UNIVERSITÀ DEGLI STUDI DI MILANO

PhD Course in Environmental Sciences

XXX Cycle

Maternal effects mediated by egg quality in the yellow-legged gull (*Larus michahellis*)

PhD Thesis

Cristina Daniela Possenti

R11007

Scientific tutor: Prof. Nicola SAINO

Scientific co-tutor: Dr. Marco PAROLINI

Academic Year: 2016-2017

SSD: BIO/07

Thesis performed at Department of Environmental Science and Policy,
University of Milan, Italy.

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'Seedlings from the same fruit, and the young of the same litter, sometimes differ considerably from each other, though both the young and the parents have apparently been exposed to exactly the same conditions of life; and this shows how unimportant the direct effects of the conditions of life are in comparison with the laws of reproduction, of growth, and of inheritance'

(Darwin, C. *On the Origin of Species*, 1859)

Abstract

The genetic component and the environmental conditions have cascading effects on individual phenotypic values. However, the most pervasive influences, which are potentially both genetic and environmental, are provided by 'maternal effects' that arise when the phenotype and environment of mothers markedly affect individual developmental trajectories of their progeny, potentially exerting carrying-over effects on the subsequent generation and maximizing their own fitness. In birds, mothers can adjust offspring phenotype by modulation of egg size and quality, in terms of variation in the concentration of quantitatively major (i.e. lipids, albumen) or minor (e.g. antioxidants, steroid hormones) components. Because antioxidants and steroid hormones are pivotal for the development of morphological and behavioural traits and their concentrations can vary according to laying order and in relation to maternal environmental experience, they are fundamental to interpret maternal allocation strategies to the eggs in an evolutionary perspective. The studies presented in this thesis aimed at assessing maternal effects mediated by egg quality in the yellow-legged gull (*Larus michahellis*), manipulating egg composition by directly injecting in the yolk a physiological dose of different maternal compounds, including vitamin E, testosterone and corticosterone. The consequences of variation in egg composition were investigated on markers of oxidative status, telomere length, morphology and behavioural lateralization of both embryos and hatchlings. Moreover, because egg antioxidants are thought to operate in an integrated way in the complex defence system against oxidative stress, I scrutinized the patterns of correlation between several egg antioxidants, markers of oxidative status in liver and brain, and morphology of embryos. In the same study, I also analysed the consequences of an experimental supplementation of vitamin E concentration on the distribution of other antioxidants and their effects on embryo phenotypic traits. Finally, the last work of this thesis aimed at testing non-consumptive effects of predation, which was experimentally increased by presenting stuffed predators to breeding adults before egg laying, on egg concentration of steroid hormones, and clutch and egg size.

The results on the administration of vitamin E in egg yolk showed a positive effect on somatic growth during embryonic development and on oxidative status soon after hatching, although the supplemental dose did not affect oxidative damage and telomere length during early life stages. From the study focused on the correlations among egg antioxidants and phenotypic embryo traits emerged that embryo morphology was positively associated with the concentration of antioxidants and negatively associated with markers of oxidative status. While antioxidant concentrations were positively correlated both within and between organs, this was not the case for markers of oxidative status; in addition, weak relationships existed between antioxidants and markers of oxidative status. Moreover, the experimental increase of vitamin E concentration did not affect the distribution of other antioxidants and their effects on embryo phenotypic traits. The evidence on the consequences of

testosterone supplementation was complex and differed in relation to phenotypic traits and ontogenetic stage. Indeed, testosterone had contrasting effects on embryo oxidative status depending on the focal organ, ameliorating oxidative status in the liver but not in the brain. In addition, although the supplemental dose of testosterone was observed to boost body size during embryonic development, it had a negative effect on embryo brain size and on somatic growth soon after hatching. Furthermore, the present thesis adds information to the scant knowledge about the organizational effects of steroid hormones on the development of lateralization; indeed, the physiological dose of testosterone was found to increase the consistency of lateralization whereas the direction of lateralization was affected by corticosterone treatment. Finally, the last work of this thesis, where non-consumptive predator effects were tested, showed that mothers exposed to increased predation risk laid larger eggs compared to control ones but did not modulate egg steroid hormone concentration and clutch size.

