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Effect of climate change on female reproductive fitness in Rock sparrow *Petronia petronia* (Aves, Passeridae) breeding in the Western Alps.

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...ai miei genitori e a Laura

ABSTRACT

Il Climate change ha profondi effetti sulle popolazioni di uccelli e, in particolare, su alcune delle più importanti componenti delle *life hystory* come (i) distribuzione geografica, (ii) date utili alla migrazione e alla deposizione delle uova, (iii) dimensioni della covata e (iv) successo d'involo. Gli ecosistemi artici e alpini, inoltre, rappresentano eccellenti modelli di studio perché, proprio alle alte latitudini e altitudini, saranno più evidenti gli effetti che il cambiamento climatico ha sull'integrità di habitat e sistemi biologici. La Passera lagia *Petronia petronia* è un passeriforme (35g di peso, 14,5cm lunghezza) distribuito dall'Europa meridionale all'Asia centrale, di abitudini parzialmente coloniali, legato ad ambienti aperti e soleggiati. Il sistema riproduttivo è complesso, con individui principalmente monogamici, ma anche poligamici e poliandrici. Le coppie usano anche la *multiple breeding* (più di un evento riproduttivo all'interno della medesima stagione) come strategia riproduttiva. In Europa meridionale si riproduce per lo più in piccoli nuclei (tipicamente in cavità) e spesso in condizioni sinantropiche (abitati e piccoli centri); ciò conferisce alle sue popolazioni una geometria spaziale piuttosto isolata e irregolare. Il Progetto *Petronia*, uno studio a lungo termine sulla biologia riproduttiva e sull'ecologia della specie, è intrapreso nel 1988 sulle Alpi occidentali italiane, dove *P. petronia* è rinvenuta nidificante, tra i 1500 e i 1800 m. s. m., in piccoli villaggi, associati a prato-pascoli del piano montano, in condizioni di marcata continentalità climatica. La popolazione in studio è localizzata al limite nord del suo areale alpino. I piccoli e isolati nuclei dell'alta Val di Susa costituiscono verosimilmente una propaggine della ben più consistente e diffusa popolazione del versante francese delle Alpi. L'obiettivo del Progetto *Petronia* è quello di studiare l'influenza che il Climate

change, in questa popolazione marginale e in condizioni ambientali estreme, ha sulla fitness individuale e della popolazione agendo su alcuni importanti parametri della *life history* quali (i) fenologia riproduttiva (avanzamento della prima data di deposizione, allargamento della finestra temporale utile alla riproduzione), (ii) status riproduttivo (numero di uova e giovani prodotti, tassi di schiusa e involo, trend delle seconde covate di successo), (iii) qualità individuale (condizione di stress degli adulti, immunocompetenza dei nidiacei), nonché sulla sopravvivenza (frequenza di ricattura) di giovani e adulti e su importanti *fitness proxies* (peso, *body condition index*). La popolazione nidificante (50-70 individui/anno) utilizza box nido appositamente progettati per la specie che, all'occorrenza, sono impiegati come box di cattura. Tutti gli individui (adulti riproduttivi e giovani), inoltre, sono individualmente riconoscibili tramite una combinazione di 4 anelli (3 colorati e 1 metallico con combinazione alfanumerica) su ambedue i tarsi. Nel presente studio, considerando i 22 anni di attività (1991 – 2013), abbiamo misurato i principali parametri riproduttivi e fenologici (date di deposizione, inizio cova, schiusa, involo, oltre a numero di covate, numero di involati/individuo/anno) insieme ai *proxies* climatici principali (temperatura dell'aria, pressione, piovosità umidità) così da meglio indagare l'associazione tra parametri climatici e popolazionistici. Abbiamo inoltre considerato la strategia riproduttiva delle femmine, in modo da identificare (i) i reali benefici riproduttivi del portare avanti una seconda covata, (ii) l'influenza di stress delle femmine e immunocompetenza dei nidiacei sul numero di covate di successo, (iii) i *possibili carry over effects* che rendono la *multiple breeding* la migliore strategia riproduttiva per ciascuna femmina. Sia lo stress delle femmine che l'immunocompetenza dei nidiacei, in fine, sono stati misurati e relazionati ai principali *fitness componets* (numero di covate e numero di involati/individuo/anno) e ad importanti *fitness proxies* (peso, *body condicion index*).

Per quanto riguarda l'influenza del *Climate change* sulla life hystory di *P. petronia*, abbiamo verificato (i) un significativo anticipo delle date d'inizio della stagione riproduttiva (deposizione del primo uovo) in relazione alle temperature primaverili (principalmente quelle di giugno), (ii) un significativo incremento in frequenza delle femmine nidificanti due volte nella medesima stagione, contemporaneamente ad una significativa anticipazione delle date della seconda covata, (iii) un significativo incremento del successo riproduttivo medio della popolazione (successo d'involo più alto). Pur non essendoci alcuna associazione tra condizioni di stress degli adulti o tra immunocompetenza dei nidiacei e la scelta della *multiple breeding* da parte delle femmine o tra gli stessi parametri fisiologico-ormonali e la loro sopravvivenza, abbiamo verificato che le femmine ricavano un significativo vantaggio nell'allevare una seconda nidiata di successo, avendo un più alto *lifetime reproductive success* (misurato come totale piccoli involati) rispetto alle femmine con una sola covata. Le condizioni fisiologiche e ormonali di giovani e aduli, in fine, risultano significativamente associati ad importanti *fitness proxies* come massa corporea e *body condition*; le femmine meno stressate, quindi, avranno nidiacei più grassi e con rapporto peso/tarso più elevato. Il Progetto *Petronia*, dunque, come studio a lungo termine, può essere considerato come utile strumento per l'identificazione e la misurazione dei possibili effetti che i cambiamenti climatici hanno sulla dinamica e la vitalità delle popolazioni. In particolare, sulla popolazione di Passera lagia oggetto dello studio, ha permesso di individuare l'impatto che le variazioni climatiche hanno sulla fitness (individuale e della popolazione), agendo positivamente sia sulla finestra temporale utile alla riproduzione, sia sulla plasticità fenotipica alla base della *multiple breeding* usata dalle femmine. In fine, ha permesso di evidenziare, in linea con la *glucocorticoid–Fitness Hypothesis*, come elevati valori di stress nelle femmine, (i) caratterizzino i soggetti in cattive condizioni fisiche e (ii) influenzino negativamente quei processi di allocazione energetica alla base del *trade-off* tra investimento nella riproduzione e sopravvivenza da cui la fitness strettamente dipende.

INTRODUCTION AND BACKGROUND

Biodiversity is transformed by an unprecedented changing of climate (Lovejoy & Hannah 2006; IPCC 2007; Mihoub *et al.* 2012). Furthermore, it is increasingly evident as this change is relentlessly and negatively affecting the distribution, reproduction and phenology of wildlife (Dunn & Winkler 1999), succeeding in some cases to modulate the structure of ecological communities worldwide (Reif & Flousek 2012). In this climatic scenario, many studies have shown numerous responses of species to increasing temperature, such as changes in geographic range, migration and phenology. Animal species can react to environmental variations and thermal fluctuations by shifting their space axis and following favorable habitat conditions (Bellard *et al.* 2012). Uphill or poleward range shifts have already been observed in more than 1000 species – especially those with high dispersal capacities like birds, insects and marine invertebrates (Parmesan 2006). Often these movements are followed by drastic effects on phenotypic plasticity (i.e. the capacity of individuals to adjust their phenology to environmental variables) and local adaptation to new habitats occupied (Davis, Shaw & Etterson 2005; Thomas 2010; Martin *et al.* 2014). However, in most unfavorable cases, these dynamics can lead to a dramatic habitat loss and local extinctions (Parmesan 2006; Sekercioglu *et al.* 2008; Lehikoinen *et al.* 2014; Lenoir & Svenning 2014). Moreover, these negative effects are more evident at high altitudes and latitudes (Rahbek 1995; La Sorte & Jetz 2010; Fletcher *et al.* 2013) where notoriously they act more quickly and dangerously on the integrity of habitats and biological systems (Graae *et al.* 2012; Oliver & Morecroft 2014). In accordance with this, it is more clear why properly alpine species, which generally on this topic received relatively little attention (Chamberlain *et al.* 2012), have shifted their distribution uphill, sometimes with interesting differences in increment of the average elevation (Popy, Bordignon & Prodon 2010; Maggini *et al.* 2011). In terms of adjusting to climate change, phenotypic plasticity in birds may involve changes also in migratory behavior (Dunn & Winkler 2010). This is an important biological consequence of climate change which will be connected to many changes in migration schedules (Lehikoinen & Sparks 2010), such as the advancement in time of departure from the wintering grounds, a variation in migration speed, influenced by ratio between flight and stopover durations (Drent, Fox & Stahl 2006; Lehikoinen & Sparks 2010), as well as a range shift of wintering ground (Both & Visser 2001; Huntley *et al.* 2006). These inappropriate behavioral and physiological responses may act against life history events, disrupting food webs by causing an early or late arrival on the breeding grounds compared to the optimal food availability (Visser, Holleman & Gienapp 2006; Mihoub *et al.* 2012; Courter *et al.* 2013). A breeding time well matched with food peak, in fact, increases fitness because adult can optimize their food intake (Varpe *et al.* 2007) and consequently can feed their young during the period with the greatest abundance of food (Gienapp & Visser 2006; Marvelde *et al.* 2011; Reed, Jenouvrier & Visser 2013). In recent years has increased the number of studies that have considered the effect that climatic changes, acting on the abundance and availability of preys, have on individual fitness (Sanz 2002; Both *et al.* 2006; Burger *et al.* 2012). Furthermore, has increased the number of studies that have considered both long-term studies on wild populations to detect and understand temporal trends in life-history traits (Visser 2008; Dunn & Winkler 2010) as well as changes in abundance of bird species increasingly seen as important pioneer indicators of population and physiological responses to habitat changes (Visser 2008; Wingfield, Visser & Williams 2008). Hence, especially in seasonal environments, and precisely from long-term studies, has been highlighted as birds, changing their phenology and advancing their date of laying together with an increment of mean spring pre-laying temperature, can optimize their reproductive output and then their fitness (Dunn & Winkler 2010; Schaper *et al.* 2012; Fletcher *et al.* 2013). Strong seasonal fluctuations in food availability and temperature can deeply affect the short time window in spring suitable for reproduction in terms of energy of nutrient and energy availability (Moller 2006; Schaper *et al.* 2012). Thus, for birds can be crucial to maximize individual fitness (Verhulst & Nilsson 2008; Møller *et al.* 2010) choosing both a correct timing for reproduction as well as a correct number of breeding attempts. In accordance with this, while annual reproductive success could have a strong demographic effect on population growth rate (Bulluck *et al.* 2013), is through an optimal frequency of multiple brooding (i.e. more than one reproductive event within the same reproductive season) that females can maximize their lifetime reproductive success (Weggler 2006; Carro, Mermoz & Fernández 2014; Hoffmann, Postma & Schaub 2014) optimizing also the important life-history trade-off between reproductive investment and survival (Ardia 2005a; Sockman, Sharp & Schwabl 2006; Carro, Mermoz & Fernández 2014; Hoffmann, Postma & Schaub 2014). For bird species, in fact, reproduction has a cost and individuals which invest too much could pay with a reduction of survival or reproductive output in the successive seasons (Bell 1980; Sockman, Sharp & Schwabl 2006; O'Brien & Dawson 2013). However, a limited reproductive investment may not allows individuals to maximize their reproductive potential (Sockman, Sharp & Schwabl 2006; Dunn & Møller 2014). Early reproduction for some bird species is associated with an high reproductive output

(Verboven & Verhulst 1996), an higher survival rate and recruitment of offspring produced (Bulluck *et al.* 2013). For this reason, just early females, being more likely to double brood, could be define as "high quality individuals" than late breeders (O'Brien & Dawson 2013). In this scenario, Rock sparrow can be considered as a good model to study the effect of climatic variations on life-history stages on the Alps for three main reasons: 1) this bird is a "southern" and heliophilous species (Mingozzi & Onrubia 1997) that, in the study area, reach its ecological (altitudinal) limits of European range (Mingozzi *et al.* 1994) and consequently exposes the breeding population to the effects of Climate change (Rahbek 1995; La Sorte & Jetz 2010; Fletcher *et al.* 2013), 2) in this species both sexes exhibit complex social mating systems, from monogamy to polygamy, and brood desertion (Griggio *et al.* 2003; Griggio & Pilastro 2007) as well as a facultative multiple brooding, 3) it is the subject of which can benefit from a knowledge gained in more than 20 years of research conducted on the same population. The *Petronia* Project, in fact, since 1988 has been studying various aspects of the natural history of *Petronia petronia*, and in particular: (i) distribution and reproductive strategies (Mingozzi *et al.* 1994; Mingozzi & Onrubia 1997), (ii) social organization (Pilastro *et al.* 2001; Pilastro *et al.* 2002; Pilastro, Griggio & Matessi 2003), (iii) trophic strategies (Biddau, Mingozzi & Fedrighini 1995; Venuto *et al.* 2002), (iv) effect of climatic parameters (Venuto *et al.* 2002), (v) local survival and dispersal (Tavecchia *et al.* 2002), (vi) social interactions and mate choice (Griggio *et al.* 2007; Griggio, Zanollo & Hoi 2010), (vii) reproductive investment (Griggio & Pilastro 2007; Griggio & Venuto 2007).

For this work we started from a main research objective:

- Study how Climate change in marginal populations and extreme environmental conditions, may favour individual and population fitness acting on (i) phenology (i.e. advance of laying dates, enlargement of the time window for reproduction), (ii) reproductive status (i.e. number of eggs and nestlings produced, hatching and fledging rates, trend of second successful clutches), (iii) the expression of different individual qualities (i.e. stress condition of adults, immunological response in offspring) and proxies of fitness (i.e. body mass, body condition index) and survival (i.e. recapture rate of adults and offspring).

Therefore, we tried to verify hour research hypotheses writing three different papers:

PAPER 1: Meteorological correlates of phenology and breeding strategy changes in a mountainous habitat: the Rock sparrow *Petronia petronia* in the Western Alps**.**

Aim: measure the effect of Climate change on important life history traits such as breeding season duration, reproductive strategy and reproductive success.

Methods: The breeding population (50-70 birds/year), during 22 years of *Petronia* Project, was studied using 40-50 artificial nest boxes/year and individual tagging of adults (n = 207) and fledglings (n = 2,469). We measured reproductive and phenology population parameters (laying, brooding, and fledging dates, *n* clutches, *n* fledged juv/season per female) and principal proxies of climatic conditions (air temperature, pressure, rainfall, and humidity).

Results: We detected a significant advance (6 days) of clutch initiation associated with warmer seasons, with an almost significant extension of the laying season. We detected a significant increase in the frequency of females that nested twice in a season, contemporary to an almost significant advance of the second deposition as well. We also detected a significant trend in the fledging success (number of fledged juveniles per laid eggs) of the second depositions, but not of the first ones. We showed that reproductive success of Rock Sparrow was positively related to increasing air temperature during the nestling period (mainly June and July).

Main conclusions: *Petronia* Project, as all long-term studies on birds, can be consider as useful indicators of biological effects and population and physiological responses to habitat modifications. Moreover, for the alpine breeding Rock sparrow population, meteorological correlates have a general positive impact on multiple brooding and then on reproductive fitness, while the rapidity of change of the reproductive performance suggests that the observed responses to climatic variations are more likely explained by phenotypic plasticity.

PAPER 2: Multiple breeding in an Alpine population of Rock Sparrow. Are there carry-over effects of rearing a second clutch?

Aim: analyze the breeding strategy of females of a Rock sparrow nesting in the Western Italian Alps in order to identify: i) the actual benefits of rearing a second clutch, (ii) the influence of stress levels of females along with the immunocompetence of fledglings on the number of breeding events, iii) the potential carry over effects for reproductive females who choose multiple breeding as an optimal breeding strategy.

Methods: We used reproductive and phenology population parameters and computed several measures of reproductive success referred both to first and second clutches in order to calculate both annual reproductive fitness (ARS) and lifetime reproductive fitness (LRF). Survival rate was measured as the recapture rate of females and of their nestlings in the following three years. Carry over effects on fledglings were estimated by comparing, (i) the survival rates (i.e. number of recaptures) of fledglings of single brooding females to those of double brooding females at 1st clutch, and (ii) the survival rate of fledglings from the 2nd clutch compared to the 1st clutches of double brooding females. We measured quality of breeding females and nestlings using two non-invasive methods: (1) we measured the individual stress as amount of Corticosterone (CORT) accumulated in the growing feathers and (2) we tested immunocompetence of fledglings measured as the T-cell-mediated immune response (CMI) towards the mitogen phytohemagglutinin (PHA).

Results: we found that Rock sparrow females retrieve a significant advantage to rear a second clutch having a higher lifetime reproductive success (measured as total number of offspring produced) than single brooding females. Furthermore, even if there is no association between stress condition of females or immunocompetence of nestlings and their survival, our results support the “cort–fitness hypothesis” and the negative influence that high CORT levels have on the energy-allocation processes and consequently on estimates of fitness (i.e. body mass and body condition).

Main conclusions: double brooding by Rock sparrow could be influenced primarily by seasonal climatic variations and particularly by warmer spring temperature. In fact, temperature warming in the first part of the breeding season leads an earlier onset of breeding as well as a significant increase in the frequency of females that nested twice in a season. Furthermore, other proximate and background factors should be considered with more detail: (i) the effect that long-term CORT, acting on metabolic and energy-allocation processes, could have on breeding phenology and reproductive strategy, but also (ii) seasonal changes in resources (e.g. phenology of preys), as well as environmental quality and (iii) the individual energy storage tactic, according to which some individuals should prepare a complete clutch of eggs from body stores gained during pre-breeding season or achieve the same result rely on exogenous sources (nutrients ingested during or just before breeding season).

PAPER 3: Long-term glucocorticoid levels reflect fitness in a marginal alpine population of Rock Sparrow *Petronia petronia*.

Aim: analyze how long-term CORT levels, acting on different individual qualities, may favour relative fitness.

Methods: Using the corticosterone Enzyme-Linked Immunosorbant Assay (ELISA) kit, we measured the CORT that accumulated in the feathers of breeding females and nestlings from a population of Rock sparrow. Then, we evaluated CORT levels along with the immunocompetence of fledglings measured as the T-cell-mediated immune response (CMI) towards the mitogen phytohemagglutinin (PHA).

Results: We found a positive association between the body condition index (BCI) of females and fledglings, and between the body mass of fledglings and their CMIs. In addition, CORT levels in both naturally moulted flank feathers and primary wing covers were negatively correlated with female and fledgling BCI, but not with CMI of fledglings.

Main conclusions: The functional relationships that we found in both adults and nestlings between GCs and important proxies of fitness (body mass and BCI) allows us to identify future research directions. These include (i) integration of measures to describe the difference between levels of CORT in plasma *vs* that in feathers, and the influence of long-term CORT on (ii) breeding strategy, or (iii) on fitness indicators such as survival rate of individuals and (iv) their Lifetime Reproductive Success (LRS).

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PAPER 1

PAPER – 1 - METEOROLOGICAL CORRELATES OF PHENOLOGY AND BREEDING STRATEGY CHANGES IN A MOUNTAINOUS HABITAT: THE ROCK SPARROW PETRONIA PETRONIA IN THE WESTERN ALPS

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SUMMARY

Climate change is known to have a pervasive effects on bird populations and their geographic range, life history traits, like timing of` migration, egg-laying dates, fledging success and clutch size. Alpine and arctic ecosystems represent excellent models to study these effects as they are sensitive to climate change showing marked oscillations following climate perturbations.

A marginal population in the Italian Cottian Alps was chosen for a 22-years long term study (1991-2013) on the relation between weather condition, phenology and reproductive success. The breeding population (50-70 birds/year) was studied using artificial nest boxes (40-50/year) and individual tagging of adults. We measured reproductive and phenology population parameters (laying, brooding, and fledging dates, *n* clutches, *n* fledged juv/season per female) and principal proxies of climatic conditions (air temperature, pressure, rainfall, and humidity) to explore associations between population parameters and climate change in 22 years. We detected: i) a significant advance of clutch initiation date, associated with warmer seasons, with an almost significant extension of the laying season, ii) a significant increase in the frequency of females that nested twice in a season, contemporary to a significant advance of their first clutch as well. We also detected a significant trend in the fledging success (number of fledged juveniles per laid eggs) of the second depositions, but not of the first ones. We showed that reproductive success of Rock sparrow was positively related to increasing air temperature during the nestling period (mainly June and July). These findings illustrate the rapidity of change of the reproductive performance and suggests that the observed responses to climatic variations are more likely explained by phenotypic plasticity.

Key-words: Climate change, *Petronia petronia*, Alps, breeding biology, temperature warming, laying date, long-term study, reproductive success, phenotypic plasticity.

