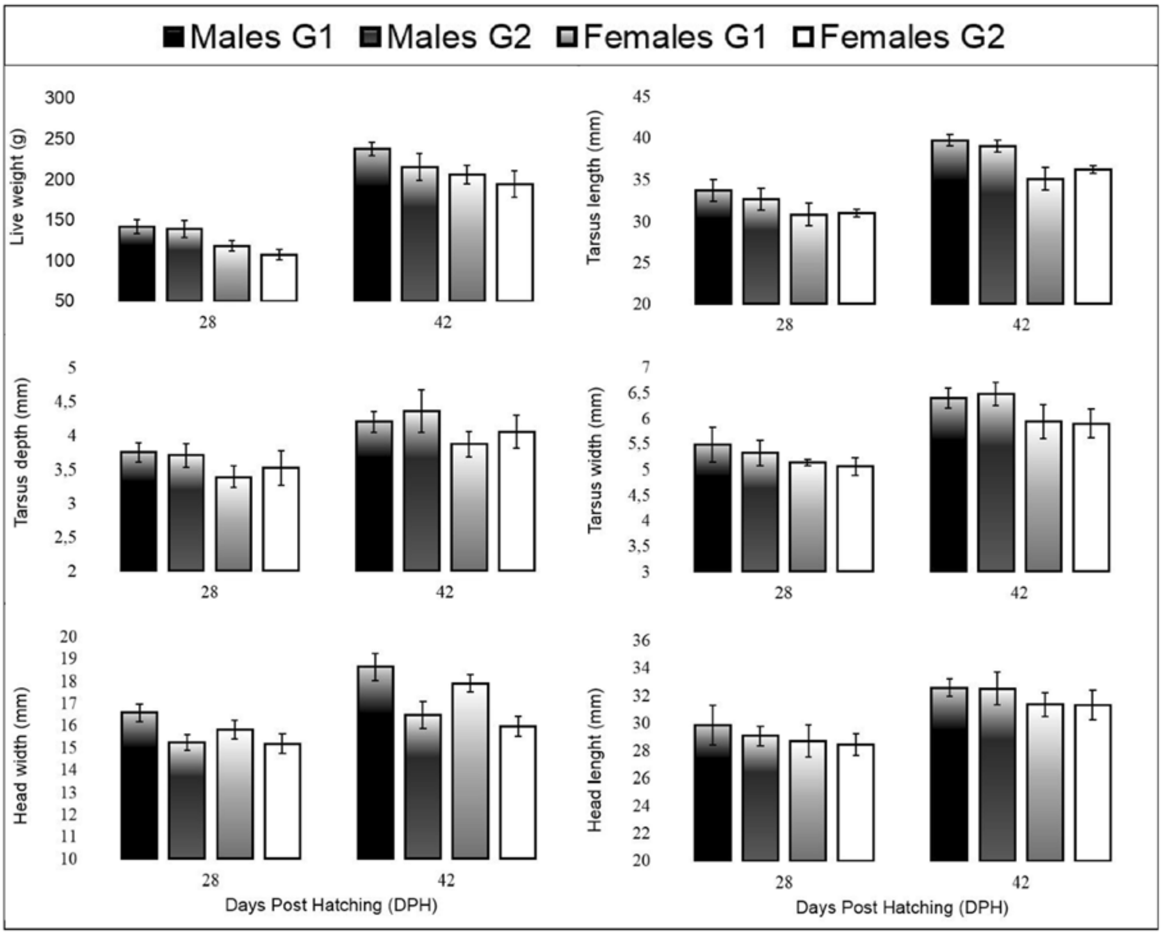
Differences between sexes in all the combinations of the groups (Table 5.2) proved that only LW, TL and HL are Discriminant between sexes Regardless the Rearing System (DRRS) in the observation period.

Table 5.2. Differences (t-test at p = 0.01) by sex between intensive reared (G1) and semi-natural birds (G2), and between sexes in all the combinations of the groups, at 28 and 42 days post hatching (DPH). Significant differences are bolded. M1: males in the G1; M2: males in the G2; F1: females in the G1; F2: females in the G2. LW: live weight; TL: tarsus length; TD: tarsus depth; TW: tarsus width; HW: head width; HL: head length. Boxed data prove the respective morphological trait immune to the effect of the rearing system (robust) and discriminant between sexes regardless the rearing system (DRRS).