In conclusion, these studies shed new light on the consequences of maternal effects mediated by egg quality during both pre- and postnatal periods in the yellow-legged gull, showing that maternal transmission to the eggs may entail substantial costs and benefits for the progeny.

Chapter 1

General Introduction

Indirect genetic effects and the specific case of maternal effects

In traditionally quantitative genetic terms, the individual phenotypic value is predicted by the combined effects between the genetic component provided by parents (‘additive genetic value’) and the consequences of environmental conditions (‘environmental value’) for genotypic expression (Fig. 1; Falconer and Mackay, 1996; Wolf et al., 1998). However, in a more complicated scenario genetic influences are the result of the interactions among genes producing epistatic, dominant or additive effects (Wolf, 2000). For environmental effects, a peculiar category of influences includes the environmental features that are provided by other individuals to the focal individual. In addition, if there is quality variation in the environment shaped by the individuals and this variation depends on genetic differences among the individuals, the environment can become heritable and individuals can be selected for the influences that they exert on others. Such effects are termed ‘indirect genetic effects’ (IGEs), and they are potentially both genetic and environmental (Mousseau and Fox, 1998a,b; Wolf et al., 1998).

Any conspecific can promote IGEs but the most important interaction, which can markedly affect individual developmental trajectories, is between a mother and her offspring. These influences are termed ‘maternal effects’ and they occur when the phenotype of the progeny is affected by any influence from maternal phenotype, besides direct genetic effects (Mousseau and Fox, 1998b; Wolf et al., 1998). Therefore, maternal effects include both maternal inheritance, which causes mother and offspring resemblance, and maternal selection, which is a direct influence on fitness of the progeny (Kirkpatrick and Lande, 1989). Changes in the mean maternal phenotypic value shift the mean phenotypic value of the progeny in the following generation. For instance, if elevated milk production in mammals provides larger progeny, an evolutionary change in milk production can lead to shift the mean phenotypic trait for offspring size (reviewed in Rossiter, 1996; Wolf et al., 1998). Hence, maternal effects can have evolutionary consequences on a population and they may act in two different, not mutually exclusive, ways. They may impact on microevolution through genetic changes in response to natural selection pressure, producing divergence, and through phenotypic plasticity, promoting different phenotypes facing changing environments (Rossiter, 1991; Mousseau and Fox, 1998b).