INTRODUCTION

The unprecedented rate of global warming (IPCC 2007) as well as the its potential effect on phenology and distribution of plant and animals has gained increasing attention in recent decade (McCarty 2001; Walther *et al.* 2002; Parmesan & Yohe 2003; Root *et al.* 2003; Parmesan 2006; Wiens & Bachelet 2010; Mihoub *et al.* 2012). Indeed, some of the most documented evidences for the effects of climate change on animal species come from long-term studies on birds (Sparks *et al.* 2002; Parmesan & Yohe 2003; Crick 2004; Møller, Berthold & Fiedler 2004; Weatherhead 2005; Visser 2008; Møller *et al.* 2010).

Climate changes may affect many life history events of bird populations, such as geographic range (Thomas & Lennon 1999; Brommer 2004; Hitch & Leberg 2007; Devictor *et al.* 2008; Brommer & Pape Moller 2010; Auer & King 2014; Lenoir & Svenning 2014; Oliver & Morecroft 2014), moult (Moller *et al.* 2011), timing of migration (Forchhammer, Post & Stenseth 2002; Sparks *et al.* 2005; Gordo 2007; Jonzén, Hedenström & Lundberg 2007; Rubolini *et al.* 2007; Dunn & Winkler 2010; Lehikonen & Sparks 2010; Rappole 2013), egg-laying dates (Crick & Sparks 1999; Root *et al.* 2003; Both *et al.* 2004; Dunn 2004; Crick & Sparks 2006; Sockman, Sharp & Schwabl 2006; Dunn & Winkler 2010; Matthysen, Adriaensen & Dhondt 2011; Dunn & Møller 2014) and even if with non-univocal responses (Dunn & Winkler 2010), clutch size and number (Sanz 2003; Visser *et al.* 2003; Both & Visser 2005; Sockman, Sharp & Schwabl 2006; Verhulst & Nilsson 2008), breeding success (Przybylo, Sheldon & Merilä 2000; Rodríguez & Bustamante 2003; Crick & Sparks 2006; Visser, Holleman & Gienapp 2006; Mihoub *et al.* 2012), recruitment (Wilson *et al.* 2007), and population settlement and dynamics (Sæther, Sutherland & Engen 2004; Sæther & Steinar 2010; Thackeray *et al.* 2010; Mihoub *et al.* 2012).

Most data on the effect of climate change on birds come from studies on phenology, such as the timing of egg laying (Crick & Sparks 2006; Møller *et al.* 2010), and most of studies are based on correlations between breeding date and temperature or other climate variables ((Dunn & Winkler 2010; Newton 2013).

Progressively earlier reproduction in the last decades was consistent with climate change (McCleery & Perrins 1998; Brown, Li & Bhagabati 1999; Crick & Sparks 1999; Sergio 2003; Both *et al.* 2004; Weatherhead 2005; Visser, Holleman & Caro 2009; Matthysen, Adriaensen & Dhondt 2011; Mihoub *et al.* 2012; Charmantier & Gienapp 2014; Dunn & Møller 2014). Around 59% of studies have shown long-term advances in laying dates (Dunn & Winkler 2010). Sometimes warmer spring are associated with earlier return from wintering grounds and onset of breeding (Jonzén, Hedenström & Lundberg 2007; Halupka, Dyrcz & Borowiec 2008; Mihoub *et al.* 2012). However, the researches revealed considerable variation in responses of breeding time to climate change within and among avian species and study sites (Visser *et al.* 2003; Dunn 2004; Visser, Holleman & Gienapp 2006; Dunn & Winkler 2010; Dunn & Møller 2014). Laying early, besides that much more frequent in multiple breeding species (Dunn & Møller 2014), it is frequently associated with larger clutch sizes and greater production of young. But the general effects of earlier laying on reproductive performance are less clear (Dunn 2004). For example, breeding output could decline if a shift in the timing of breeding disrupts synchrony between timing of food peak abundance and food demand of nestlings or brooding adults (Weatherhead 2005; Jonzén, Hedenström & Lundberg 2007; Both 2010). In some bird species, indeed, the timing of reproduction is synchronised with peaks of insect abundance and with the period of favourable conditions for raising chicks (Perrins & McCleery 1989; Burger *et al.* 2012; Reed, Jenouvrier & Visser 2013).

Local weather has been shown to affect reproduction through its effects not only on laying phenology, but also on many parts of food chain (Both, Bijlsma & Visser 2005), breeding output (Kostrzewa & Kostrzewa 1990; Li & Brown 1999; Redpath *et al.* 2002; Sanz 2002; Van Heezik *et al.* 2002; Hussell 2003; Rodríguez & Bustamante 2003; Chase *et al.* 2005; Weatherhead 2005; Halupka, Dyrcz & Borowiec 2008; Novoa *et al.* 2008), especially at the northern edge of the range and in environments with relatively cold winters (Hitch & Leberg 2007; Novoa *et al.* 2008; Lehikoinen *et al.* 2014). Indirect effects of climate on birds reproduction are probably very frequent, e.g. adverse temperature and rainfall can influence the availability of food for chicks and adults by affecting, in addition to body condition (Van Heezik *et al.* 2002),the timing and rate of plant growth in spring, and the availability of arthropods in early summer (Moss, Oswald & Baines 2001; Weatherhead 2005; White 2008). Also, weather conditions can influence prey activity and accessibility (Steenhof *et al.* 1999; Tyler & Green 2004; Parmesan 2006; Burger *et al.* 2012) , and can reduce the foraging capacity of individuals (Olsen & Olsen 1992; García-Navas & Sanz 2011).

It is worthwhile to note that most of research on the effects of climatic variation on timing and reproductive success of birds come from a few well-studied species, most of them living in boreal and temperate forests and at low altitudes (Virkkala *et al.* 2008; Dunn & Winkler 2010; Gonzalez *et al.* 2010). A very few studies on such effects have been conducted on grassland bird species in alpine habitats (Martin & Wiebe 2004; Chamberlain *et al.* 2012). The Rock Sparrow *Petronia petronia* is a loosely colonial and cavity nesting passerine, distributed from Southern Europe to Central Asia (see Fig. 2A APPENDIX), in open habitats and under dry and sunny climatic conditions (Cramp & Perrins 1994; Mingozzi & Onrubia 1997). In Southern Europe it breeds at low density in small groups near villages which gives a natural small and patchy structure to the related populations (Mingozzi & Onrubia 1997; Brichetti & Fracasso 2013).

This species is a good model to study the effect of climatic variations on population dynamics for three main reasons: (1) the altitude at which it is located, makes relevant the effects of climate change (Rahbek 1995; La Sorte & Jetz 2010; Fletcher *et al.* 2013), (2) few studies have demonstrated as temperature affects reproductive success (Burger *et al.* 2012; Mihoub *et al.* 2012) and how weather variations (e.g. temperature and rainfall), especially during spring, may act on deposition dates (Matthysen, Adriaensen & Dhondt 2011) it is the subject of study which can benefit from a knowledge gained in more than 20 years of research conducted on the same population.

In fact, a long-term study on the breeding biology and ecology of the species was undertaken in 1991 in the Western Italian Alps (see Fig. 1 and Fig. 2B APPENDIX), where the Rock Sparrow was found in small villages surrounded by pastures and montane meadows, at the northern, and altitudinally higher, limits of its European range (Mingozzi *et al.* 1994).

MATERIALS AND METHODS

STUDY POPULATION

The study population is located in the Upper Susa Valley (Italian Cottian Alps, province of Torino), between 1,550 and 1,800 m a.s.l. in three small villages (San Sicario, 1,570 m a.s.l.; Champlas Janvier, 1,784 m a.s.l.; and Thures, 1,750 m a.s.l., see Fig. 1 and 5 APPENDIX), that are not more than 4 km apart one from each other. Birds bred in artificial nest boxes (N = 40-50/year) especially designed for Rock Sparrows (11 × 12 cm and 50 cm high, see Fig. 6 APPENDIX), and when necessary, as trapping devices (Mingozzi *et al.* 1994). Within the study area, three are the main habitats frequented by Rock sparrow during the breeding season; hay cutting, pasture, meadow (see Fig. 4 APPENDIX).

All adults and fledglings have been individually marked with numbered aluminium rings and colour plastic ring combinations (Mingozzi *et al.* 1994). Similarly, chicks were individually marked at 12±1 days after hatching date. A total of 4,137 birds were ringed from 1991 to 2013. We assumed that unringed birds that were found breeding were all first-year immigrants because virtually all young are ringed at the nest each year.

COLLECTED DATA AND BREEDING PARAMETERS

All birds were measured and weighed; adults immediately after capture, nestlings at 12±1 days after hatching date. We used a Pesola (Micro-Line Spring Scale; PESOLA AG, Baar, Switzerland; cat# 20100) to measure body mass, and a metric digital calliper (Trimtec Sistemi S.r.l., Milano, Italy; cat# 35200030) to measure tarsus, bill and wing length (see Fig. 8C APPENDIX). From mid-May until the end of August, every 2-3 days, we identified the birds, pair bonds, and parental feeding activity during early morning and late afternoons when the activity is highest. During the whole breeding season, for each breeding pair we recorded the laying date, the brooding date (see Fig. 8A-B APPENDIX), and the fledging date along with brood size, number of eggs hatched and number of young fledged. The observations lasted until all the young fledged. In the overall period (1991 to 2013), we recorded 586 breeding events (N = 453 first clutches, N = 114 second clutches, N = 19 third clutches) and the breeding population ranged between 33 and 77 individuals (from 12 to 30 breeding pairs).

Using raw data, we computed several measures of reproductive success referred both to first and second clutches, and to the whole season. In particular, for each nest we calculated the clutch size, the hatching success (number of hatched eggs over laid eggs), the fledging success (number of fledged juveniles over the laid eggs), weight and tarsus length of fledglings at 12±1 days, and, for each female, an overall seasonal success (number of fledged juveniles per female per season). Carry over effects of the double breeding strategy were estimated by calculating the survival rates of Double Breeding Females (hereafter DBFs) and of their fledglings, comparing them to Single Breeding Females (hereafter SBFs).

We also computed, on individual basis, the duration of laying, of brooding, and of fledging, along with the overall breeding season duration. We used the 10th percentile of the start of laying of each nest to estimate the seasonal start of laying, and then the mean laying dates to detect the association between climate parameters and reproductive phenology (Sanz 2003; Halupka, Dyrcz & Borowiec 2008; Visser, Holleman & Caro 2009). Moreover, to investigate the reproductive consequences of a change in laying date, we analysed annual trend in clutch size (Sergio 2003; Moller 2006).

WEATHER DATA

Climate data series (1991-2013; on daily basis) were obtained from ARPA Piemonte (www.regione.piemonte.it/ambiente/aria/rilev/ariaday/annali/meteorologici) and referred to a regional meteorological station located in Oulx-Gad (1,135 m a.s.l.), 9 km north of the study area. We calculated monthly and 15-days composites (minimum, mean and maximum) from daily values of air temperature (in degrees Celsius), precipitation (in millimetres) and humidity before the breeding season, during the laying period (April and May), and during the breeding season (from June to August).

These climatic parameters have been chosen as they are known to affect the reproduction of population (Venuto *et al.* 2002; Venuto *et al.* 2005). After a preliminary Pearson correlation analysis that highlighted a strong association between temperature and humidity we focused on the variations of air temperature and on the rain intensity the indicators of weather conditions.

DATA ANALYSIS

The temporal trends were tested using both linear and non-linear regression analyses, while linear associations were first estimated by Pearson moment correlation coefficient and then modelled using a linear/non-linear regression models as well (Sokal & Rohlf 1994; Skinner *et al.* 1998; Li & Brown 1999). Regression fit was estimated using an ADJUSTED R^2 (R^2_{Adj}):

$$R^2_{Adj} = R^2 - \frac{(1-R^2)p}{C-p*}$$

Where C is the number of cases, P THE number of independent variables, P* THE Number of coefficients in the model. p* = p if the intercept is not included, otherwise p* = p + 1. Comparison between reproductive success parameters of SBFs and DBFs was conducted using an ANOVA (Sokal & Rohlf 1994). Due to relatively small sample size, the difference in survival rates were tested using a Wilcoxon test for paired samples, using a complete randomization method (permutation or exact test; PExact) or by a Monte Carlo simulations (Good 2000) based on 100,000 sampled tables (PMC) when computation by the permutation method was not possible (Mehta & Patel 2011). The permutation approach increases statistical power to the level of the corresponding parametric statistics computed when all the assumptions are met.

Statistical analyses have been carried out using SPSS 21.0 (Statistical Package for Social Sciences, © Copyright 1989, 2013 SPSS, Inc., an IBM Company) and Statistica 8.0 (©Statsoft Inc., USA) was used for non-linear regression analysis.

RESULTS

During the study period (1991 - 2013), a clear trend in the weather conditions has been detected particular related to air temperature, while no similar trend was detected for rain intensity. The monthly air temperature of April ($R^2_{adj} = 0.148$, $F_{1,15} = 4.811$, $P = 0.020$; $\text{Beta}_{YEAR} = 0.432$), May ($R^2_{adj} = -0.010$, $F_{1,15} = 0.774$, $P = 0.194$; $\text{Beta}_{YEAR} = 0.189$) and June ($R^2_{adj} = 0.166$, $F_{1,15} = 5.370$, $P = 0.015$; $\text{Beta}_{YEAR} = 0.451$) increased significantly, whilst no increase was detected in July ($R^2_{adj} = -0.023$, $F_{1,15} = 0.523$, $P = 0.239$; $\text{Beta}_{YEAR} = 0.160$).

When focusing the attention on phenology and on the breeding parameters, we observed a significant advance of the laying date ($R^2_{adj} = 0.142$, $F_{1,15} = 4.312$, $P = 0.026$; $\text{Beta}_{YEAR} = -0.345$) whose shift was of about 6 days (10th perc 1991 = June 17th, 10th perc 2013 = June 11th) with a range of 18 days (min: May 29th 2000, max: June 17th 1991; Fig. 2A). This trend determined an almost significant widening of the laying period ($R^2_{adj} = 0.173$, $F_{1,15} = 7.179$, $P = 0.055$; $\text{Beta}_{YEAR} = 0.474$) that ranged from 12 (1991) to 32 days (2013) during the study period with a minimum of 10 days in 1994 and a maximum of 51 days in 2005. As a possible consequence of the widening of the laying period, we detected a significant increase in the frequency of DBFs ($R^2_{adj} = 0.410$, $F_{1,15} = 14.926$, $P < 0.001$; $\text{Beta}_{YEAR} = 0.663$; Fig. 3A) as well as a significant advance of their first clutch ($R^2_{adj} = 0.151$, $F_{1,15} = 68.735$, $P < 0.001$; Fig. 6). We did not find any significant advance of the second deposition ($R^2_{adj} = -0.052$, $F_{1,15} = 0.004$, $P = 0.476$; $\text{Beta}_{YEAR} = 0.014$). We also detected a significant trend in the fledging success (number of fledged juveniles per laid eggs) of the second depositions ($R^2_{adj} = 0.238$, $F_{1,58} = 7.259$, $P = 0.007$; Beta$_{YEAR} =$ 0.526), but not of the first ones ($R^2_{adj} = 0.035$, $F_{1,283} =$ 1.729, $P =$ 0.102; Beta$_{YEAR} =$ 0.289).

Furthermore, the overall nesting success (% of reproductive females with > 1 fledged juvenile per season) increased significantly during the 17-years period season ($R^2_{adj} =$ 0.395, $F_{1,15}$ =14.074, P < P < 0.001; Beta$_{YEAR}$ = 0.652; Fig. 3B) along with a significant, increasing trend of the mean fledged juveniles per female per season ($R^2_{adj} =$ 0.184, $F_{1,283}$ = 5.503, $P = 0.015$; Beta$_{YEAR}$ = 0.474). This increment is likely to be due to the effect of second depositions (Tab. 1).

On average, DBFs had a greater reproductive success than SBFs (respectively 9.00±0.35 *vs*. 4.90±0.15 fledged juveniles per season; $F_1 = 129.889$, $P < 0.001$), and this difference did not refer to differences in the success of the first depositions as the first clutch laid by DBFs did not differ from those laid by SBFs for both fledging success (respectively 0.844±0.14 *vs*. 0.795±0.033 fledged juveniles per laid eggs; $F_1 = 1.828$, $P = 0.184$), and total fledged juveniles (respectively 4.77±0.17 *vs*. 4.58±0.161 fledged juveniles per clutch; $F_1 = 0.655$, $P = 0.421$).

The slight increase in the occurrence of the DBFs during the study period (Fig. 3A), along with their better reproductive performances, may have determined the tendency to the increase of the seasonal individual reproductive success (mean fledged juveniles per female per season).

When looking for association between the weather conditions and the phenological and reproductive parameters, we observed a strong association of the 10th percentile of the deposition date and the minimum temperature in the first half of June ($R^2_{adj} =$ 0.240, $F_{1,15}$

= 6.365, $P = 0.011$; Beta$_{MinTJune1}$= -0.533; Fig. 4A), whilst the maximum temperature in the second half of June seemed to influence the mean number of fledged juveniles per female per season ($R^2_{adj} = 0.251$, $F_{1,15} = 8.031$, $P = 0.005$; Beta$_{MaxTJune2}$= 0.535; Fig. 4B). Interestingly, despite the absence of a clear trend in the fledging success (FS) of the first depositions, the maximum air temperature in the second half of June was non linearly associated (polynomial regression model: $FS = -3.723 + 0.380*MaxTJune2 - 0.008*[MaxTJune2]^2$; $R^2_{adj} = 0.641$, $F_{1,15} = 19.765$, $P = <0.001$; Fig. 5A) with fledging success of the first deposition, suggesting that an excessive increase of the maximum temperature in the second half of June may exert a negative effect on the fledging success. Conversely, the fledging success of the second depositions seemed to be linearly and strongly influenced by the increment of the mean July air temperature ($R^2_{adj} = 0.193$, $F_{1,15} = 5.545$, $P = 0.015$; Beta$_{MeanTJuly}$= 0.485; Fig. 5B).

We did not detect any significant difference in weight between fledglings (at 12 days) of SBFs and those of DBFs at 1st clutch (28.88 gr vs. 28.84 gr, respectively; $t = 0.173$, df = 1154, $p = 0.862$), nor in tarsus length (18.40 mm vs. 18.70 mm, respectively; $t = -4.938$, df = 1156, $p < 0.001$).

As for the potential carry-over effects of laying 2 clutches per season, we did not detect any significant difference in recapture rate of fledglings from SBFs and those from the first clutch of DBFs (15.6% vs. 14.6%, respectively; $P_{Exact} = 0.370$), whilst we detected a significant decrement of survival (i.e., reduced recapture rate) of second clutch fledglings compared to the first clutches of DBFs (3.5% vs. 14.6% respectively; $P_{Exact} < 0.001$).

DISCUSSION

REPRODUCTIVE SUCCESS

Our data showed that reproductive success of Rock Sparrow increased with increasing air temperature during the period of hatchlings rearing (mainly June and July). Mean air temperature in June was the best predictor of reproductive performance. Rainfall appeared to have no detectable effect on reproduction. Temperature certainly acts through complex mechanisms, but we suggest that it may influence annual reproductive success in two general ways: influencing nest survival and the number of young fledged (Kostrzewa & Kostrzewa 1991; Chase *et al.* 2005; Wilson & Martin 2010; Marvelde *et al.* 2011; Marvelde *et al.* 2012). Nestlings are most vulnerable during their first days of life because are unable to thermo-regulate efficiently and their survival depends a lot on the temperature and brooding behaviour of their parents (Beintema & Visser 1989; Redpath *et al.* 2002; McKinnon, Nol & Juillet 2013). Breeding in cold spring cold temperature, to minimize the problem associated with heat loss, some species modified their behaviour during the nestling stage and female brooding time increased (Redpath *et al.* 2002; Wilson & Martin 2010). Then, cold can affect the survival of chicks because increases the thermoregulation costs and restricts also the time of the parents available for foraging (Tyler & Green 2004; Marvelde *et al.* 2012). On the contrary, fair weather reduces the energy needs of the adults, especially for thermoregulation during the night, thus making more energy and nutrients available for feeding young (Wingfield 1984). It has been found that chicks mortality is negatively related to temperature (Redpath *et al.* 2002).