		LW		TL		TD		TW		HW		HL	
DPH	Comparisons	t	p	t	p	t	p	t	p	t	p	t	p
28	M1 vs M2	0.38	0.703	2.00	0.090	0.37	0.710	1.53	0.147	4.39	0.000	1.68	0.114
	F1 vs F2	1.47	0.165	0.22	0.822	1.10	0.290	0.41	0.684	2.88	0.013	0.46	0.649
	M1 vs F1	4.76	**0.000**	5.33	**0.000**	4.16	0.000	2.64	0.019	2.95	0.010	3.02	0.009
	M1 vs F2	5.23	**0.000**	8.32	**0.000**	2.18	0.049	3.38	0.005	4.71	0.000	3.05	0.002
	M2 vs F1	3.06	**0.008**	3.31	**0.005**	2.35	0.033	1.37	0.191	2.29	0.038	0.73	0.477
	M2 vs F2	3.65	**0.003**	4.91	**0.000**	1.16	0.267	1.94	0.075	0.24	0.807	1.01	0.329
42	M1 vs M2	2.28	0.030	1.59	0.133	1.38	0.186	0.54	0.595	9.16	0.000	0.22	0.826
	F1 vs F2	1.00	0.334	1.69	0.119	1.50	0.158	0.25	0.806	5.83	0.000	0.12	0.902
	M1 vs F1	3.54	0.003	7.13	**0.000**	3.16	0.006	3.17	0.006	2.36	0.030	4.73	**0.000**
	M1 vs F2	3.05	0.009	6.32	**0.000**	1.07	0.303	2.54	0.025	13.59	0.000	3.71	**0.002**
	M2 vs F1	1.16	0.261	7.04	**0.000**	5.10	0.000	4.10	0.001	4.35	0.000	4.45	**0.000**
	M2 vs F2	1.60	0.135	6.68	**0.000**	2.35	0.036	5.89	0.008	2.11	0.056	3.51	**0.004**

Among all the morphological traits tested in the present study only LW, HL and TL were contemporary DRRS and *robust* enough for early sexing purpose: LW at 28 DPH, HL at 42 DPH and TL at both time. In the successive step only robust and DRRS morphological traits were processed by DA with the aim to assess eventual improvement in sex determination accuracy.

Fig. 5.1 Histograms show the averages values (means) of each morphological trait, detected at 28 and 42 days post hatching (DPH) on each sex of each group. Vertical bars denote standard deviations (TD).



IMPROVEMENT IN EXPORTABILITY

The internal and the external validations of the discriminant function developed at 28 DPH with only *robust* and contemporary DRRS morphological traits (TL and LW excluding HL) (Table 5.3) do not prove an obvious improvement in sex prediction accuracy (Table 5.1) in the G2. The discriminant function developed at 42 DPH with TL and HL (excluding LW)

(Table 5.3) improved sex prediction accuracy in the G2 (Table 5.1) even if the percentage of individuals correctly classified did not change overall (96.7%).

Table 5.3. Discriminant functions developed at 28 and 42 days post hatching (DPH), by Discriminant Analysis (DA), with data recorded on intensive reared birds (G1) considering only morphological traits *robust* and Discriminant Regardless the Rearing System (DRRS). Percent (%) of correctly sexed males and females of the intensive group (G1) and of the semi-natural (G2) one, are reported in the last four columns. TL: tarsus length; LW: live weight; HL: head length; Wilk's: Wilks' Lambda; r: canonical correlation. Values of D > 0 indicate female cases. (n): number of individuals in each sample.

DPH	Discriminant function	r	Wilk's	p	% males correct		% females correct	
					G1 (n)	G2 (n)	G1 (n)	G2 (n)
28	D = - 0.636 (TL) - 0.056 (LW) + 27.77	0.86	0.26	0.000	100.00 (8)	75.00 (8)	100.00 (8)	100.00 (6)
42	D = - 0.672 (TL) - 1.137 (HL) + 61.661	0.92	0.14	0.000	100.00 (8)	100.00 (8)	87.50 (8)	100.00 (6)

POOLED APPROACH

In the pooled dataset, at 28 DPH, DA retained both TL and LW as variable in the analysis and assessed a discriminant function with r = 0.79 and Wilk's = 0.36 (Table 5.4). This function correctly classified the 91.7% of the males and the 90.0 % of the females in the sample used to assess the model. All the males and the females in the test sample were correctly classified and overall, 93.3% of the individuals were assigned to the correct class of sex.

At 42 DPH, DA retained only TL as variable in the analysis, and developed a univariate discriminant function with r equal to 0.89 and Wilk's 0.21. This function correctly classified 100% of the cases in both the original and the test sample.

Table 5.4. Discriminant functions developed at 28 and 42 days post hatching (DPH), by Discriminant Analysis (DA), with data organized in the pooled dataset (70% of the cases of each sex randomly extracted from each group of birds) considering only morphological traits *robust*, and Discriminant Regardless the Rearing System (DRRS). TL: tarsus length; LW: live weight; Wilk's: Wilks' Lambda; r: canonical correlation. The probability of correct sex determination (accuracy) of each model was estimated: a) on the sample used to develop the model, b) on the test sample (30% of the cases of each sex randomly extracted from each group of birds) by external validation. Values of D > 0 indicate female cases. (n): number of individuals in each sample.