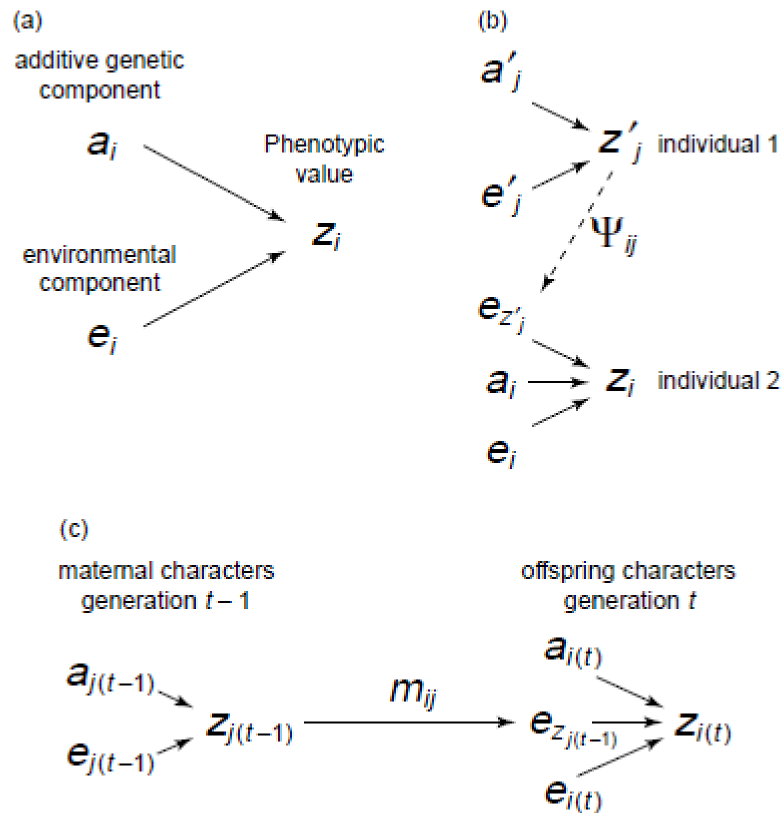

Figure 1. a) The traditionally quantitative genetic partitioning of the phenotype (z_i) into additive genetic (a_i) and environmental (e_i) values. (b) The indirect genetic effect ($e_{z'j}$) of phenotype (z'_j) in individual 1 on the phenotype (z_i) of individual 2. Ψ_{ij} is a coefficient that measures the effect. (c) The specific case of maternal effects. (*t*-1) is the previous mother's generation; *t* is the current offspring's generation. The coefficient m_{ij} represents the degree to which the maternal trait z_j contributes to the expression of the offspring trait z_i (modified from Wolf et al., 1998).

Phenotypic plasticity, whereby the environment experienced by mothers is translated into offspring phenotype variation, is traditionally represented as a reaction norm underlying the natural selection modulation (Mousseau and Dingle, 1991; Mousseau and Fox, 1998a), and, when the environmental cues are predicable, it results in the evolution of transgenerational plasticity (Rossiter, 1996; Mousseau and Fox, 1998a). Indeed, it has been suggested that mothers may have evolved a mechanism for 'adapting' offspring phenotype according to ecological predictable cues to anticipate the environmental conditions that their progeny will experience, ultimately for the optimization of maternal fitness (Bernardo, 1996a; Mousseau and Fox, 1998a). However, not all maternal effects are to be considered as an 'adaptation' because some of them can have maladaptive effects, depending on ecological and physiological constraints that may prevent the adoption of optimal maternal strategies (Groothuis et al., 2006; Groothuis and Schwabl, 2008; see Mousseau et al., 2009). This case occurs more frequently when the environment suddenly changes and mothers are not able to shape offspring phenotype in a context-specific way (Laaksonen, 2004; Marshall and Uller, 2007). From an

ecological and evolutionary perspective, maternal effects are considered as an evolutionary compromise between maternal and offspring fitness in response to the same selection pressure (Mousseau and Fox, 1998b). For instance, mothers face a trade-off among offspring quality and number, and future reproductive success by adopting different strategies. Generally, females in better condition (e.g. having large resource availability) can spend more energy in reproduction, investing in both offspring number and quality, than those in poorer condition, which may invest more resources in the future rather than in the current reproductive event (Mousseau and Fox, 1998b).

The consequences of maternal effects have attracted notable attention in studies of evolutionary ecology, showing different mechanisms underlying maternal strategies, which induce indirectly or directly phenotypic variation in the progeny in a context-specific way (Fig. 2; Badyaev, 2008). Each pattern of offspring developmental variation can represent either a by-product of a maternal adaptation passively transferred to the offspring generation or an active maternal strategy to adjust the phenotype of the progeny (Badyaev, 2002a). For instance, mothers may directionally adjust offspring development by increasing or reducing the frequency of some phenotypes when temporal/spatial gradient in the allocation of maternal compounds (e.g. hormones and antioxidants) occurs during the breeding period (Fig. 2a,c; Schwabl, 1996; Eising et al., 2001; Lipar, 2001; Badyaev et al., 2002; Badyaev, 2005; Love et al., 2005; Tschirren et al., 2006). Maternal effects can also produce divergent offspring phenotypes by providing variation in morph- or sex-specific resources, which depends on temporal/spatial requirements of the progeny (Fig. 2b; Burke and Sharp, 1989; Adkins-Regan et al., 1995; Velando, 2002). The adaptive value of these effects consists in setting maternal compounds to the differential sensitivity and needs of simultaneously growing embryos (Badyaev et al., 2005). Finally, mothers may adjust the phenotype of the progeny by compensating for other developmental inputs determined, for example, by environment conditions or mate quality (Fig. 2d; Michl et al., 2004; Groothuis et al., 2006; Oh and Badyaev, 2008). Among these maternal mechanisms, natural selection is expected to favour the optimal strategies that maximize maternal fitness in a context-specific way (Williams, 1966; Plaistow et al., 2007).