At the same time, the air temperatures during the rearing and hatching periods are most important for chicks survival, because they can affect the trophic sources (Dunn 2004; Mihoub *et al.* 2012) that for Rock Sparrow during breeding period were constituted by grasshoppers, the 76.5% of the prey carried to the nest (Biddau, Mingozzi & Fedrighini 1995). In warm springs, high temperatures can determine a greater seasonal abundance of prey (Mihoub *et al.* 2012). Moreover, the emergence of insects and other invertebrates is generally allowed and advanced in warmer than in colder springs (Visser, Holleman & Gienapp 2006), as temperature speeds up insect cycles (Wingfield 1984; Barrios & Rodriguez 2004; Visser & Both 2004), with a clear advantage for daily feeding activity of adults Thus, insectivorous species that feed on invertebrates and their larvae, during spring and early summer are particularly susceptible to changes in weather (Wingfield 1984; Dunn 2004; Visser, Holleman & Gienapp 2006). No association between reproductive success and rainfall represents a data that differs from the results emerged by analogous studies. Rainfall during the nestling period has a negative influence on nest success rate (Bradley, Court & Duncan 1997; Rodríguez & Bustamante 2003; Tyler & Green 2004; Novoa *et al.* 2008). It is likely that rainy days at the time of hatching cause temporal declines in prey availability (Noordwijk, McCleery & Perrins 1995) or foraging activity (Cowie & Hinsley 1987), causing mortality of complete clutches of recently hatched chicks (Peach *et al.* 2008).

LAYING PHENOLOGY

During the 22 years of the study, we detected an advance in laying dates. This advance was associated with the air temperatures of the half of June. Our results conform to other studies which have linked similar long-term advances in laying dates to climate change. Many species of birds show a significant negative relationship between the date of laying and air temperature. They start to lay eggs earlier in years with warmer temperature (McCleery & Perrins 1998; Brown, Li & Bhagabati 1999; Crick & Sparks 1999; Dunn & Winkler 1999; Sanz 2002; Hussell 2003; Sanz 2003; Sergio 2003; Visser *et al.* 2003; Gienapp & Visser 2006; Halupka, Dyrcz & Borowiec 2008; Visser, Holleman & Caro 2009; Møller *et al.* 2010; Matthysen, Adriaensen & Dhondt 2011; Dunn & Møller 2014). Although in recent years there have been many studies that have analysed this phenomenon (Wesolowski & Cholewa 2009; Dunn & Winkler 2010; Matthysen, Adriaensen & Dhondt 2011; Dunn & Møller 2014), the proximate and background causes that lead this process are at present not completely clear (Sergio 2003).

But to predict the effect of climate change on timing of reproduction we need to know how temperature affects this reproductive parameter. In general terms, many proximate factors determine laying date, including precipitation (Leitner, Van't Hof & Gahr 2003; Rodríguez & Bustamante 2003), photoperiod (Wingfield *et al.* 2003), local climate, food availability and abundance, nesting site (Dunn & Winkler 2010), endocrine stress (Goutte *et al.* 2010; Berglund *et al.* 2014), body condition of the individual (Waye & Mason 2008a), behaviour of the mate (Wingfield 1984; Lambrechts *et al.* 1997). The relative importance of these factors in the determination of variation in a bird’s onset of egg laying has not been experimentally established (Lambrechts *et al.* 1997).

Besides to influence the gonadal development , which could indirectly affect the timing of laying (Williams 2012), several ways can be hypothesized in which the correlation between laying date and temperature may occur. Above all, there may be a direct effect of temperature on the energetic demands of female and this can influence their timing of laying. Two studies (Meijer *et al.* 1999; Marvelde *et al.* 2012), respectively on Great tits (*Parus major*) and European Starlings (*Sturnus vulgaris*), for example, showed how temperature, acting on Daily energy expenditure, has a direct effect on the timing of laying independent of food supply and photoperiod. Also, temperature can influence food availability, especially invertebrates (Tyler & Green 2004; McKinnon, Nol & Juillet 2013), which can reduce the capability of female to produce eggs (Reynolds, Schoech & Bowman 2003; Wilson & Martin 2010). These mechanisms are likely to act together to influence timing of laying and are not exclusive (Dunn 2004). In accord with McCleery and Perrins (1998) we think that Rock Sparrows can lay earlier because likely food supply becomes available earlier. Perrins and McCleery (1989) and Burger *et al.* (2012) showed that tits or pied flycatchers vary their breeding season in ways related to their food supplies, breeding earlier when the caterpillars are early. In Rock Sparrows, mean air temperatures of June likely could predict seasonal trend of grasshopper’s availability, and could synchronize eggs laying, and following hatching, to greater availability of prey. On the long period, the trend for earlier laying is determined by the increase of the spring temperature. It can be hypothesized that birds dependent for their feeding on ectothermic organisms, such as grasshoppers and insect in general, which could be more affected by climate change than endotherm organisms, can show more rapid responses to climate change than birds dependent on endotherm prey. As suggested by other authors (Przybylo, Sheldon & Merilä 2000; Charmantier & Gienapp 2014), the phenological shift induce to wonder whether any observed change results from evolutionary processes, or from phenotypic plasticity. The rapidity of change of the reproductive performance suggests that the observed responses to climatic variations can be explained probably as a result of phenotypic plasticity (Przybylo, Sheldon & Merilä 2000; Sergio 2003; Lewis *et al.* 2012).

It's known that laying date is an important factor in determining the productivity and nesting success of most passerine bird species (Sanz 2003; Gienapp & Visser 2006; Reed, Jenouvrier & Visser 2013). In our study area, the progressive advance of laying date produces some reproductive consequences in the Rock Sparrow population: the number of second clutches is more frequent across years, and this cause an overall increase in the individual and population reproductive success. Timing of reproduction, in fact, represent an important component of life history variation with deep effect on individual trade-off between reproductive investment and survival (Ardia 2005a). Thus, should be important for Rock sparrow, during pre-breeding season, optimize nutritional quality of diet (Reynolds, Schoech & Bowman 2003) as well as those energy-allocation processes directly involved to help efficiently maintain a physiological response to environmental and social challenges (Sapolsky, Romero & Munck 2000), such that maximize individual fitness (Varpe *et al.* 2009).

CONCLUSIONS

Ongoing unprecedented climatic changes are altering and modifying, as pervasive forces, environment, biological systems and, consequently, temporal patterns of populations (Hurrell & Trenberth 2010). Thus, *Petronia* Project, as all long-term studies on birds, can be consider as useful indicators of biological effects and population and physiological responses to habitat modifications (Dunn 2004; Wingfield, Visser & Williams 2008; Charmantier & Gienapp 2014). In this climate scenario and in contrast with studies showing an uncontrollable decline on distribution pattern and breeding phenology (Both *et al.* 2006; Burger *et al.* 2012), especially in mountain habitats (Martin & Wiebe 2004; Sekercioglu *et al.* 2008; Lehikoinen *et al.* 2014), for the alpine breeding Rock sparrow population, we found a general positive impact of meteorological correlates on multiple brooding and then on reproductive fitness. This phenotypically plastic trait, in fact, is part of life history theory according to which, every year, individuals have to cope with the energy expenditure characterizing the compromise between current and future reproduction (Husby, Kruuk & Visser 2009b). This energetic trade-off is optimized by Rock sparrow in Upper Susa Valley being at the northern limits of its European range, so this position can also be an important parameter to understand effects of climatic variations on life history stages, crucial for more comparisons between populations. In this regard, is still much to be done to obtain a full understanding about the impact that temperature warming could have on individual fitness, acting on abundance and availability of prey. Furthermore, although there is a large number of studies on climate effect on bird phenology (Charmantier & Gienapp 2014), this *Petronia* population provides a substantial support to the small fraction of studies based on individual data, which could quantify the relationship between evolutionary and plastic responses in models of population persistence.

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TABLE LEGENDS

Table 1: comparisons of reproductive success parameters between 1 clutch per season versus 2 clutches season females in Rock sparrow *Petronia petronia* during a 22-years study (1991-2013) conducted in the Susa Valley (Piedmont Region, Western Alps, Italy).

FIGURE LEGENDS

Figure 1 See APPENDIX

Figure 2: Trends of start (A) and duration (B) of the egg laying period in a Rock sparrow *Petronia petronia* population studied in the Susa Valley (Piedmont Region, Western Alps, Italy) during a 22-years study (1991-2013).

Figure 3: Trends in the percentage of second depositions (A) and of successful clutches (B, N fledged juveniles > 1) in a Rock sparrow *Petronia petronia* population studied in the Susa Valley (Piedmont Region, Western Alps, Italy) during a 22-years study (1991-2013).

Figure 4: Relation between (A) the minimum air temperature (horizontal axis, in °C) in the first half of June (1st - 15th June) and the beginning of the depositions (vertical axis: 10th percentile of first laying date in Julian date), and between (B) the maximum air temperature (horizontal axis, in °C) in the second half of June (16th - 30th June) and the seasonal reproductive success per year (mean number of fledged juveniles per female per season) in a Rock sparrow *Petronia petronia* population studied in the Susa Valley (Piedmont Region, Western Alps, Italy) during a 22-years study (1991-2013).

Figure 5: Relation between (A) the fledging success (number of fledglings over laid eggs) in first clutches and the maximum air temperature (horizontal axis, in °C) in the second half of June (15th – 30th June), and between (B) the fledging success of second clutches and the mean air temperature (horizontal axis, in °C) in July, in a Rock sparrow *Petronia petronia* population studied in the Susa Valley (Piedmont Region, Western Alps, Italy) during a 22-years study (1991-2013).

Figure 6: Temporal distribution of laying dates of SBFs (white boxes) and the first clutch of DBFs (grey boxes) in a Rock sparrow *Petronia petronia* population studied in the Susa Valley (Piedmont Region, Western Alps, Italy) during a 22-years study (1991-2013). Boxes represent inter-quartile distance; thick line, median; whiskers, extreme values; grey circles, outliers.

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Rep. success per female	Clutch/female	N	Mean	SD	Min.	Max.	df	F	P
1ST CLUTCH PATTERN									
N Eggs	1	273	5.81	0.998	3	8	1	3.854	0.5
	2	92	6.05	1.02	4	9			
N Chicks	1	273	5.08	1.286	1	8	1	0.017	0.897
	2	92	5.1	1.471	1	9			
N Fledged Juveniles	1	273	4.58	1.348	1	8	1	0.655	0.421
	2	92	4.77	1.65	1	9			
Brood success (Hatched/laid eggs)	1	273	0.87	0.163	0	1	1	2.48	0.116
	2	92	0.84	0.198	0	1			
Breeding success (fledged/Hatched juveniles)	1	273	0.91	0.242	0	1	1	1.943	0.246
	2	92	0.9	0.214	0	1			
Reproduction success (fledged juveniles/laid eggs)	1	273	0.75	0.316	0	1	1	1.828	0.184
	2	92	0	0.28	0	1			
SEASONAL PATTERN									
N Eggs per season	1	273	5.83	0.534	5	7	1	342.471	0
	2	92	11.19	1.23	9	13			
N Chicks per season	1	273	5.06	0.584	4	6	1	202.689	0
	2	92	9.45	1.304	7	12			
N Fledged Juveniles per season	1	273	4.9	0.712	3	6	1	129.889	0
	2	92	9.05	1.53	6	12			
Brood success (Hatched/laid eggs per season)	1	273	0.86	0.042	0	1	1	0.701	0.408
	2	92	0.84	0.099	0	1			
Breeding success (fledged/Hatched juveniles	1	273	0.96	0.046	0	1	1	0.146	0.704
	2	92	0.95	0.08	0.14	1			
Reproduction success (fledged juveniles/laid eggs	1	273	0.83	0.069	0	1	1	1.228	0.275
	2	92	0.8	0.097	0	1			

Table 1

LIST OF FIGURES

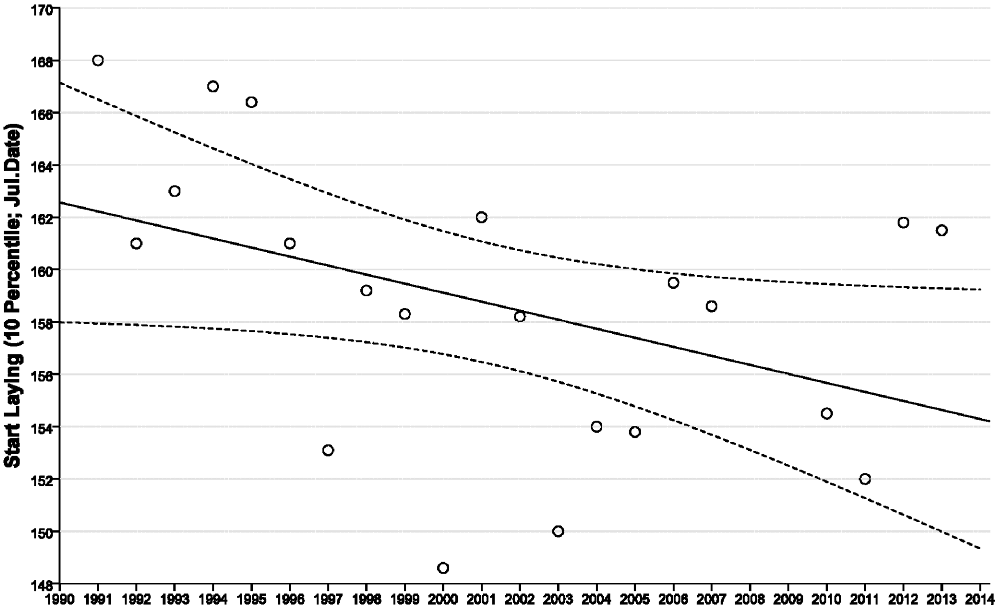
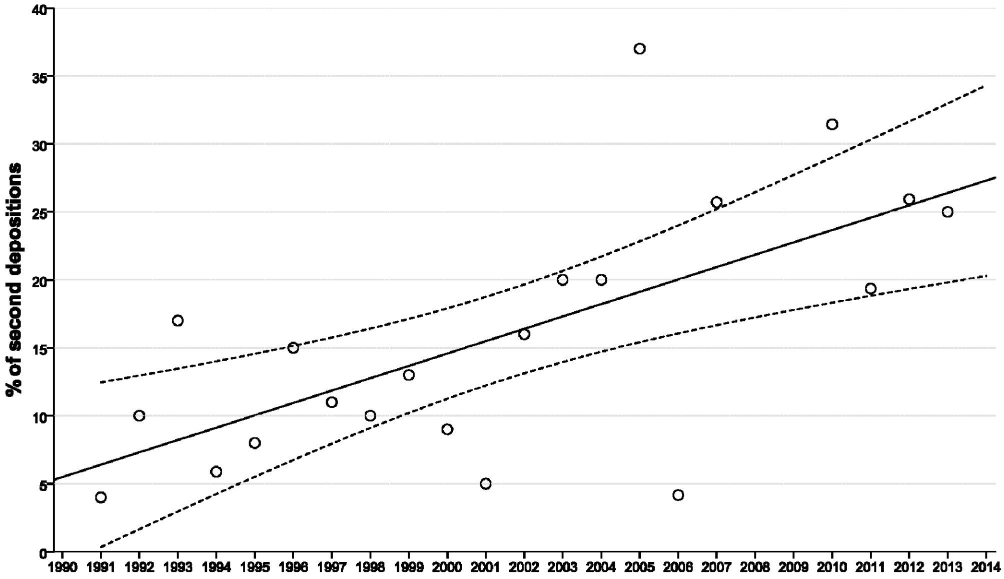


Figure 2A



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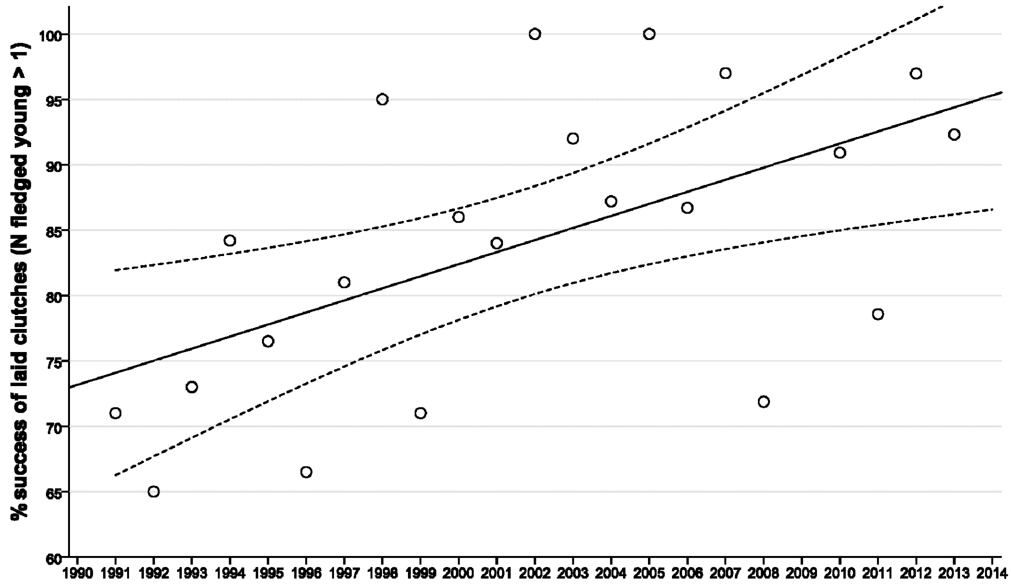

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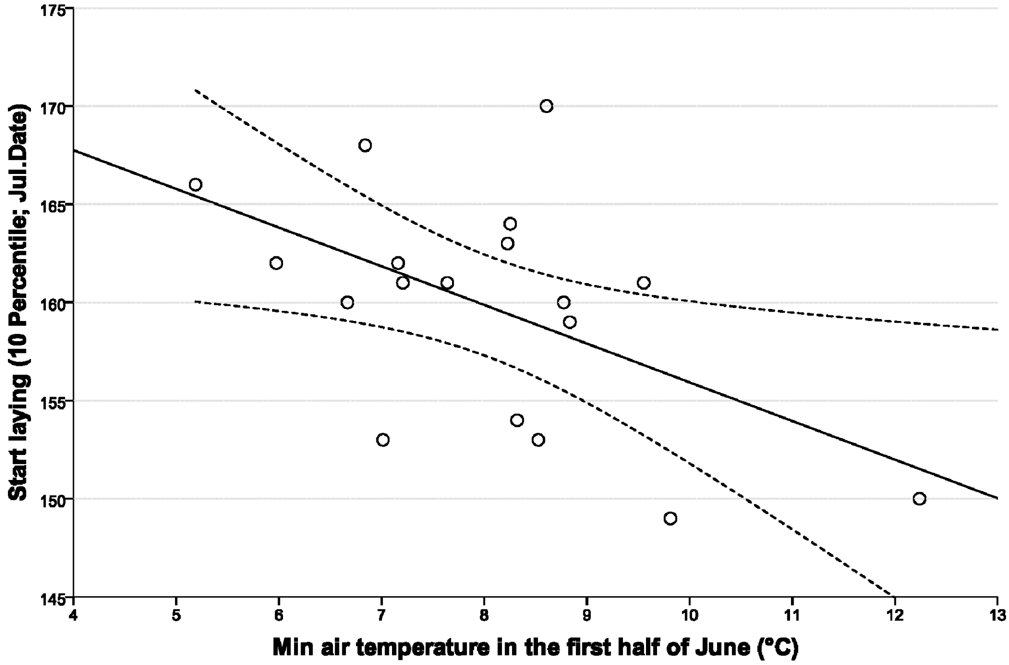

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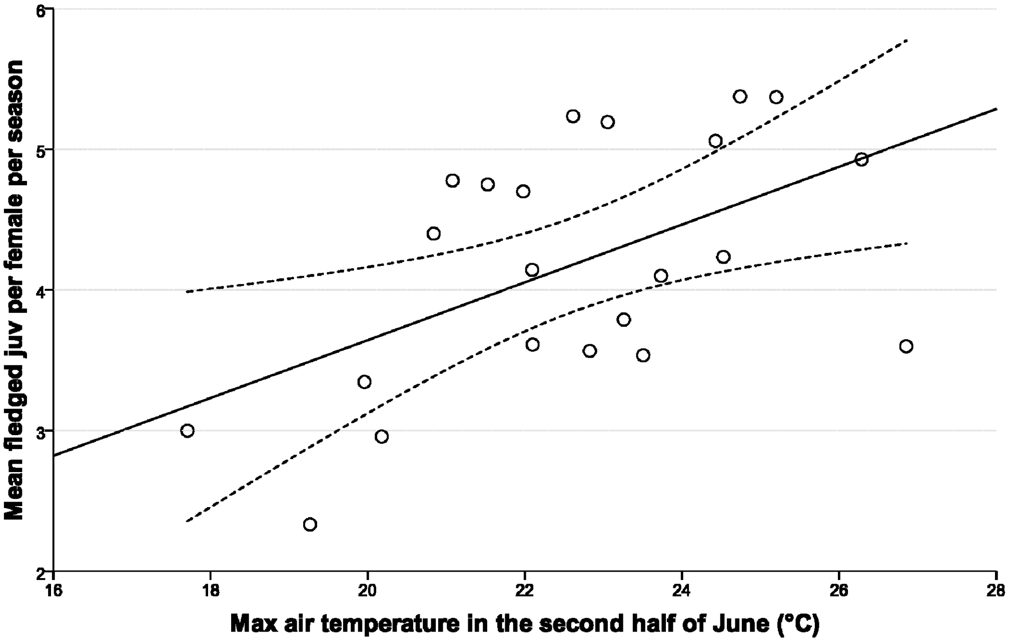