DPH	Discriminant functions	r	Wilk's	p	% males correct		% females correct	
					a	b	a	b
28	D = - 0.715 (TL) - 0.037(LW) + 27.746	0.79	0.36	0.000	91.66 (12)	100.00 (4)	90.00 (10)	100.00 (4)
42	D = - 1.063 (TL) + 40.224	0.89	0.21	0.000	100.00 (12)	100.00 (4)	100.00 (10)	100.00 (4)

Discussion

Morphological criteria and models for sexing hand-reared partridges have been proposed in the past (Weaver and Haskell 1968, Pépin 1985, Woodard *et al.*1986, Bernard-Laurent *et al.* 2003).

Bernard-Laurent *et al.* (2003) tested the exportability of discriminant models developed on hand-reared rock partridges to a wild sample without success, suggesting the occurrence of morphological changes between conspecifics living under antipodal environmental stimuli as also reported by Price (2002) and O'Regan and Kitchener (2005).

Based on the available literature, the non-exportability hypothesis of sex determination methods seemed plausible.

Contrary to expectations, our results reject this hypothesis highlighting that not all the morphological traits are affected by the rearing system, at least at an early stage.

Our data suggest that beak length and wing length are not reliable criteria for sex determination because these traits are subjected to wear in intensive rearing conditions, as previously reported by others (Bernard-Laurent at al. 2003; see Chapter 4).

In the present study LW, HL and TL were not affected by the rearing system and were considered *robust* enough for sex determination before 56 DPH.

Furthermore, LW and TL were also DRRS at 28 DPH, as well as TL and HL at 42 DPH. Accordingly, Woodard *et al.* (1986) detected in *A. chukar* chicks significant differences between sexes in TL and LW starting from 28 DPH.

In the G1, difference between sexes in HL was significant starting from 28 DPH and it was maintained thereafter according to Çağlayan *et al.* (2011) but in the G2, it occurs later corresponding to 42 DPH.

Comparisons between groups by sex and between sexes in all the possible combinations of the groups showed, at 28 and 42 DPH, results in agreement with that obtained in a previous paper (see chapter 4) in which comparisons between groups and between sexes by group were performed. This evidence allowed excluding the occurrence of masked differences due to variations between groups in sexual dimorphism.

Conclusions

With our approach, we proved LW, TL and HL exportable criteria for sex determination in *A. g. graeca* at least at an early stage (before 56 days post hatching). With these morphological traits, we developed valuable and simple methods for sexing young rock partridges with high accuracy regardless their rearing system at 42 DPH. These tools may allow for effective conservation strategies making possible an early translocation of sex balanced groups of birds from captivity to the wild.

However, it should be stressed that our results are true in the respect of the assumptions they are based on and consequently further research will be necessary to understand if the sexing models proposed in the present paper can be exported with the same accuracy to not related birds or to different *A. graeca* sub-species and populations.

More broadly, we present evidence that a preliminary identification of robust and DRRS morphological traits makes possible the development of early exportable sexing models and that the most accurate sex determination regardless the rearing system is obtained when all the variability of samples reared under antipodal conditions is considered in a pooled dataset.

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Ethical approval: "All applicable international, national, and/or institutional guidelines for the care and use of animals were followed."

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Chapter 6

General conclusions

In the present PhD thesis, we present evidences that in different suitable areas of the Apennine distribution range, the rock partridge persists with very low-density populations (Chapter 2), confirming the tangible risk of local "*extinction vortices*" due to any stochastic environmental variations. This risk was highlighted also in the national and regional (Lazio) action plans for the conservation of the species. The most plausible causes of the local critical conservation status are that reported in many other studies (Chapter 1): loss of suitable habitat, fragmentation of the metapopulation in small isolated populations, predation and direct/indirect impacts connected to different human activities, hunting included.

On the bases of the available knowledge, effective *in situ* conservation strategies should focus on: 1) improving the availability of suitable habitats; 2) restoring predator-prey balance; 3) restoring Minimal Viable Population (MVP) sizes and 4) recovering distribution gaps.

In this perspective, the instrumental start-up of *ex situ* conservation programs is crucial to preserve the genetic heritage and to produce individuals for reinforcing wild populations. In fact, to reinforce wild populations, the translocation of wild birds is dangerous because it reduces further the effective sizes of the source population. However, in captivity, behavioural, physiological and morphological changes need attentions because they make animals unable to survive in a natural environment. Coherently, in a specific survey (Chapter 4), we proved that some morphological deficiencies occur in rock partridges subjected to intensive rearing after 70 days post hatching. This evidence suggest that the intensive rearing could be successfully adopted as long as it is guaranteed the translocation of the birds from tight captivity to pre-release complex semi-natural environments within 70 days post hatching, when the effect of intensive rearing is not yet effective on the animal morphology. Optimally, the translocation should be performed before 56 days post hatching to ensure an adequate period of exercise necessary for the compensative development of muscles, tendons and brain (cognitive ability).