Maternal contribution to offspring phenotypic expression occurs from early embryo development, whereby maternal nutritional and physiological conditions during the breeding period can affect gamete size and quality (Mousseau and Fox, 1998b and references therein). These prezygotic maternal effects can act as epigenetic influences of newly formed somatic tissues during embryo development, by affecting germ cells soon after their segregation (Badyaev, 2008). Maternal effects can directly influence meiosis and mitosis (see review by Rutkowska and Badyaev, 2008), take the form of selective apoptosis and affect maturation processes of germ cell clusters (Gilbert et al., 1983; Bahr and Johnson, 1984; Yoshimura et al., 1993; Johnson 2000, 2003). These effects may represent

a maternal tool to select germ cells and their fate during embryonic development (Badyaev, 2008). Moreover, mothers can provide mRNAs and cytoplasmatic gradients, which modulate cell differentiation and protein synthesis during early morphogenesis (e.g. Dworkin and Dworkin-Rastl, 1990; see Badyaev, 2008), and allocate immune factors, antioxidants and steroid hormones that are involved in developmental pathways (e.g. Gatford et al., 1998; Surai and Speake, 1998). This maternal allocation of growth-affecting compounds can either affect development directly by transferring resources for the offspring or promote the offspring own production of developmental substances (Badyaev, 2002b). Although the mechanisms underlying these maternal effects are unknown, different physiological pathways may act according to differences in the breeding context and among species (e.g. Johnson, 1996; Zakaria, 1999; see Badyaev, 2008). Furthermore, postzygotic maternal effects exist, and they can develop during the prenatal stage, resulting in maternal provisioning of the progeny, and during the postnatal phase, being expressed as parental care or ecological and environment inheritance (Mousseau and Fox, 1998b).