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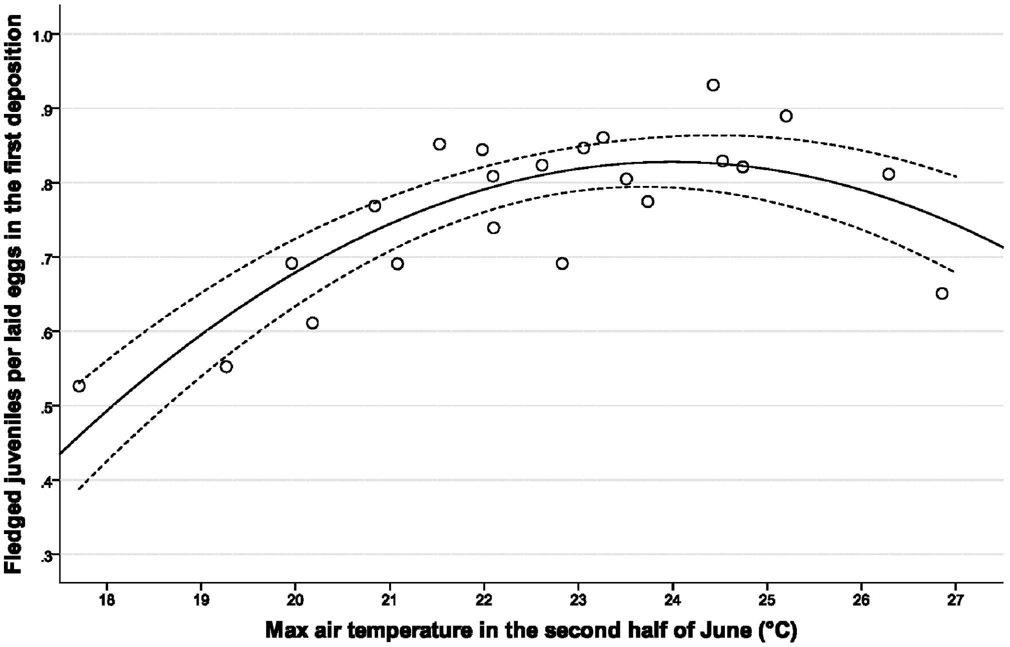

Figure 5A

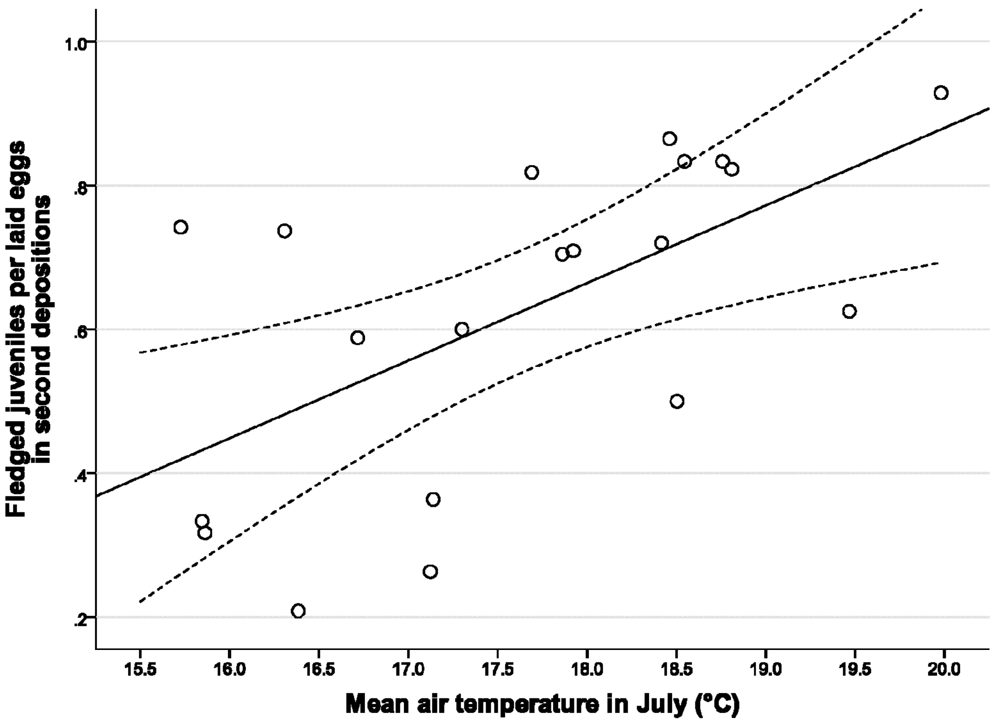

Figure 5B

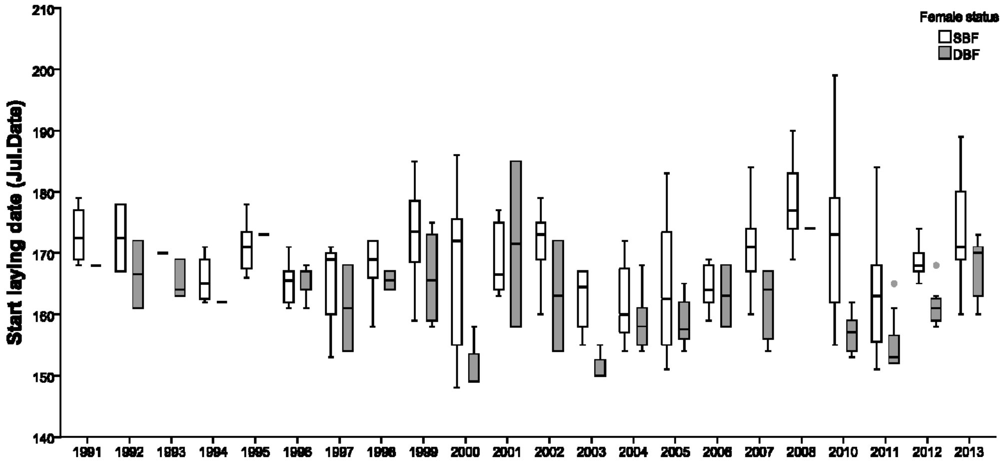

Figure 6

PAPER 2

<u>PEPER – 2 -</u> MULTIPLE BREEDING IN AN ALPINE POPULATION OF ROCK SPARROW. ARE THERE CARRY-OVER EFFECTS OF REARING A SECOND CLUTCH?

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SUMMARY

1. The number of successful breeding attempts within a season is for birds a good strategy to maximize seasonal fecundity, their lifetime reproductive success and then their fitness.

2. Fitness of multiple-brooding bird species, according to life-history theory, is influenced by the trade-off between costs and benefits of reproduction. Thus, each further reproductive event carries an additional energetic and physiological costs which could be paid by individuals with a sacrifice of self-maintenance and a reduction of reproductive output or survival in the successive seasons.

3. In this paper we analyse the breeding strategy of females of a Rock sparrow population nesting in the Western Italian Alps in order to identify: i) the actual benefits of rearing a second clutch, (ii) the influence on the number of breeding events of baseline glucocorticoid (CORT) levels along with the immunocompetence of fledglings measured as the T-cell-mediated immune response (CMI) towards the mitogen phytohemagglutinin (PHA), and iii) the potential carry over effects for reproductive females who choose multiple breeding as an optimal breeding strategy.

4. We found that, in Rock sparrow, even without physiological and hormonal differences among them, double-brooding females retrieve a significant advantage to rear a second clutch having a higher lifetime reproductive success (measured as total number of offspring produced) than single brooding females.

Key-words: Alps, breeding biology, double brooding, feather corticosterone, lifetime productivity, mating system, *Petronia petronia*, phytohaemagglutinin assay, reproductive effort, reproductive success.

INTRODUCTION

For many bird species, annual reproductive success (hereafter ARS) of individuals within a population varies not only in the number of fledglings produced, but also in the number of broods attempted (Verhulst, Tinbergen & Daan 1997; Husby, Kruuk & Visser 2009a; Bulluck *et al.* 2013; Carro, Mermoz & Fernández 2014). In accordance with this and especially for short-lived species (Nagy & Holmes 2005; Carro, Mermoz & Fernández 2014; Hoffmann, Postma & Schaub 2014), while ARS could have a strong demographic effect on population growth rate (Bulluck *et al.* 2013), is through an optimal frequency of multiple brooding (i.e. more than one reproductive event within the same reproductive season) that females can maximize their lifetime reproductive success (hereafter LRS) (Weggler 2006; Carro, Mermoz & Fernández 2014; Hoffmann, Postma & Schaub 2014).

Thus, phenotypic plasticity of individuals may involve several changes (i.e. diet, habitat selection, migratory behaviour) (Dunn & Møller 2014) in order to optimize the trade-off between reproductive investment in any one attempt of reproduction and survival (Ardia 2005a; Sockman, Sharp & Schwabl 2006; Carro, Mermoz &

Fernández 2014; Hoffmann, Postma & Schaub 2014). This energetic compromise that parents must deal, following the reproductive trade-off hypothesis (Parejo & Danchin 2006), is an important part of life-history theory and is at the same time one of the major challenge for the evolutionary theory (Pryke *et al.* 2012). For all species, in fact, reproduction has a cost and individuals which invest too much could pay with a reduction of survival or reproductive output in the successive seasons (Bell 1980; Sockman, Sharp & Schwabl 2006; O'Brien & Dawson 2013). However, a limited reproductive investment may not allow individuals to maximize their reproductive potential (Sockman, Sharp & Schwabl 2006; Dunn & Møller 2014).

Early reproduction for some bird species is associated with an high reproductive output (Verboven & Verhulst 1996), an higher survival rate and recruitment of offspring produced (Bulluck *et al.* 2013). For this reason, just early females, being more likely to double brood, could be define as "high quality individuals" than late breeders (O'Brien & Dawson 2013). Hence, in this scenario is important to know how many proximate (i.e. climate change, abundance and availability of prays, oxidative stress) or background factors (i.e. individual energy storage tactic, cooperative breeding strategy) are behind this important life-history stage (Varpe *et al.* 2009; Cox *et al.* 2010; Crespi *et al.* 2013; Legagneux *et al.* 2013). According to the parental quality hypothesis, individual quality is linked with the negative association between the start of reproductive season and the physical and physiological condition of the parents (Grüebler & Naef-Daenzer 2010). This behavioural plasticity could be constrained by the length of the breeding season relative to the differences in when females initiate first broods (Weggler 2006; Verhulst & Nilsson 2008; Carro, Mermoz & Fernández 2014), but also by important mediators of Darwinian fitness, such as stress hormones (e.g. circulating Glucocorticoids GCs). GCs, acting on essential metabolic and energy allocation process (Koren *et al.* 2012; Sternalski, Mougeot & Bretagnolle 2012) and fitness proxies (e.g. Body mass and Body condition) facilitate the adaptation of a distinct emergency state in order to maximize fitness through stress management (Blas *et al.* 2007). For example, lipids, a major source of energy storage in vertebrates, may support individuals with the best body condition index (BCI). This is expressed as the allometric relationship between body mass (i.e. fat content) and linear measure of body size (Jakob, Marshall & Uetz 1996; Kotiaho 1999; Green 2001; Peig & Green 2009). A favourable BCI supports survival to reproductive maturity, conferring a fitness advantage (Schneider 2004; Ardia 2005b; Schulte-Hostedde *et al.* 2005; Beauplet & Guinet 2007).

Hence, environmental and energetic constraints (e.g. scarcity or decrease of food resources, predation) that individuals have to cope with, causing an activation of the hypothalamic-pituitary-adrenal (HPA) axis (Wingfield *et al.* 1998; Wingfield & Sapolsky 2003; Blas *et al.* 2007; Schmid *et al.* 2013; Whirledge & Cidlowski 2013), drive a rapid release of GCs in to the bloodstream. The concentration of corticosterone (CORT), the primary glucocorticoid in birds (Schoech *et al.* 2009; Koren *et al.* 2012; Fairhurst *et al.* 2013; Legagneux *et al.* 2013), gradually accumulate in the growing feathers, provides an integrated, alternative and non-invasive matrix to measure the HPA activity and the baseline and stress-induced CORT secreted during the period of feather growth (Bortolotti *et al.* 2008; Legagneux *et al.* 2013) (see Fig. 16 APPENDIX). In accordance with this, during an emergency life history state induced by internal or external perturbations, an inefficient management of mediators of allostasis (i.e. adrenal hormones, neurotransmitters, etc.) (Korte *et al.* 2005), acting negatively on strength and responsiveness of the immune system (Ardia 2005a; Tella *et al.* 2008; Sternalski, Mougeot & Bretagnolle 2012) (e.g. immunosuppression) has a negative influence on modulation of trade-off between reproduction and survival (Patterson *et al.* 2014) and then on energetic state and therefore on all components (i.e. fecundity, survival) and proxies (i.e. energy intake, body size, BCI) of fitness (Westneat & Fox 2010; Wilson & Nussey 2010; Koren *et al.* 2012).

Climate change and its unprecedented rate (IPCC 2007) could be considered like another important proximate factor having a pervasive effect on reproductive phenology and on multibrood status of females (McCarty 2001; Walther *et al.* 2002; Parmesan & Yohe 2003; Root *et al.* 2003; Parmesan 2006; Wiens & Bachelet 2010; Mihoub *et al.* 2012). Many species of birds advanced their laying dates as a consequences of warming spring temperatures (Lepage, Gauthier & Menu 2000; Newton 2008; Møller, Fiedler & Berthold 2010; Mingozzi *et al.* in prep). This phenomenon is even more evident for species whose fitness is influenced by ecological constraints at high elevations, such as colder temperatures, greater seasonality and shorter breeding seasons (Møller, Fiedler & Berthold 2010; Fletcher *et al.* 2013). In these conditions, organisms have a short but highly productive window they use for reproduction (Verhulst & Nilsson 2008), than they must utilize their limited resources to growth and maintenance, optimizing their tactics of resource use (e.g. differences between capital or income breeders) (Langin *et al.* 2006; Varpe *et al.* 2009).

In this climatic scenario, long-term studies are some of the most documented evidences which allowed to understand the association between climatic or environmental variations and frequency of multiple brooding or population growth (Sparks *et al.* 2002; Parmesan & Yohe 2003; Crick 2004; Møller, Fiedler & Berthold 2004; Weatherhead 2005; Visser 2008; Møller, Fiedler & Berthold 2010; Bulluck *et al.* 2013).

Parental quality and the multibrood status of females are usually explained also by the association between fitness components and differences in phenotype (i.e. age or breeding experience) (Reed, Jenouvrier & Visser 2013). In fact, other studies have shown that older females, nesting in high quality habitat (i.e. optimal food availability) (Møller 2008) and breeding early (Verboven & Verhulst 1996; Verhulst & Nilsson 2008; Goutte *et al.* 2010; Mihoub *et al.* 2012), are more likely to double brooding. Moreover the environmental quality (Verboven & Visser 1998; Visser, Holleman & Gienapp 2006) shows that the synchrony of breeding with the peak of preys, in addition to promoting the double brooding triggering the advancement of the mean first-egg date (Matthysen, Adriaensen & Dhondt 2011), is the primary determinant of reproductive success (Tomás 2014).

The Rock Sparrow *Petronia petronia* is a loosely colonial and cavity nesting passerine, distributed from Southern Europe to Central Asia (see Fig. 2A APPENDIX), in open habitats, under dry and sunny climatic conditions (Cramp & Perrins 1994; Mingozzi & Onrubia 1997). In Southern Europe it breeds at low density in small groups near villages which gives a natural small and patchy structure to the related populations (Mingozzi & Onrubia 1997; Brichetti & Fracasso 2013).

This species could be consider as a good study model because (i) is located at the northern, and altitudinally higher, limits of its European range (Mingozzi *et al.* 1994), (ii) because, as upland species, should be more vulnerable to the effects of climate change (Fletcher *et al.* 2013), and (iii) because other studies have already shown the effect of climatic variations on many traits of his population dynamics (i.e. advance of laying dates, increment in trend of both second clutches and overall breeding success) (Mingozzi *et al.* in prep).

With this paper we aimed to describe, in a marginal population and in extreme environmental conditions, the physiological (e.g. stress condition) and reproductive costs of rearing a second clutch in females of Rock sparrow nesting in North-Western Alps in Italy.

In order to identify the actual benefits and potential carry over effects for reproductive females who choose multiple breeding as optimal reproductive strategy, we investigated the association between their seasonal productivity (clutch size, annual number of broods and nestlings) and the carry over effects such their own survival, nestling survival and overall fitness as expected on the basis of life-history theory (Brinkhof *et al.* 2002).

Specifically, we wanted to test whether (i) there are females that tend to lay two clutches per year and females that never lay more than one clutch per year. Therefore, by comparing the seasonal reproductive success of single or double brooding females (hereafter, respectively SBFs and DBFs), their nestlings' survival rates, as well as their overall fitness, we expect that (ii) DBFs and SBFs have comparable survival rates, (iii) fledglings from SBFs and those from the first clutch of DBFs have comparable survival probabilities, whereas fledglings from second clutch compared to the first clutches of DBFs have lower survival rates and that (iv) DBFs have an overall higher output fitness (i.e. total number of fledglings produced) than SBFs.

In addition, to quantify the relative importance of plastic versus evolutionary responses (Charmantier & Gienapp 2014), we examined whether stress condition has an effect on fitness, acting on metabolic and energy-allocation processes and then on important fitness proxies such as energy intake (i.e. lipids) and body size. Thus, we expect that (i) at the end of the first clutch, DBFs have lower baseline glucocorticoid levels than SBFs, or (ii) nestlings of DBFs have a stronger immune system showing a stronger cell-mediated immune response to PHA (stronger tissue swelling) than nestlings of SBFs.

MATERIALS AND METHODS

STUDY POPULATION

The study population is located in the Upper Susa Valley (Italian Cottian Alps, province of Torino; see Fig. 1 APPENDIX), between 1,550 and 1,800 m a.s.l. in three small villages (San Sicario, 1,570 m a.s.l.; Champlas Janvier, 1,784 m a.s.l.; and Thures, 1,750 m a.s.l.), that are not more than 4 km apart one from each other. Birds bred in artificial nest boxes (N = 40-50/year) especially designed for Rock Sparrows (11 × 12 cm and 50 cm high, see Fig. 6 APPENDIX), and when necessary, as trapping devices (Mingozzi *et al.* 1994). Within the study area, three are the main habitats frequented by Rock sparrow during the breeding season; hay cutting, pasture, meadow (see Fig. 4 APPENDIX).

All adults and fledglings have been individually marked with numbered aluminium rings and colour plastic ring combinations (Mingozzi *et al.* 1994) (see Fig. 4B and Fig. 5). Similarly, nestlings were individually marked at 12±1 days after hatching date (see Fig. 7D APPENDIX). A total of 4,137 birds were ringed from 1991 to 2013. We assumed that unringed birds that were found breeding were all first-year immigrants because virtually all young are ringed at the nest each year.

COLLECTED DATA AND BREEDING PARAMETERS

During 22 years (1991-2013) of *Petronia* Project, all birds were measured and weighed; adults immediately after capture, nestlings at 12±1 days after hatching date. We used a Pesola (Micro-Line Spring Scale; PESOLA AG, Baar, Switzerland; cat#

20100) to measure body mass, and a metric digital calliper (Trimtec Sistemi S.r.l., Milano, Italy; cat# 35200030) to measure tarsus, bill and wing length (see Fig. 7A APPENDIX). From mid-May until the end of August, every 2-3 days, we identified the birds, pair bonds, and parental feeding activity during early morning and late afternoons when the activity is highest. Every day from mid-May until the end of August, for each nest box we recorded the identity of the breeding birds and parent feeding activity, through direct observation during early morning and late afternoon, when the activity is highest. For each reproductive bird, we collected data on laying and brooding phenology, number of laid eggs, number of hatched eggs (see Fig. 8A-B APPENDIX), fledging success, and for each bird, his mating system according to the number of nest and partners detected during the whole season.

We computed several measures of reproductive success referred both to first and second clutches, and to the whole season. In particular, for each nest we calculated the clutch size, the hatching success (number of hatched eggs over laid eggs), the fledging success (number of fledged juveniles over the laid eggs), and, for each female, an overall seasonal success (number of fledged juveniles per female per season).