Rock partridges are monogamous birds. Coherently, at the appropriate time, sex balanced groups should be transferred from tight captivity to acclimatization sites. The species is monomorphic in plumage and does not show obvious sexual size dimorphism, therefore, the development of inexpensive and expeditious morphological methods for sex determination is crucial. Furthermore, partridges are reared with different techniques and under the effect of different ecological stimuli affecting animal's morphology potentially making a morphological method for sex determination accurate within the framework in which it was developed but not exportable with the same accuracy to different conditions.

In the Chapter 5, we present evidences that a preliminary identification of robust (not affected by antipodal rearing systems) and DRRS (discriminant between sexes regardless the rearing system) morphological traits, makes possible the development of early exportable sexing models. With our approach, we proved Live Weight (LW), Tarsus Length (TL) and Head Length (HL) exportable morphological criteria for sex determination in *A. g. graeca* at least at an early stage (before 56 days post hatching). With these morphological traits, we developed valuable and simple methods for sexing young rock partridges with high accuracy regardless their rearing system at 42 days post hatching.

About the threat of genetic pollution, we investigated the spontaneous colonization of the north of Lazio region by the non-native red-legged partridge (chapter 3). Our results highlighted that it does not represent an immediate risk but also that the assessment of its potential dispersion is necessary to prevent possible future contacts, considering also the interest of the local hunting association (ATC VT1) to favour the affirmation of this non-native species.

Chapter 7

Perspective

What done and what to do

In the lack of knowledge section (Chapter 1), I highlighted the necessity to increase knowledge on different incomplete *key aspects* concerning genetic, morphology, demography, ecology, *ex situ* conservation and breeding. In this thesis, I presented several results concerning only some of these incomplete *key aspects* concerning local demography, genetic pollution risk, morphology, effect of intensive rearing on the functional morphology and sex determination methods. In the near future, further interregional researches must be planned on the entire Apennine distribution range to give a complete, organic description of the Apennine metapopulation. In particular, the definition of the main population's parameters (density, structure, reproductive success, mortality, annual increase) is necessary to identify small and isolated populations and to perform population viability analysis (PVA). Furthermore, widespread on field surveys planned on the medium – long term, will allow for the mapping of the distribution gaps and for collecting non-invasive biological samples necessary for the definition of the Apennine genetic pool and the degree of genetic flow and hybridization. Many data of presence, collected in all the different phonological phases and well-distributed in the entire Apennine distribution range, are also necessary for performing ecological niche factors analysis (ENFA) and developing seasonal habitat suitability models necessary to orient monitoring and management. Further research aimed to well understand autecology, movements, dispersion and spatial use strategies, should be performed trough satellite monitoring

Work in progress

In the year 2003[1], a survey inferred the evolutionary history of 332 rock partridges sampled (non-invasive feather samples) throughout the entire species' distribution range using mitochondrial DNA control-region and microsatellite data. In this survey, 22 mtDNA haplotypes were identified and phylogenetic analyses allowed separating samples collected in Sicily from all the others. Furthermore, population assignment analyses of microsatellites suggested a subdivision between Alpine population and all the others in the Apennines, Albania and Greece.

In the year 2013, we started a non-invasive biological sampling (feathers and faeces) within the Apennine distribution range in order to increase the scarce size of the previous Apennine sample, searching for eventual unknown haplotypes. At the present, we have collected 54 biological samples and we are waiting for some others. Meanwhile, with the help of a geneticist, we sequenced four haplotypes (H3, H8, H23, and H13) one of which (H13) previously not found in the Apennine population. At the end of the sample collection, we will carry out further analysis considering both mitochondrial DNA control region, and microsatellite, to define the Apennine genetic pool and to assess genetic flow and richness, introgressive hybridization and, possible consequences on the phylo-geographical assessment.

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Future planned researches

In November 2015, the wildlife research team coordinated by Professor Andrea Amici of the Department of Agricultural and Forestry Sciences (DAFNE), I belong to, was awarded a grant for the "monitoring of rock partridge (*Alectoris graeca*) and grey partridge (*Perdix perdix*) in Sibillini mountains National Park". The project proposal, that I contributed to draw, includes the study of 1) factors influencing the choice of wintering area, 2) auto-ecology, 3) dispersion and 4) spatial use strategy, through satellite monitoring of some captured wild birds.