Fitness was calculated for each individual as the total number of fledged nestlings during a minimum of 2 reproductive seasons. To account for different recapture rates (some birds have been recaptured for more than 6 reproductive seasons, others for only 3), the overall fitness was expressed as total number of fledged nestlings over the total number of recapture years (including only those animals that were captured in at least 2 different reproductive seasons).

Survival rate was measured as the recapture rate of females and of their nestlings in the following three years. Carry over effects on fledglings were estimated by comparing, (i) the survival rates (i.e. number of recaptures) of fledglings of SBFs to those of DBFs at 1st clutch, and (ii) the survival rate of fledglings from the 2nd clutch compared to the 1st clutches of DBFs.

In the overall study period, we recorded 586 breeding events (N = 453 first clutches, N = 114 second clutches) and the breeding population ranged between 33 and 77 individuals (from 12 to 30 breeding pairs).

FEATHERS SOURCES

Feathers were obtained from wild-caught Rock sparrows (see Fig. 7B and 17A APPENDIX) and stored in paper envelopes measuring 6.0 cm by 10.0 cm (Paradox Company, Kraków, Poland; Cat#TRB 060) at ambient indoor emperature in Arcavacata di Rende, Italy, before analysis. In the last 2 seasons (2012-2013), between late June and mid-July, during the ringing activity of the first clutch, on day 11 post-hatch date and for each reproductive female (N = 40), we collected flanks feathers and four primary covers (two from each wing). Besides that, in order to compare their CORT levels with adults, only during 2013 reproductive season, we collected flank feathers and wings covers also from nestlings (N = 35).

FEATHER SAMPLES PREPARATION AND CORT EXTRACTION

Feather CORT assays were performed at the Department of Ecosystem and Public Health, Faculty of Veterinary Medicine, University of Calgary (Alberta, Canada). Feathers were prepared and CORT was extracted using a methanol-based technique (Sauvé *et al.* 2007; Bortolotti *et al.* 2008).

The CORT in the samples was measured using the corticosterone Enzyme-Linked Immunosorbant Assay (ELISA) kit (Enzo Life Sciences Inc., Farmingdale, NY, USA; cat #ENZO-900-097 – 96 wells) and the analysis, in order to minimize external interferences, was performed in a single analytical run. Dried extracts were reconstituted first in a small volume of EIA buffer and then in absolute methanol (see Fig. 14A-B APPENDIX). Afterwards, the reconstituted samples were vortexed for 3min, re-suspended with pipette tips, sonicated in a water bath for 30min and then transferred into Eppendorf tubes and centrifuged 10 minutes at room temperature at 14,000 rpm (see Fig. 13 APPENDIX). Finally, the supernatant was carefully collected with pipette tips and, following the manufacturer's directions with the ELISA kit provided, put into the plate for the final competitive reaction (see Fig. 15A APPENDIX). After 1 hour incubation at room temperature, the enzyme reaction within each well was stopped and the yellow color generated was read on a microplate reader 450±10nm (SpectraMax Plus384, Molecular Devices, LLC, Downingtown, Pennsylvania, USA, see Fig. 15B APPENDIX) and the concentrations were interpreted from the standard curves in the SoftMax Plus384. CORT values obtained were expressed as pg CORT per mg of feather.

IMMUNE CHALLENGE

Considering the protocol of (Smits, Bortolotti & Tella 1999), we tested the proliferative response of T lymphocytes to phytohaemagglutinin (hereafter PHA-P) in 181 nestlings from 78 nests during 2010-2013 reproductive seasons. Birds with the two intermediate weights were retrieved from each nest box on day 11 post-hatch date and, on their right wing, a 1cm patch of skin on mid-patagium was clean of feathers and down. Afterwards, we injected 30µl PHA-P (Sigma-Aldrich Co. LLC, St. Louis, USA; cat# L8754-5MG) dissolved in sterile PBS (Sigma-Aldrich Co. LLC, St. Louis, USA; cat# P3813) in the wing-web previously swabbed with alcohol (see Fig. 9A APPENDIX). We measured the thickness of the patagium at the point of injection three times immediately prior to and 24h after injection, using a micrometer caliper (Min-Wall Thickness Gage 313 Series; McGovernville Rd., Lancaster, Pennsylvania, USA cat# 313-401, see Fig. 9B APPENDIX). The T-cell-mediated immune (CMI) response was estimated as the difference between the second and the first patagium thickness value. In order to minimize operator error, all measurements were done by the same individual.

STATISTICAL ANALYSIS

The comparison of fledgling survival rates between SBFs and DBFs was conducted using Pearson's chi-squared test (Sokal & Rohlf 1994; Menzel *et al.* 2006). The difference in survival rates between fledglings of SBFs and those of DBFs (1991-2013) were tested using a Wilcoxon signed rank test for paired samples (Siegel & Castellan 1988).

The difference in reproductive success among SBFs and DBFs (1991-2013) was tested using an independent-samples t-test. The difference in CORT levels, body mass, BCI and CMI between females laying one or two clutches per season was performed using a one-way ANOVA with reproductive status of females as factor. A paired *t*-test was performed to determine whether or not patagium skin swelling increased significantly after PHA injection (pre- and post-treatment). Means and standard errors of the means (SEM) are reported throughout the text.Statistical analyses have been carried out using SPSS 21.0 (Statistical Package for Social Sciences, © Copyright 1989, 2013 SPSS, Inc., an IBM Company).

RESULTS

The frequency of recapture among reproductive females comparing survival in SBFs, DBFs with both clutches successfully completed and DBFs with both clutches failed (respectively SBF, DBF, DBF/0), did not differed significantly ($N_{SBF} = 138$ vs.

$N_{DBF} = 26$ vs. $N_{DBF/0} = 26$, respectively; Pearson's chi-squared test $X^2 = 3.602$; $P_{Exact} = 0.573$; Table 1).

We did not detect any significant difference in survival rate between fledglings of SBFs and those of DBFs at 1st clutch (N = 1,259 vs. N = 327, 17.1% vs. 15.1% respectively; Pearson's chi-squared test $X^2 = 0.224$; df = 1 ; $P_{Exact} = 0.396$; Table 2), whereas we detected a significant decrement in survival of fledglings of 2nd clutch compared to the 1st clutches of DBFs (N = 217 vs. N = 327, respectively 4.7% vs. 15.1%; Pearson's chi-squared test $X^2 = 17.243$; df = 1; $P_{Exact} < 0.001$; Table 2)

Overall, comparing the re-capture rate of fledglings from SBFs and DBFs we detected a significant difference (N = 215 *vs.* N = 60, respectively 17.1% *vs.* 11.0% respectively; Pearson's chi-squared test $X^2 = 8.696$ $P_{Exact} = 0.002$; Table 2).

When looking at the differences in reproductive success (average number of fledglings/year) between SBFs and DBFs recaptured for more than two different reproductive seasons (N = 10 *vs.* N = 36, respectively), we detected a significant higher fitness in DBFs (N = 7.55 Fledged/year) than SBFs (N = 4.65 Fledged/year) (independent-samples t-test $P < 0.001$; df = 9.981; Fig. 2);

Even if no significant difference was detected between females with different breeding strategies (i.e. DBFs and SBFs) and their CORT levels ($F = 0.048$, df = 1, $P = 0.828$; Fig. 3), at the end of first clutch, feather CORT levels in both SBFs and DBFs were negatively associated with their BCI ($N = 37$, $r = -0.368$, $P = 0.025$; Fig. 4). Furthermore, if we consider the association between feather CORT and reproductive success of females, i.e., fledging success (number of fledged juveniles per laid eggs) or hatching success (number of hatched eggs over laid eggs), there were no significant associations (for fledging success: $N = 37$, $r = -0.028$, $P = 0.862$; for hatching success: $N = 37$, $r = -0.021$, $P = 0.897$). Nor could the immune (CMI) response to the PHA challenge of nestlings be considered influenced by reproductive status of the mothers (*N* = 181, *F* = 0.073, df = 1, *P* = 0.787; Fig. 5).

DISCUSSION AND CONCLUSIONS

Rock sparrow females nesting in the Upper Susa Valley have a benefit to choose the multibrood status, because through this reproductive strategy they can maximize both ARS and LRS. This finding is in accord with results from many previous studies (Nilsson & Svensson 1996; Verhulst 1998; Bulluck *et al.* 2013; Carro, Mermoz & Fernández 2014; Hoffmann, Postma & Schaub 2014). During 22 years of study, 37.4% of females raised a successful second brood and their percentage varied among years from 3.6 to 71.5%. The individual management and allocation of time to optimize important life-history stages like reproduction (Dunn & Møller 2014) as well as important proxies of fitness such as LRS (Wilson & Nussey 2010), are for Rock sparrow lead by some proximate factors like spring temperature (Mingozzi *et al.* in prep). Thus, for this species and in accord with other studies (Bell 1980; Cresswell & Mccleery 2003; Matthysen, Adriaensen & Dhondt 2011; Bulluck *et al.* 2013; O'Brien & Dawson 2013), temperature warming in the first part of the breeding season is linked with an earlier onset of breeding as well as a significant increase in the frequency of females that nested twice in a season (Schaper *et al.* 2012; Fletcher *et al.* 2013; Hoffmann, Postma & Schaub 2014). This effect is much more evident in altricial species (i.e species where offspring tend to be born with no feathers) for which temperature has an effect on reproductive output of females and their fledging success (Cresswell & Mccleery 2003; Lehikonen & Sparks 2010; Lehikoinen *et al.* 2014) because acts positively on thermoregulation efficiency of nestlings. In addition to the lower thermoregulatory costs, higher spring temperatures acting on the advancement of the seasonal abundance and availability of preys, helps females to easily obtain and optimize food intake through an optimal foraging activity (Lepage, Gauthier & Menu 2000; Fletcher *et al.* 2013; Hoffmann, Postma & Schaub 2014). For this reason, in accord with other studies (Matthysen, Adriaensen & Dhondt 2011; Schaper *et al.* 2012; Reed, Jenouvrier & Visser 2013), females of Rock sparrow could be able to optimize their reproductive output synchronizing their time of egg laying with phenology of preys. Moreover, with this phenotypic plasticity, insectivorous birds, like Rock sparrow, may have a significant consequences on time of reproduction and fitness, because the synchronisation of their offspring needs with abundance of preys is an important selection pressure (Lepage, Gauthier & Menu 2000; Verboven, Tinbergen & Verhulst 2001; Sockman, Sharp & Schwabl 2006).

As reported by Nilsson and Svensson (1996) and conversely to the short-lived species having an high mortality risk and a small chance of survival (Hoffmann, Postma & Schaub 2014), invest much more in their current breeding season choosing multiple brooding (Carro, Mermoz & Fernández 2014), in Rock sparrow we observed that DBFs had the same survival chances than SBFs (Table 1). In addition, while for those species that choose the multiple brooding to optimize their ARS and LRS, the higher energy expenditure to rear a second clutch might also negatively affect survival of their offspring (Verhulst, Tinbergen & Daan 1997), in Rock sparrow did not found any difference in chance of survival between fledglings of SBFs and those of DBFs at the 1st clutch (Table 2). Hence, these findings confirm that Rock sparrow DBFs, as we can see in other species (Grüebler & Naef-Daenzer 2010; O'Brien & Dawson 2013), take advantage from multiple brooding because have a higher LRS (measured as total number of offspring produced; Fig. 2) than SBFs, although they have, in accord with other species (Verboven & Visser 1998; Verhulst & Nilsson 2008), a decrement in survival of fledglings from the 2nd clutch compared to those from the 1st clutch. Late-hatched birds, in fact, have less time to achieve an optimal body development because food is less abundant for them, they fledge with a lower body condition and later than early-hatched birds (Verboven & Verhulst 1996).

However, as detected by Schmid *et al.* (2013), even if we have not evidenced any clear association between stress condition of females or immunocompetence of nestlings and their survival, our results support the cort–fitness hypothesis (Bonier *et al.* 2009a; Bonier *et al.* 2009b; Angelier *et al.* 2010; Koren *et al.* 2012) and the negative influence that high CORT levels have on the energy-allocation processes and consequently on estimates of fitness (e.g. BCI) (Bonier *et al.* 2009a; Bonier *et al.* 2009b; Bonier, Ignacio & Robertson 2011; Rivers *et al.* 2012). The negative correlation that we found between CORT levels and important life-history proxies (Westneat & Fox 2010) like body mass and body condition (Smith, Wingfield & Veit 1994; Schoech, Mumme & Wingfield 1997; Fairhurst *et al.* 2013) can be considered for multiple-brooding females, in addition to the parental quality hypothesis, like an integrative measurement of their individual quality and fitness (Waye & Mason 2008b; Bonier *et al.* 2009a; Bonier *et al.* 2009b; Angelier *et al.* 2010).

In sum, double brooding by Rock sparrow could be influenced primarily by seasonal climatic variations and particularly by warmer spring temperature (Mingozzi *et al.* in prep). Thus, understand if this reproductive strategy and the multi-brood status is adaptive and then changeless for each individual during every breeding season, will require additional study. Further studies should test in depth other proximate factors as such oxidative stress. The relationship between secretion of GCs or immune function in the contest of life-history trade-offs, for example, need to consider with more detail how individual respond to immunochallenges. While CORT levels in breeding females, representing the amount of stress gained during the most recent post-moult, likely influences success of reproductive effort, long-term CORT acting on metabolic and energy-allocation processes, could have important effects on breeding phenology and reproductive strategy. In accordance with this, seeing double brooding is linked to the basis of individual optimization (Brinkhof *et al.* 2002), it is likely that the ability of some individuals to lay earlier than others is connected, in addition to individual quality (Carro, Mermoz & Fernández 2014), also to seasonal changes in resources (e.g. phenology of preys), as well as to environmental quality (O'Brien & Dawson 2013; Tomás 2014). This could explain why not all Rock sparrow females, as well as we can see in other bird species (Hoffmann, Postma & Schaub 2014), even if they would have enough time to do it, attempt a second brood after a successful first one. Lastly, multi-brood status of females could be driven by other background factors such as the individual energy storage tactic, according to which some individuals should prepare a complete clutch of eggs from body stores gained during pre-breeding season (Klaassen *et al.* 2001; Varpe *et al.* 2009) or achieve the same result rely on exogenous sources (nutrients ingested during or just before breeding season).

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TABLES LEGENDS

Table 1. Lifetime Reproductive Success (LRS, average number of fledglings per year) of SBFs (white bar) and DBFs (grey bar), including only those birds captured for more than 2 different reproductive seasons. The error bars represent 95% confidence intervals on the estimates of the means.

Table 2. Survival measured as recapture rate in the first three years of life of nestlings from: (i) SBFs or DBFs, (ii) from SBFs and from the first clutch of DBFs (respectively SBF and DBF/1) and (iii) from second clutch compared to the first clutch of DBFs, during 1991 – 2013 breeding seasons.

FIGURE LEGENDS

Figure 1 See APPENDIX

Figure 2. Lifetime Reproductive Success (LRS, average number of fledglings per year) of SBFs (white bar) and DBFs (grey bar), including only those birds captured for more than 2 different reproductive seasons, during 1991 – 2013 breeding seasons. The error bars represent 95% confidence intervals on the estimates of the means.

Figure 3. Amounts of feather CORT (pg/mg) between 1 clutch per season (SBF, white bar) versus 2 clutches per season females (SBF, grey bar) in Rock sparrow, during 2012 – 2013 breeding seasons. The error bars represent 95% confidence intervals on the estimates of the means.

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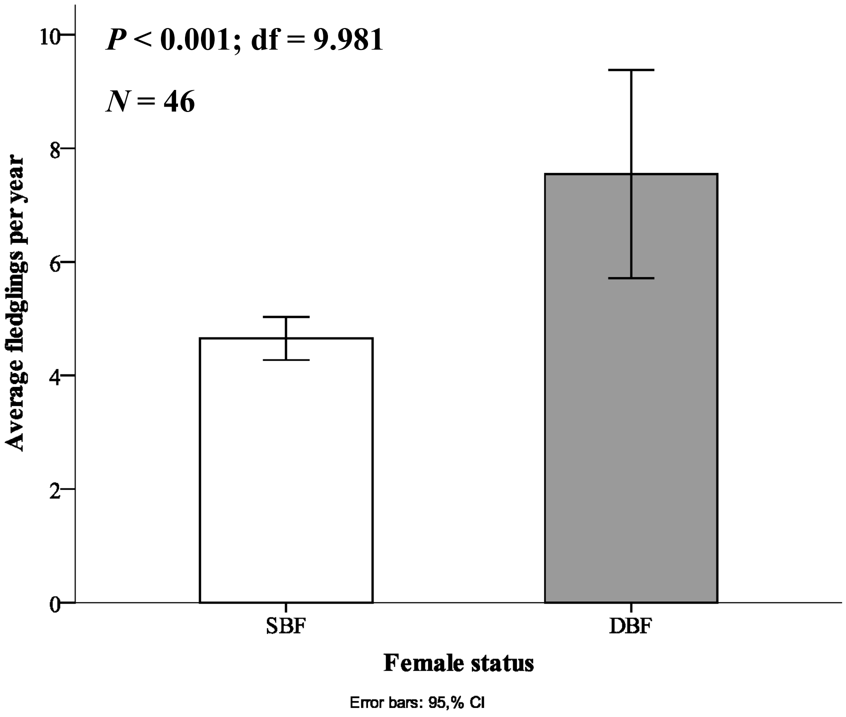
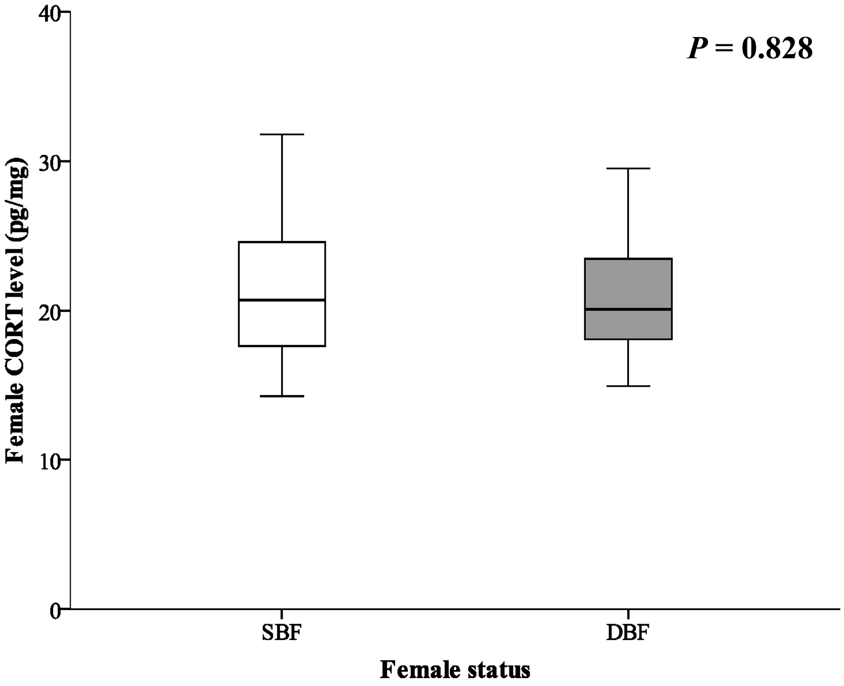


Figure 2



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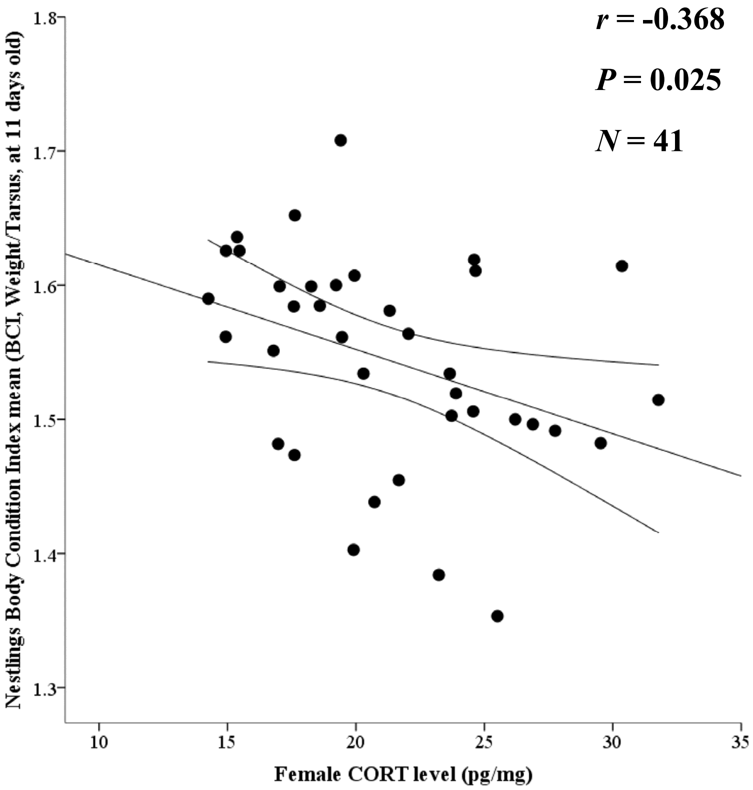

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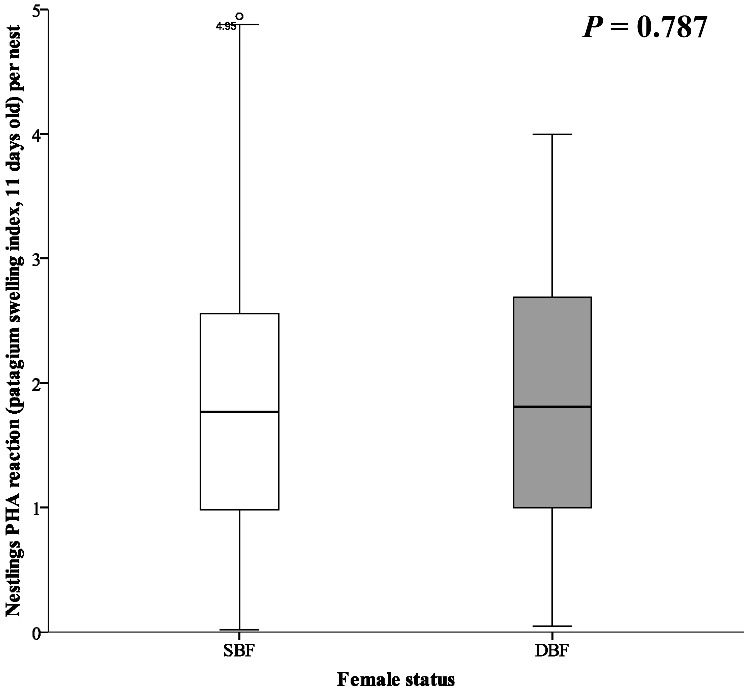

Figure 5

PAPER 3

PAPER – 3 - LONG-TERM GLUCOCORTICOID LEVELS REFLECT FITNESS IN A MARGINAL ALPINE POPULATION OF ROCK SPARROW *PETRONIA PETRONIA*

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SUMMARY

1. Variation in baseline glucocorticoid (CORT) levels reflects any physical, physiological or social process that has a pervasive effect on life-history trade-offs and on the expression of individual quality in animals. Trade-offs are driven by glucocorticoids hormones (GCs), mediators of metabolic and energy-allocation processes which affect individual fitness acting on reproduction and survival, and related proxies such as energy intake (i.e. lipids) and body size.

2. Using feathers as a novel and non-invasive method to determine endogenous hormone levels, we investigated how long-term CORT levels, acting on different individual qualities, may favour relative fitness. Using the corticosterone Enzyme-Linked Immunosorbant Assay (ELISA) kit, we measured the CORT that accumulated in the feathers of breeding females and nestlings from a population of Rock sparrow (*Petronia petronia*), which is the subject of a long-term study in North-West Alps, at the geographical and altitudinal edge of their Italian breeding range. Thus, we evaluated

CORT levels along with the immunocompetence of fledglings measured as the T-cell-mediated immune response (CMI) towards the mitogen phytohemagglutinin (PHA).

3. We found a positive association between the body condition index (BCI) of females and fledglings, and between the body mass of fledglings and their CMIs. In addition, CORT levels in both naturally moulted flank feathers and primary wing covers were negatively correlated with female and fledgling BCI, but not with CMI of fledglings.

4. Our results provide further support to the glucocorticoid–Fitness Hypothesis which suggests that elevated baseline CORT levels; i) signal individuals in poorer condition, and ii) suggest a critical influence of glucocorticoid on essential metabolic and energy-allocation processes, consequently affecting individual fitness. In the current study on Rock sparrows, the functional relationships that we found in both adults and nestlings between GCs and important proxies of fitness (body mass and BCI) allows us to identify future research directions. These include (i) integration of measures to describe the difference between levels of CORT in plasma *vs* that in feathers, and the influence of long-term CORT on (ii) breeding strategy, or (iii) on fitness indicators such as survival rate of individuals and (iv) their Lifetime Reproductive Success (LRS).

Key-words Glucocorticoids, baseline Corticosterone, proxies of fitness, feather Corticosterone, cell-mediated immunocompetence, phytohaemagglutinin assay, birds, Alps.

INTRODUCTION

Physiological and hormonal mechanisms have an effect on a central principle of life-history theory: investment in reproduction can compromise survival (Ardia 2005a; Cox *et al.* 2010) as well as individual quality. Thus, these mechanisms drive transitions between important life history stages (e.g. migration and reproduction) (Festa-Bianchet 1998; Pierce & McWilliams 2004; Wada 2008; Crespi *et al.* 2013) among vertebrates through those evolutionarily stable strategies which allow organisms to cope with stress (Korte *et al.* 2005). For this reason, important mediators of Darwinian fitness, such as stress hormones (e.g. circulating glucocorticoids GCs) acting on essential metabolic and energy allocation process (Koren *et al.* 2012; Sternalski, Mougeot & Bretagnolle 2012) facilitate the adaptation of a distinct emergency state to maximize fitness through stress management (Blas *et al.* 2007). In this scenario, individual variation in the trade-off between self-maintenance, growth, reproduction and survival is a central topic of natural selection and life-history theories (Stearns 1977; Ardia 2005a; Koren *et al.* 2012; Patterson *et al.* 2014). Thus, environmental and energetic constraints (e.g. scarcity or decrease of food resources, predation) that individuals have to cope with, causing an activation of the hypothalamic-pituitary-adrenal (HPA) axis (Wingfield *et al.* 1998; Wingfield & Sapolsky 2003; Blas *et al.* 2007; Schmid *et al.* 2013), drive a rapid release of GCs in to the bloodstream. The concentration of corticosterone (CORT), the primary glucocorticoid in birds (Schoech *et al.* 2009; Koren *et al.* 2012; Fairhurst *et al.* 2013; Legagneux *et al.* 2013), gradually accumulates in the growing feathers, providing an integrated, alternative and non-invasive matrix to measure the HPA activity and the sum of baseline plus stress-induced CORT secreted during the period of feather growth (Bortolotti *et al.* 2008; Legagneux *et al.* 2013) (see Fig. 16 APPENDIX). Birds are excellent indicators of population and physiological responses to habitat and climatic changes (Wingfield, Visser & Williams 2008; Newton 2013). Moreover, feather hormone levels should be considered more relevant than instantaneous blood samples (Bortolotti *et al.* 2008) as powerful biomarkers to study and predict fitness of free-ranging animals (Koren *et al.* 2012). Whereas in some cases (Darlington *et al.* 1990; Romero 2002) higher GC levels are crucial to survive noxious stimuli, for other wild species following the glucocorticoid–Fitness Hypothesis, elevated baseline CORT levels are assumed to indicate individuals in poor conditions (Marra & Holberton 1998; Thiel *et al.* 2008; Bonier *et al.* 2009b; Angelier *et al.* 2010) which would have difficulty optimizing metabolic and energy-allocation processes to maintain a physiological response to environmental and social challenges (Sapolsky, Romero & Munck 2000). During this emergency physiological state, individuals cope with internal and external perturbations (Blas *et al.* 2007) by consuming their energetic resources, in part through inefficient mediators of allostasis (i.e. adrenal hormones, neurotransmitters, etc.) (Korte *et al.* 2005). As well, there may be negative effects on the immune response (Ardia 2005a; Tella *et al.* 2008; Sternalski, Mougeot & Bretagnolle 2012) on energetic state, and therefore, on all components (i.e. fecundity, survival) and proxies (i.e. energy intake, body size) of fitness (Westneat & Fox 2010; Wilson & Nussey 2010; Koren *et al.* 2012).

Following the synthetic theory of evolution (Schneider 2004), energy balance and life-history trade-offs, indicated through morphological, biochemical or physiological responses in individuals, are used to estimate their fitness (Thompson, Flux & Tetzlaff 1993; Brommer, Merilä & Kokko 2002; Ardia 2005b; Peig & Green 2009). For example, lipids, a major source of energy storage in vertebrates, may support individuals with the best BCI. This is expressed as the allometric relationship between body mass (i.e. fat content) and linear measure of body size (Jakob, Marshall & Uetz 1996; Kotiaho 1999; Green 2001; Peig & Green 2009). A favourable BCI supports survival to reproductive maturity, conferring a fitness advantage (Schneider 2004; Ardia 2005b; Schulte-Hostedde *et al.* 2005; Beauplet & Guinet 2007). Therefore, BCIs, while varying temporally with resource availability, environment, season, habitat and population density (Beauplet & Guinet 2007), should be considered proxies of healthy endocrine and immune systems (Tella, Scheuerlein & Ricklefs 2002; Wayland *et al.* 2002; Legagneux *et al.* 2013) reflecting quality of individuals (Peig & Green 2009) and assumed to be related to their Lifetime Reproductive Success (LRS).

To assess the fitness correlates of GCs we used wing and flank feathers from wild Rock sparrows (*Petronia petronia*) within a long-term study in Northern Italy (Mingozzi *et al.* 1994). The birds, sexually monomorphic in plumage pattern (Shaobin & Lu 2012), achieve a complete prebasic moult (also known as post-breeding moult) (Hahn *et al.* 1992) once a year during summer (Svensson 1992).

The Rock sparrow is a loosely colonial and cavity nesting passerine, distributed from Southern Europe to Central Asia, in open habitats under dry and sunny climatic conditions (Cramp & Perrins 1994). In Italy this species is a widespread resident across central southern Apennines, including Sicily and Sardinia while in the northern part of the Peninsula the species nests in isolated patches in the Western Alps (Meschini & Frugis 1993; Brichetti & Fracasso 2013). These isolated populations are considered as part of the more broad French range (Mingozzi *et al.* 1994). This *Petronia* Project that since 1991 has been investigating the influence of climatic changes on breeding variables and individual fitness, in recent years has investigated how oxidative stress in this marginal population in extreme environmental conditions, may favour individual fitness acting on (i) reproductive status and investment, (ii) individual quality (of adults and nestlings) and (iii) survival.

MATERIALS AND METHODS

STUDY POPULATION

The study population is located in the Upper Susa Valley (Western Italian Alps, province of Torino (Fig. 1), between 1,550 and 1,800 m a.s.l. in three small villages (San Sicario, Champlas Janvier, and Thures). Birds bred in artificial nest boxes (N = 40-50/year) specifically designed for Rock sparrows (11 × 12 cm and 50 cm deep), which were used to trap the birds when necessary (Mingozzi *et al.* 1994).

Most of adult birds were individually marked from previous years of research with numbered aluminium rings and colour plastic ring combinations. During 2010-2013 seasons, some new adults (N = 48) and all nestlings (N = 670), were individually marked.

COLLECTED DATA AND BREEDING PARAMETERS

All birds were measured and weighed; adults immediately after capture, nestlings at 11±1 days after hatching date (see 17A APPENDIX). We used a Pesola (Micro-Line Spring Scale; PESOLA AG, Baar, Switzerland; cat# 20100) to measure body mass, and a metric digital calliper (Trimtec Sistemi S.r.l., Milano, Italy; cat# 35200030) to measure tarsus, bill and wing length (see Fig. 7A APPENDIX). A Body Condition Index (BCI) was calculated as body weight (g) divided by tarsus length (mm) (Jakob, Marshall & Uetz 1996). From mid-May until the end of August, every 2-3 days, we identified the birds, pair bonds, and parental feeding activity during early morning and late afternoons when the activity is highest. During the entire breeding season, for each breeding pair we recorded the date of clutch initiation, the brooding date, and the fledging date, along with brood size, number of eggs hatched and number of fledglings. The observations continued until all the young fledged. In the overall period (2010 to 2013), we recorded 146 breeding events (104 first clutches and 42 second clutches) and the breeding population ranged between 44 and 53 individuals.

We computed several measures of reproductive success considering both first and second clutches during one season. For each nest we calculated the clutch size, the hatching success (number of hatched eggs / laid eggs), the fledging success (number of fledged juveniles/ laid eggs) and, for each female, an overall seasonal success (number of fledged juveniles per season).

FEATHERS SOURCES

Between late June and mid-July 2012 and 2013, during the ringing activity for the first clutch, we collected flanks feathers and four primary covers (two from each wing) from reproductive females (N = 40) with different breeding strategies (N = 13 double breeders and N = 27 single breeders). Similarly, in order to compare their CORT levels with adults, during 2013, we collected flank feathers and wings covers also from the nestlings (*N* = 35) on day 11 post-hatch date.

Samples were stored in paper envelopes measuring 6.0 by 10.0 cm (Paradox Company, Kraków, Poland; Cat#TRB 060) at ambient indoor temperature in Arcavacata di Rende, Italy, until analysis.

FEATHER SAMPLES PREPARATION

Feather CORT assays were performed at the Department of Ecosystem and Public Health, Faculty of Veterinary Medicine, University of Calgary (Alberta, Canada). Feathers were prepared and CORT was extracted using a methanol-based technique (Sauvé et al. 2007; Bortolotti et al. 2008). To express feather CORT value also as a function of feather length (pg mm), after the first measure, the calamus was cut from each feather. The remaining vane portion was re-measured (see Fig. 10 APPENDIX), placed in a 20mL glass scintillation vial (see Fig. 11A APPENDIX) and then minced with dissecting scissors (4.5”, VWR International LLC, Mississauga, Ontario, Canada; Cat#82027-578) into small pieces (<3mm). After the addition of 1mL of 100% methanol (HPLC grade, Sigma-Aldrich Co. LLC, Oakville, Ontario Canada; cat# 34860) per 20mg of feather (see Fig. 16B and 11B APPENDIX), samples were placed in a sonicating water bath (VWR B1500A-MT Ultrasonic Cleaner; VWR International LLC, Mississauga, Ontario, Canada) at room temperature for 30 min (see Fig. 12 APPENDIX) and finally incubated at 50°C overnight in a microplate shaker (VWR 3500 Advanced Orbital Shaker; VWR International LLC, Mississauga, Ontario, Canada) at 160rpm (see Fig. 13A APPENDIX). Compared to other extraction methods (e.g. isopropanol), methanol extraction was preferred for its ability to penetrate feather shaft (Davenport et al. 2006). The supernatant was then separated from feather material and transferred in 2mL labelled Eppendorf tubes, than centrifuged (Spectrafuge 16M, Labnet International, Inc., Edison, New Jersey, USA) 20 minutes at room temperature at 14,000 rpm. The remaining feather residue in the incubation vials was recovered by washing with 1mL 100% methanol, vortexed for 1 min and then added to the samples. The supernatant was suctioned with pipette, transferred to a new 13mm glass tubes, dried under a nitrogen steam in a sample concentrator (Bibby Scientific Ltd, Staffordshire, United Kingdom, Cat#FSC496D) (see Fig. 14A-C APPENDIX) at 45ºC and finally frozen at –20°C until analysed using a Corticosterone Enzyme Immunoassay (hereafter EIA).

FEATHER CORT EXTRACTION

The CORT in the samples was measured using the corticosterone Enzyme-Linked Immunosorbant Assay (ELISA) kit (Enzo Life Sciences Inc., Farmingdale, NY, USA; cat #ENZO-900-097 – 96 wells) and the analysis, to minimize external interferences, was performed in a single analytical run. Dried extracts were reconstituted first in a small volume of EIA buffer and then in 100% methanol. Afterwards, the reconstituted samples were vortexed for 3min, re-suspended with pipette tips, sonicated in a water bath for 30min and then transferred into Eppendorf tubes and centrifuged 10 minutes at room temperature at 14,000 rpm (see Fig. 13C APPENDIX). Finally, the supernatant was carefully collected by pipette and, following the manufacturer's directions with the ELISA kit provided, put into the plate for the final competitive reaction. After 1 hour incubation at room temperature, the enzyme reaction within each well was stopped, the yellow color generated by the reaction was read on a microplate reader 450±10nm (SpectraMax Plus384, Molecular Devices, LLC, Downingtown, Pennsylvania, USA see Fig. 15B APPENDIX) and the concentrations were interpreted from the standard curves using the SoftMax Plus384. CORT values obtained were expressed as pg CORT per mg of feather.

IMMUNE CHALLENGE

Considering the protocol described by (Smits, Bortolotti & Tella 1999), during 2010 – 2013 reproductive seasons, we tested the proliferative response of T lymphocytes to phytohaemagglutinin (hereafter PHA-P) in Rock sparrow nestlings. On day 11 post-hatch date, two nestlings with intermediate weights were selected and, on their right wing, a 1cm patch of skin on mid-patagium was plucked clean of feathers and down. Afterwards, we injected 30µg in 30µl PHA-P (Sigma-Aldrich Co. LLC, St. Louis, USA; cat# L8754-5MG) dissolved in sterile PBS (Sigma-Aldrich Co. LLC, St. Louis, USA; cat# P3813) into the wing-web previously swabbed with alcohol (see Fig. 9A APPENDIX). We measured the thickness of the patagium at the point of injection three times immediately prior to and 24 h after injection (see Fig. 9B APPENDIX), using a dial micrometer (Min-Wall Thickness Gage 313 Series; McGovernville Rd., Lancaster, Pennsylvania, USA cat# 313-401, see Fig. 9B APPENDIX). The T-cell-mediated immune (CMI) response was expressed as the difference between the second and the first patagium thickness values. To minimize operator error, all measurements were done by the same individual.

STATISTICAL ANALYSIS

We performed Pearson's product moment correlation analysis (Sokal & Rohlf 1994; Menzel *et al.* 2006) to evaluate the association between baseline CORT values and physical (body mass or BCI) or physiological traits (CORT level, CMI) of females and nestlings or for the association between CORT and life history stages (e.g. egg-laying date, breeding success, fledging success). The difference in CORT levels, body mass, BCI and CMI between females laying one or two clutches per season was performed using a one-way ANOVA with reproductive status of females as factor. A paired *t*-test was performed to determine whether or not patagium skin swelling increased significantly after PHA injection (pre- and post-treatment). Means and standard errors of the means (SEM) are reported throughout the text. Statistical analyses were carried out using SPSS 21.0 (Statistical Package for Social Sciences, © Copyright 1989, 2013 SPSS, Inc., an IBM Company).

RESULTS

At the end of first clutch, feather CORT levels in Rock sparrow females (both single and double breeders) were negatively associated with their BCI (N = 37, r = -0.468, P = 0.002; Fig. 2) and with BCI of nestlings (N = 37, r = -0.368, P = 0.025; Fig. 3). Furthermore, if we consider the association between feather CORT and reproductive success of females, i.e., fledging success (number of fledged juveniles per laid eggs) or hatching success (number of hatched eggs over laid eggs), there were no significant associations (for fledging success: N = 37, r = -0.028, P = 0.862; for hatching success: N = 37, r = -0.021, P = 0.897). There was a 3 fold increase in patagium skin thickness 24 hrs after PHA testing (1.21 mm ± 0.31) relative to preinjection thickenss (0.44 mm ± 0.008), which was statistically significant (t = -19.865, P<0.001). The immune (CMI) response to the PHA challenge varied positively with mass of nestlings (N = 181, r = 0.252, P < 0.001; Fig. 4), negatively with their CORT levels (N = 15; r = -0.521, P = 0.046; Fig. 5), but was not affected by the BCI of females (N = 37; R^2 = 0.009; r = 0.291; P = -0.093) or with CORT levels of the mothers (N = 37; R^2 = 0.001; r = 0.023; P = 0.447; Fig. 7).

Although a positive association was found between BCIs of females and nestlings (N = 93, r = 0.366, P = 0.014; Fig. 6), no significant difference was detected between females with different breeding strategies (single or double breeding females) and their BCI (F = 0.025, df = 1, P = 0.876), CORT levels (F = 0.048, df = 1, P = 0.828), or with BCI of their nestlings ($F = 0.692$, df = 1, $P = 0.411$). Breeding strategy of the females also had no effect on the CMI of the nestlings ($F = 0.073$, df = 1, $P = 0.787$).

The ELISA kit was validated for use with Rock sparrow feathers by demonstrating: (1) parallelism between serial dilutions ($N = 5$; 2x – 4x – 8x – 16x – 32x) of feathers and the CORT standards ($R^2 = 1$); (2) significant value ($r = 0.999$, with 9.81E-06 and 1.54E-06 *P*-values for the intercept and the variables) of regression analysis on undiluted and serially diluted samples; (these results support a parallel relationship between the standard curve and the feather CORT samples); and (3) good precision of intra and inter-assay variability (coefficient of variation values were CV = 13.6% and CV = 11.6% respectively, the limit is CV<15%); with a recovery efficiency of 94.35%.

DISCUSSION AND CONCLUSIONS

Our results support the glucocorticoid–Fitness Hypothesis (Bonier *et al.* 2009a; Bonier *et al.* 2009b; Angelier *et al.* 2010; Koren *et al.* 2012) referring to the negative influence that high CORT levels have on the energy-allocation processes and consequently on fitness (Bonier *et al.* 2009a; Bonier *et al.* 2009b; Bonier, Ignacio & Robertson 2011; Rivers *et al.* 2012). In reproductive Rock sparrow females, birds with higher CORT are in poorer body condition and have nestlings with poorer body condition. This supports that CORT levels provide valuable insight into quality and fitness of individuals (Waye & Mason 2008b; Bonier *et al.* 2009a; Bonier *et al.* 2009b; Angelier *et al.* 2010), by reflecting important life-history proxies(Westneat & Fox 2010), such as body mass and BCI (Smith, Wingfield & Veit 1994; Schoech, Mumme & Wingfield 1997; Fairhurst *et al.* 2013).

As found in other studies (Smith & Moore 2003; Schneider 2004; Ardia 2005b) and supported by the positive association we found between BCI of females and nestlings (Fig. 6), it is likely that in Rock sparrows, energy stores (lipid or fat) and the physiological mechanisms that control energy balance, have a critical influence on regulation of energy homoeostasis (Ardia 2005b; Crespi *et al.* 2013). This influence is usually expressed in other important life-history traits such as migration strategies (Pierce & McWilliams 2004), clutch size, eggs size (Smith & Moore 2003) offspring quality and individual survival (King, Festa-Bianchet & Hatfield 1991; Tinbergen & Dietz 1994; Festa-Bianchet 1998). This may explain why in Rock sparrow, in agreement with some studies (Salvante & Williams 2003; Schoech *et al.* 2009; Hau *et al.* 2010; Crespi *et al.* 2013) but in contrast with others (Goutte *et al.* 2010), elevated long-term CORT levels, negatively influence essential metabolic and energy-allocation mechanisms, and are inversely related with body conditions of females and nestlings (Fig. 2 and 3). That is why, in reproductive females with lower BCI, this physiological state may be an adaptive response to food stress caused by nestlings attempting to gain more resources from parents as suggested by other researchers (Jenni-Eiermann *et al.* 2008; Harms *et al.* 2010). Furthermore, an indicatory of quality of the offspring reflected by their CMI (Tella *et al.* 2000; Salaberria *et al.* 2013), is positively associated with their body mass (Fig. 4), which can also be used as an indication of parental care (Tella *et al.* 2000; Fairhurst *et al.* 2011). It would show optimization of the resource-transfer process from parent to offspring, but of course is also a function of external factors such as food availability, infection challenge (Tella *et al.* 2000; Jenni-Eiermann *et al.* 2008), predators, weather conditions and environmental contaminants (Harms *et al.* 2010; Polo-Cavia *et al.*

2010). Our results open interesting future directions for research. The relationship between secretion of GCs or immune function in the contest of life-history trade-offs, for example, need to consider with more detail how individual respond to immunochallenges. While CORT levels in breeding females, representing perception of stress during the most recent post-moult, likely influences success of reproductive effort, long-term CORT acting on metabolic and energy-allocation processes, could have important effects on breeding phenology and reproductive strategy. While other studies on altricial species have shown a negative correlation between oxidative stress in reproductive females and number of yearly breeding attempts (Angelier *et al.* 2006; Williams 2012).

Based on CORT levels and body condition indices, we could not distinguish between, or predict breeding females that would produce and raise one or two clutches. Nor could the nestlings from first and second clutches be distinguished based on their physiological measures of immune response or long-term stress levels.

Because the use of feathers to better understand the hormonal profiles of individuals is relatively new, there were several challenges during the first two breeding seasons (2010 – 2011). The mass of feathers needed to detect CORT in this species, plus validation of the protocol, all were in development stages. Now that we have a method to gain insight into long-term stress, there is the opportunity to investigate further, the hypothalamic-pituitary-adrenal axis (HPA). We can now examine the complex relationship between glucocorticoids and fitness across Rock sparrow life history traits. We may be able to retrospectively associate immune responses and CORT levels with data on life-history stages, demography, climate and environmental change collected during the last 25 years of this project. Furthermore, using blood samples as well as feathers, we could conduct experimental studies, mapping out the relationship between blood CORT and feather CORT to see if, or how they are reflecting each other, and how the individual variation of HPA axis activity during different life-history stages may be a predictor of individual fitness.

ACKNOWLEDGEMENTS

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FIGURES LEGENDS

Fig. 1 See APPENDIX

Fig. 2 Association between CORT level (pg/mg) and Body Condition Index (BCI, measured as Weight/Tarsus ratio) of females with different breeding strategy (single and double breeders) during 2010 – 2013 breeding seasons

Fig. 3 Association between CORT level (pg/mg) of females with different breeding strategy (single and double breeders) and Body Condition Index of their nestlings (BCI, Weight/Tarsus ratio) during 2012 – 2013 breeding seasons.

Fig. 4 Association between values of body mass and T-Cell-Mediated Immune response (CMI, measured as patagium swelling) towards the mitogen phytohemagglutinin (PHA) of nestlings from the first clutch, during 2010 – 2013 breeding seasons.

Fig. 5 Association between CORT level (pg/mg) and T-Cell-Mediated Immune response (CMI, measured as patagium swelling) towards the mitogen phytohemagglutinin (PHA) of nestlings from the first clutch, during 2013 breeding season.

Fig. 6 Association between values of Body Condition Index (BCI, Weight/Tarsus ratio) in females with different breeding strategies (single and double breeders) and nestlings from the first clutch (mean per nest) during 2010 – 2013 breeding seasons.

Fig. 7 Association between values of Body Condition Index (BCI, Weight/Tarsus ratio) of females with different breeding strategies (single or double breeders) and T-Cell-Mediated Immune response (CMI, measured as patagium swelling) towards the mitogen phytohemagglutinin (PHA) of nestlings from the first clutch (mean per nest), during 2012 – 2013 breeding seasons.

LIST OF FIGURES

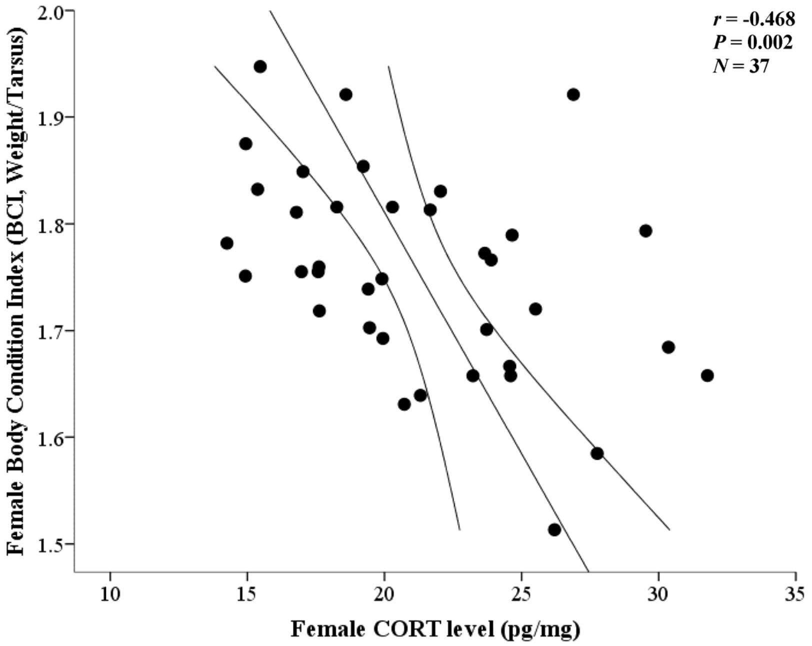


Figure 2

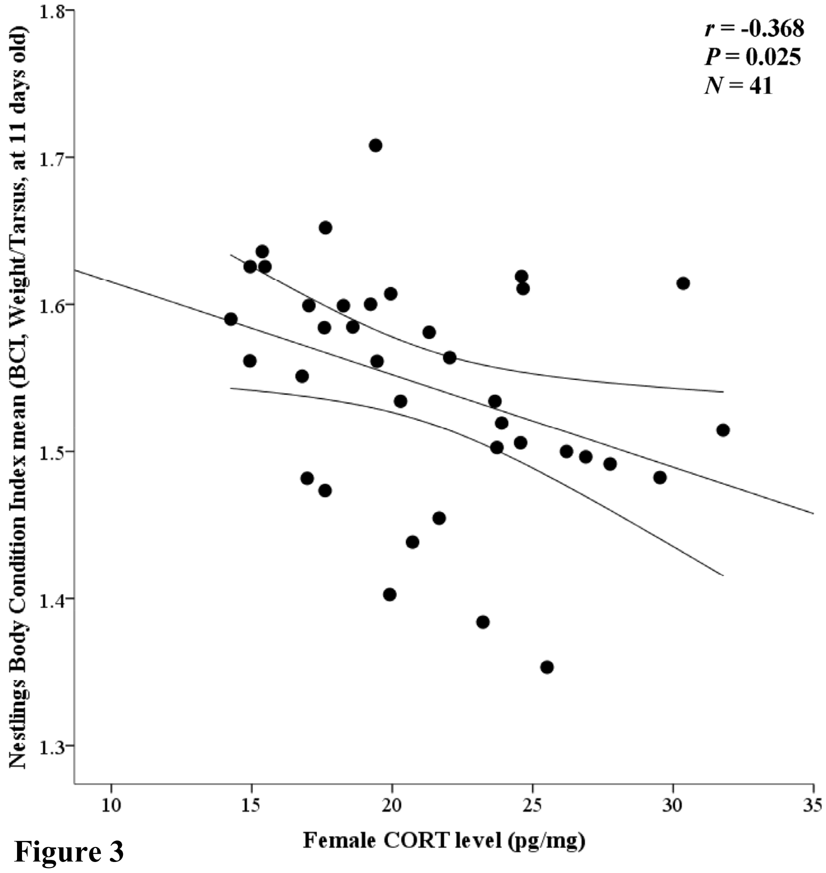
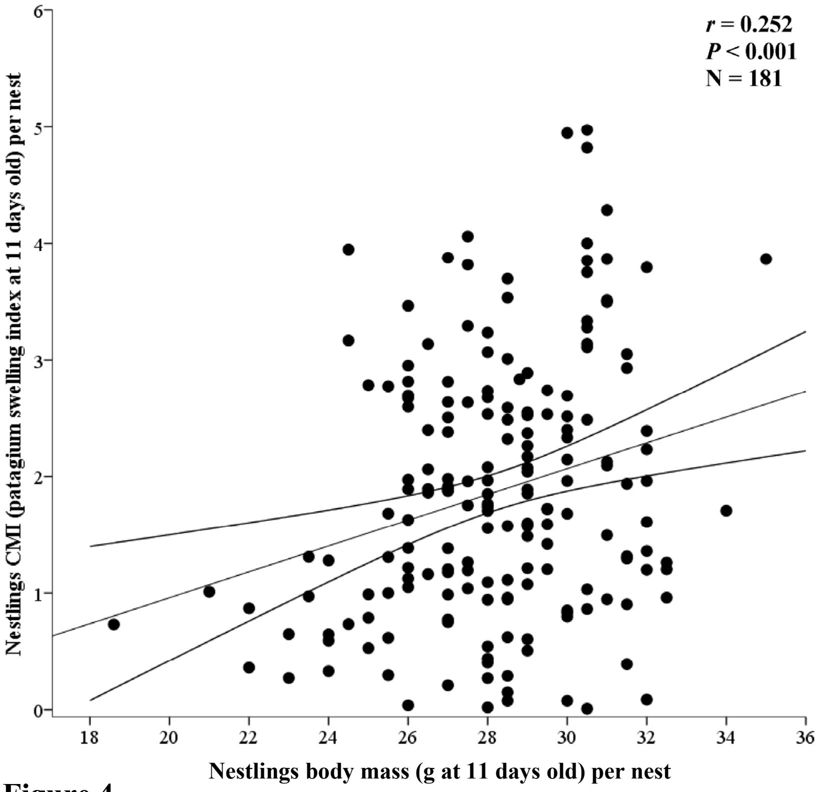


Figure 3



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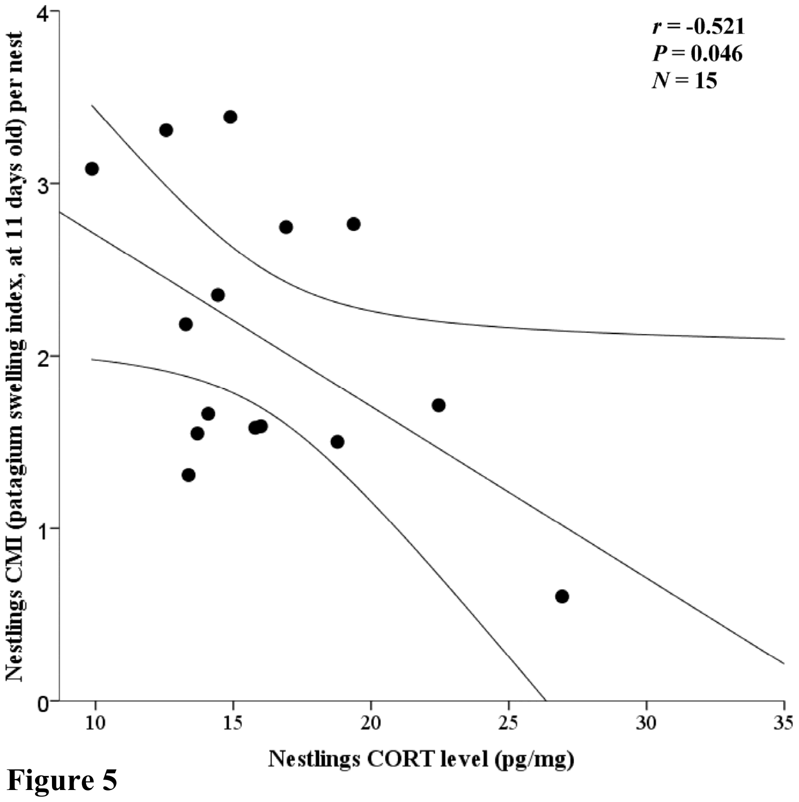

Figure 5

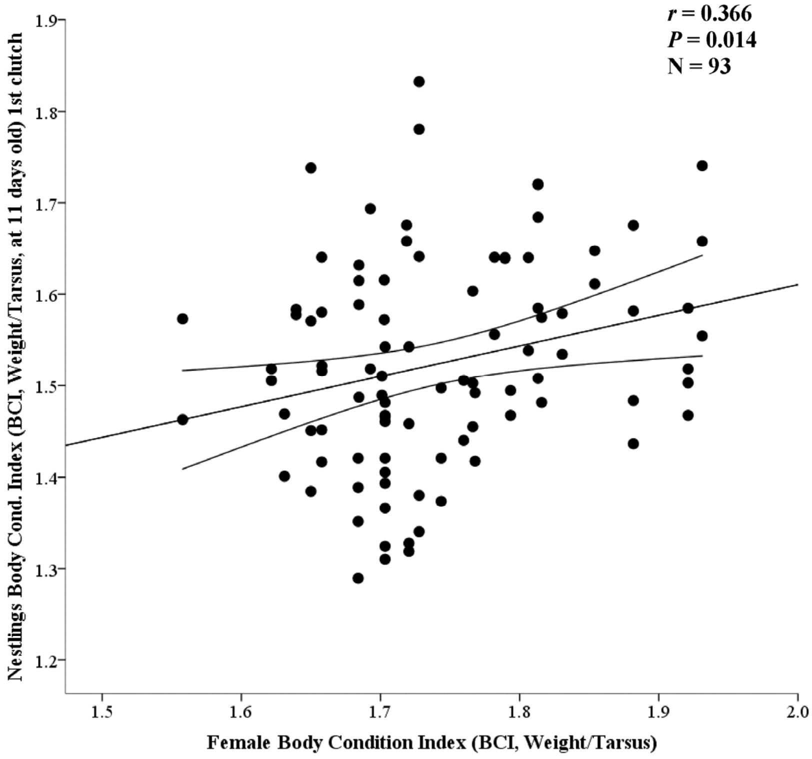

Figure 6

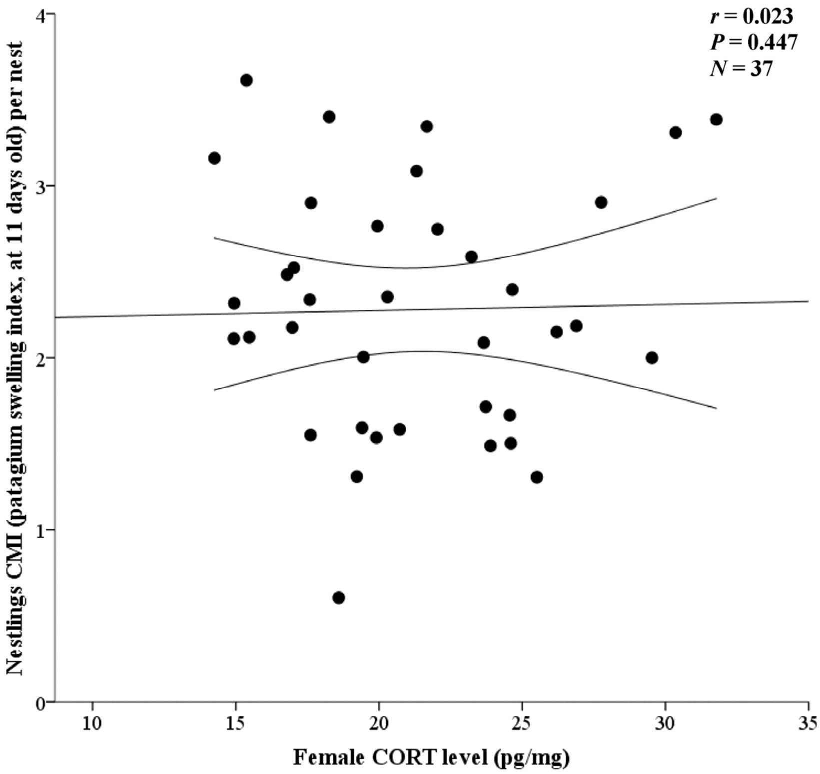

Figure 7

APPENDIX

APPENDIX

STUDY AREA IN ITALY AND EUROPEAN DISTRIBUTION

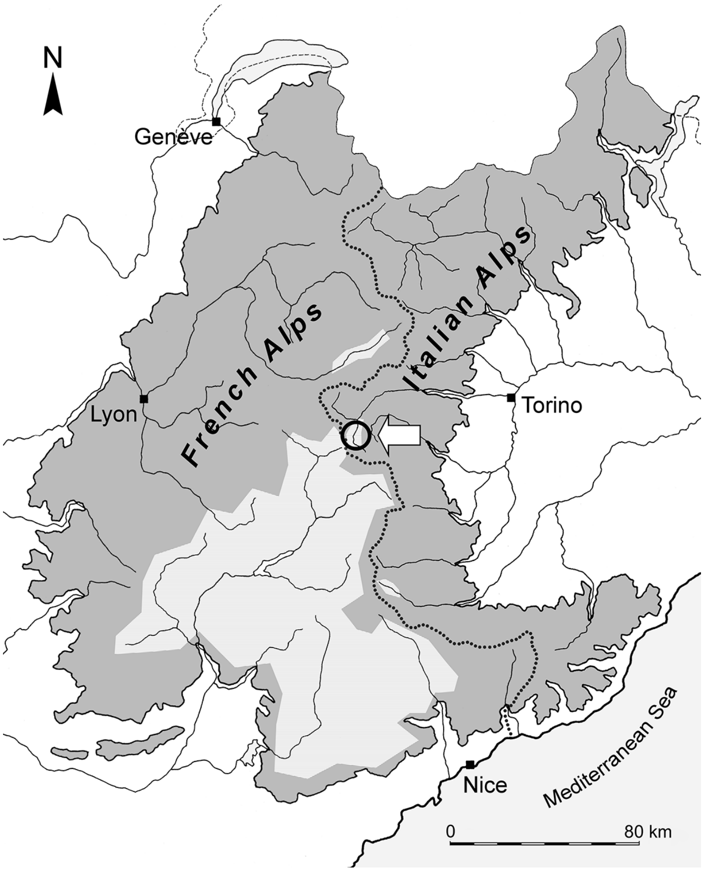

Figure 1 Breeding range (light grey) of the Rock sparrow in the Western Alps (dark grey) (redrawn by Mingozzi et al. 1994). The study area (black circle) is highlighted by an arrow.

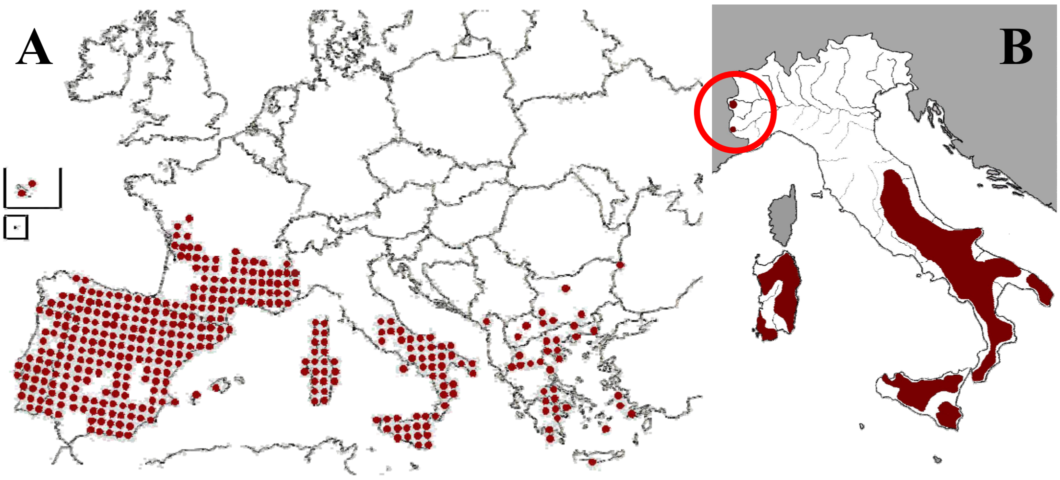

Figure 2 Breeding range (red patch) of the Rock sparrow in Europe (A) and Italy (B). The isolated breeding patches in North-West Alps are highlighted by a red circle.

BIOLOGICAL MODEL

Figure 3 Rock sparrow (Gouache 2011), by Paschalis Dougalis.

Figure 4 Main habitats in the *Petronia* Project study area: hay cutting (A), pasture (B), meadow (C)

Figure 5 A general view of one of three nesting site in the study area in Upper Susa Valley (Champlas Janvier, photo by A. Mingozzi 2007)

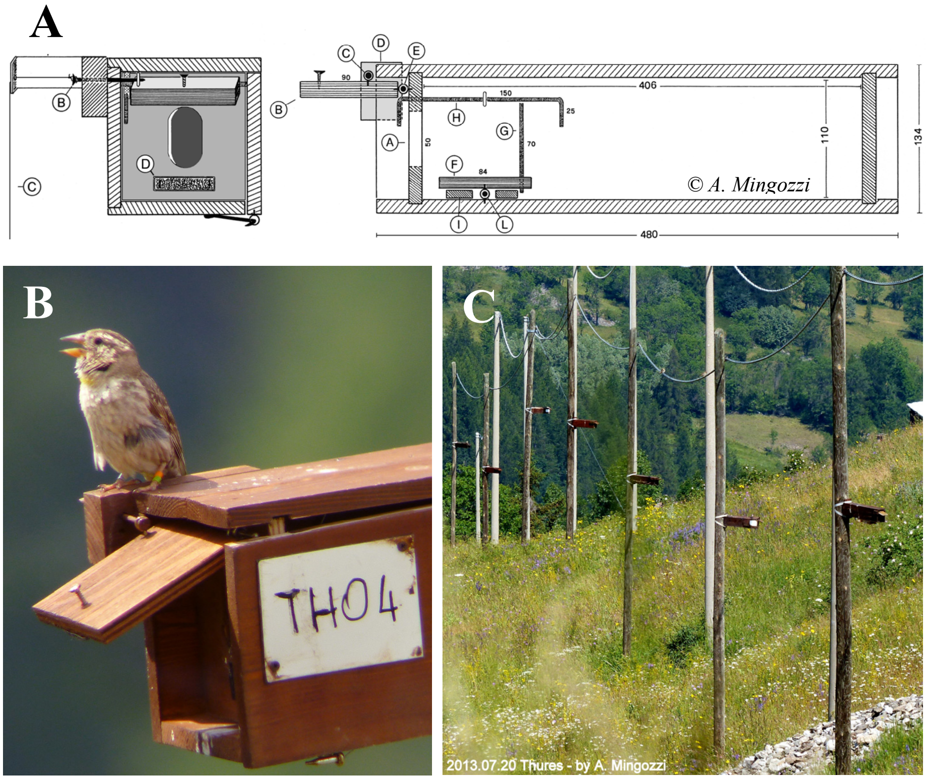

Figure 6 Technical drawings of a nest box (A), adult singing on top (B), nest boxes on a row of poles (C) in one of three nesting site in Upper Susa Valley (Thures, photo by A. Mingozzi 2013)

FIELD ACTIVITY

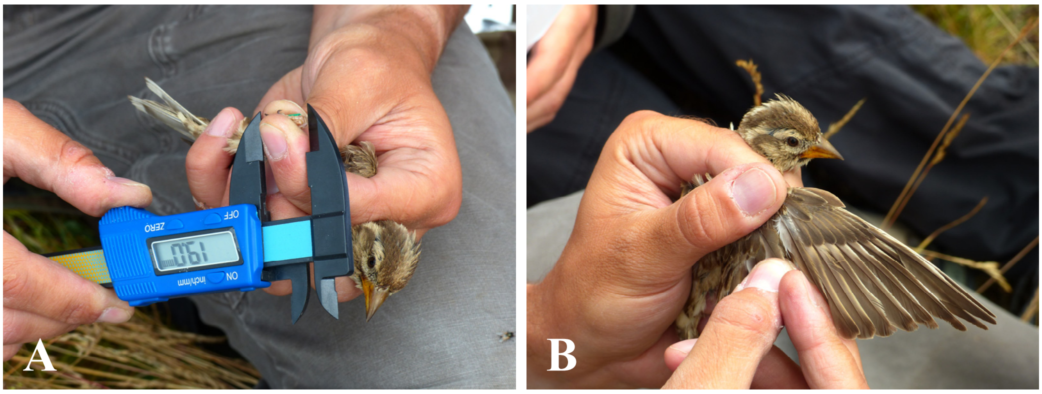

Figure 7 length tarsus measuring (A) and feathers sampling (B) (photos by A. Mingozzi 2013

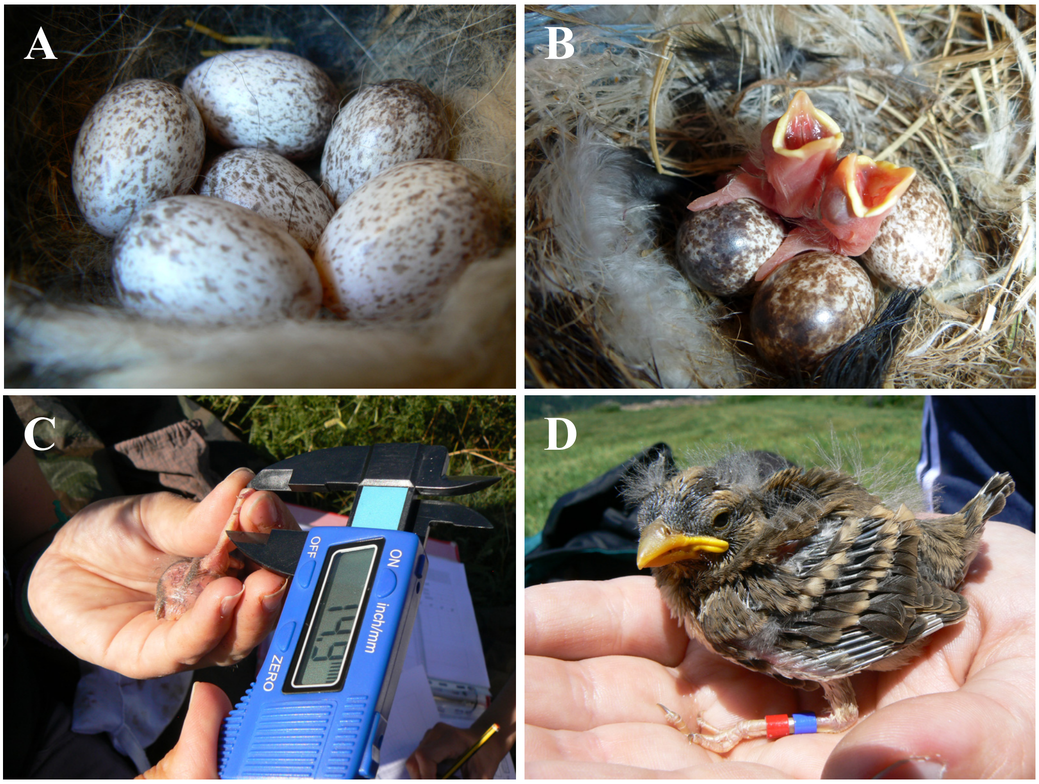

Figure 8 Laid (A) and hatched (B) eggs, length tarsus measuring (C) and a ringed nestling (D)

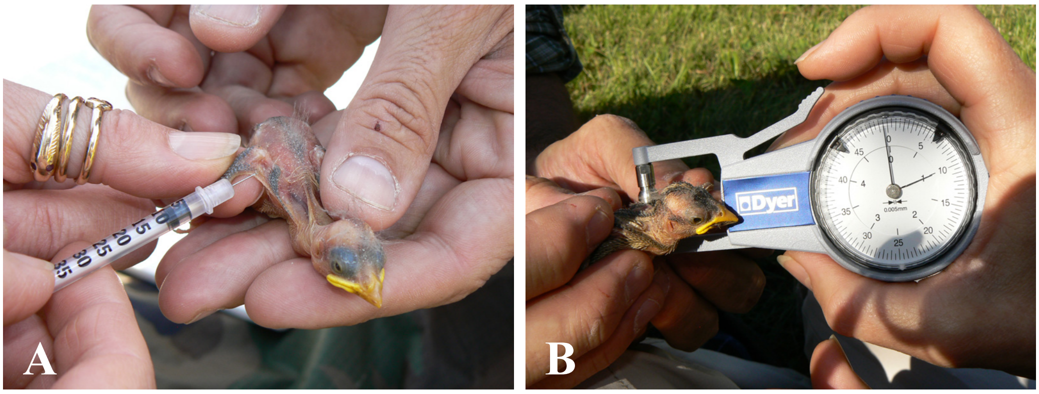

Figure 9 PHA (phytohemagglutinin) injection in the wing-web (A) and measuring of the thickness of the patagium at the point of injection whith micrometer caliper (B)

LABORATORY ACTIVITY

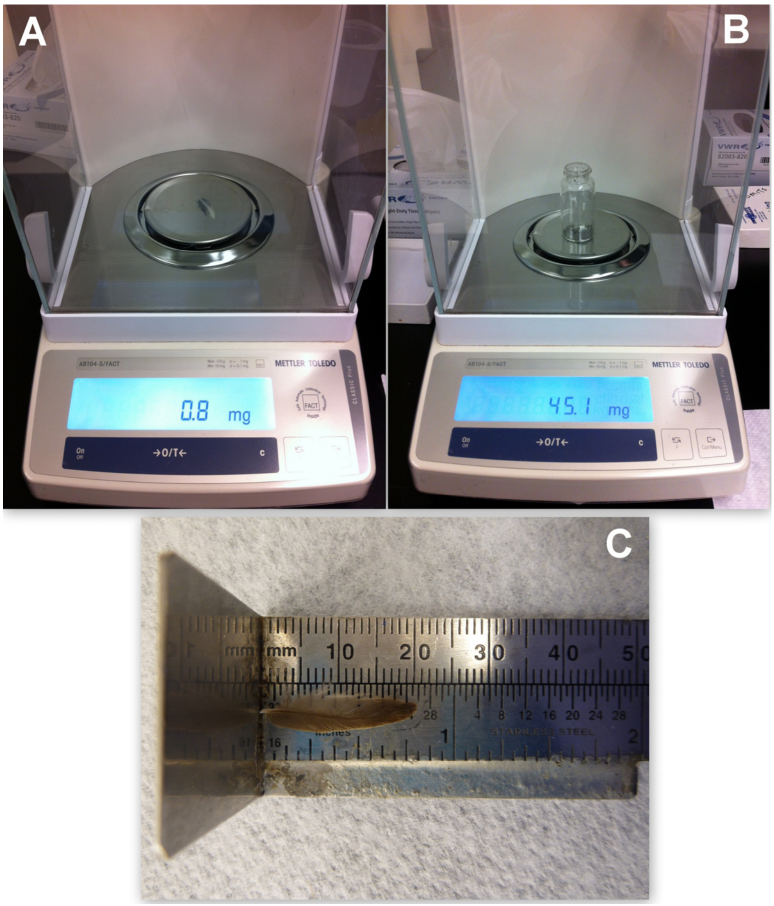

Figure 10 Weighting (A-B) and measuring of the vane (C) of the feathers

Figure 11 Whole (A) and minced (B) feathers placed in a 20mL glass scintillation vial

Figure 12 Samples before (A) and during (B) the sonication phase

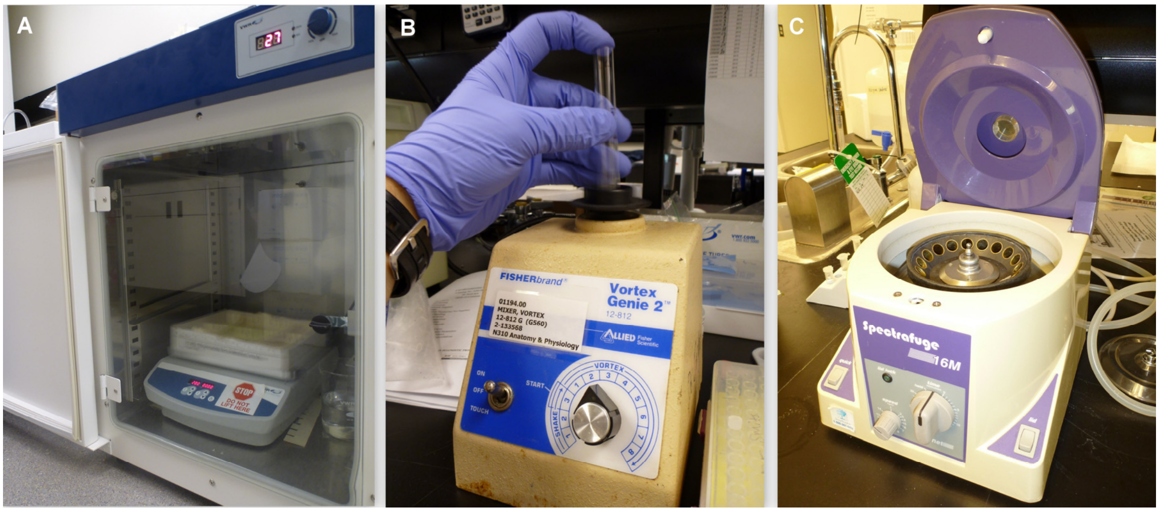

Figure 13 Preparation of the samples before CORT extraction: vortex (B), centrifuge (C) and microplate shaker (A)

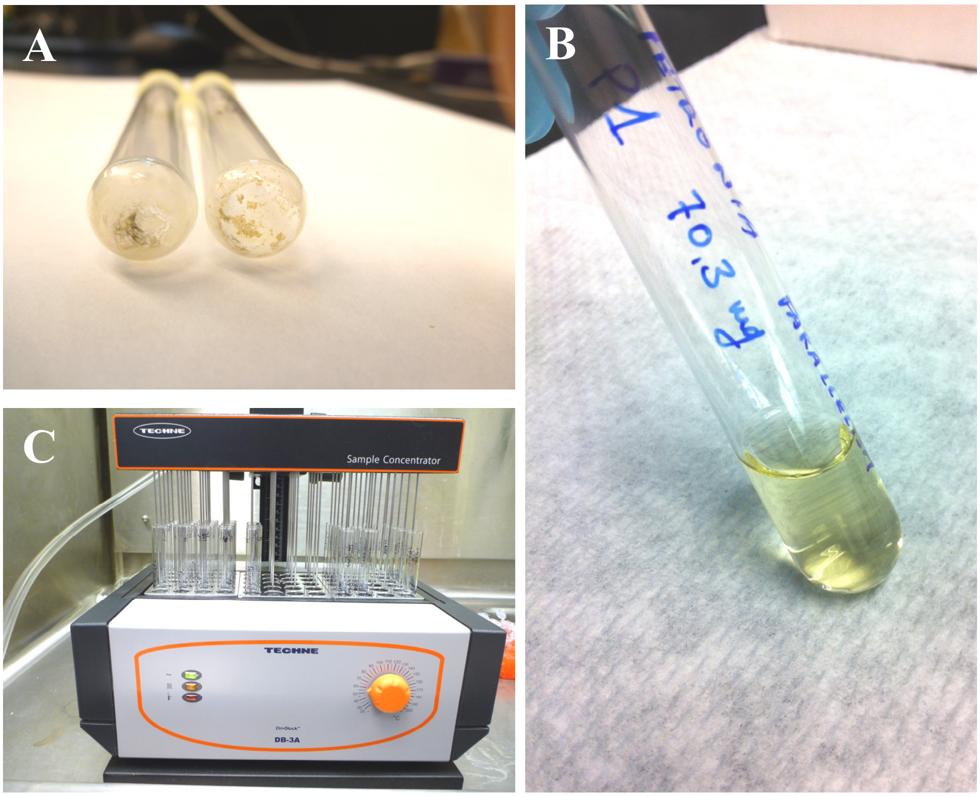

Figure 14 Dried extract (A), reconstitution of dried extract (B) and nitrogen drying phase in the sample concentrator

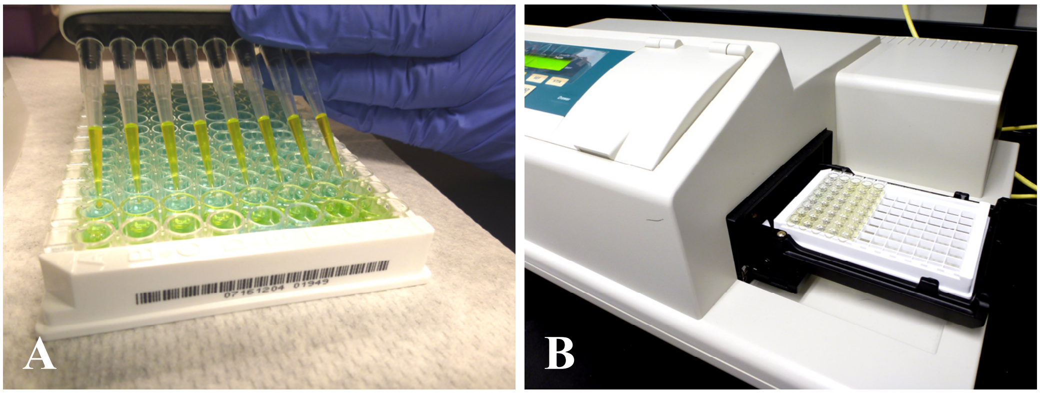

Figure 15 Before the last ELISA competitive reaction: pipetting of the samples within the reaction wells (A) and reading phase on a 450±10nm microplate reader (B)

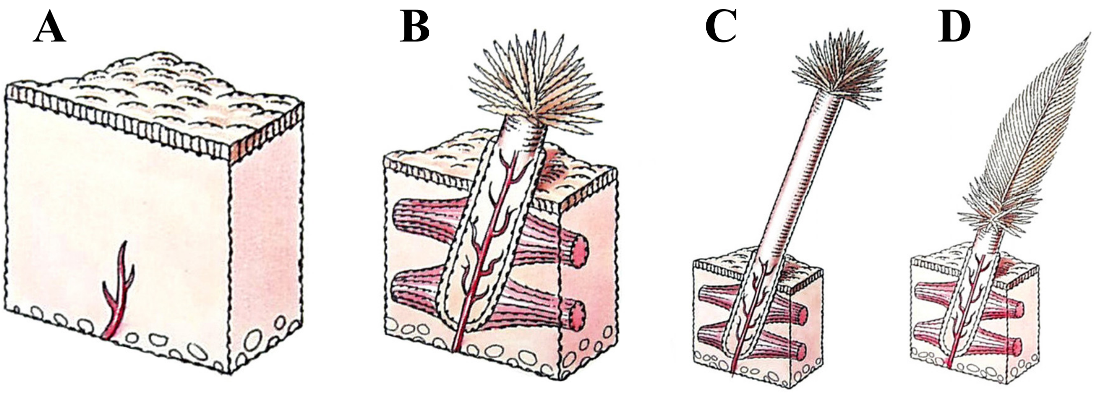

Figure 16 Development of a feather. Development of blood vessel (A), of the feather papilla through the proliferation of dermal cells (B), of a newly emerging vane sheath whit a short bud (C) and of the final structure without sheath but whit shaft and rachis (D). By Dorling Kindersley, 2009

Figure 17 Field (A) and laboratory (B) activities