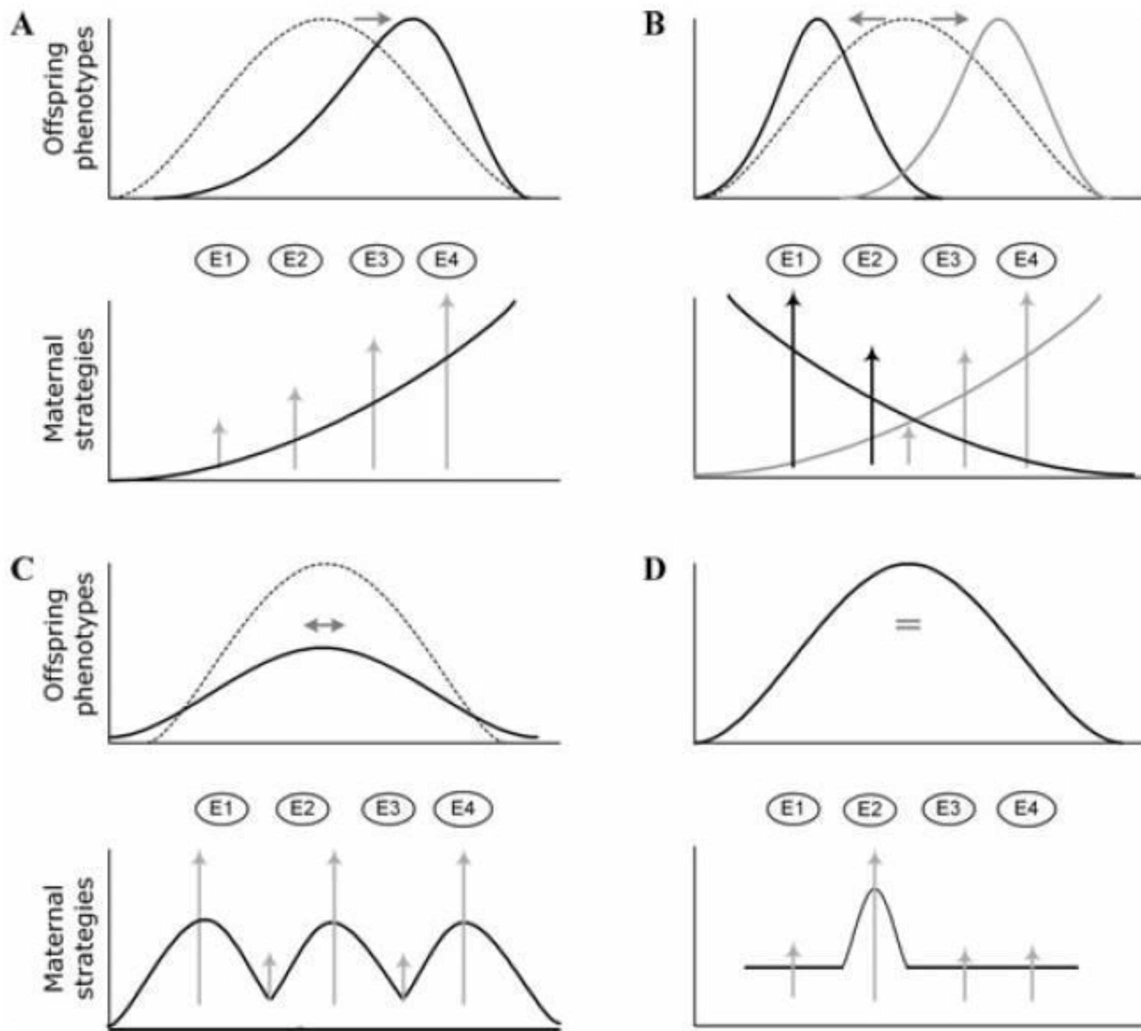

Figure 2. Maternal effects on offspring phenotypic variation. Each panel shows offspring distribution (upper graph) and the corresponding distribution of maternal strategies (lower graph). Solid line or dashed line indicate the presence or the absence of maternal effects (vertical arrows), respectively. Horizontal grey arrows show the direction of distribution change. E1-E4 represents the progeny. A) Directional adjustment of offspring variation by temporal or spatial gradients in maternal strategies. B) Maternal induction of offspring phenotypic variation by morph- or sex-specific allocation. C) Maternal effects in relation to variable amount of resources. D) Compensatory maternal effects by context-specific allocation (modified from Badvaey, 2008).

Maternal effects in birds

Maternal effects are an ubiquitous phenomenon broadly widespread across *taxa* (Bernardo, 1996a; Mousseau and Fox, 1998a,b). For example in many plants, the probability of seed dormancy is determined by photoperiod, interspecific competition and size of the maternal plant (Roach and Wulff, 1987; Donohue and Schmitt, 1998). Similarly, the probability of offspring diapause in some insect species is determined by host availability, light and temperature experienced by females during oviposition (Mousseau and Dingle, 1991). Moreover, offspring sex ratio may be influenced by host size and local female-density (insects; Hamilton, 1967; King, 1996), temperature (reptiles; Janzen, 1995; Roosenburg and Niewiarowski, 1998) as well as mate quality (birds; Sheldon et al., 1997). Postnatal maternal effects show their largest expression in mammals, showing the longest period devoted to postnatal parental care (e.g. lactation; Maestripieri and Mateo, 2009).

Although maternal effects have been universally disclosed among *taxa*, birds are commonly employed as a model in the physiological and evolutionary study of maternal effects. The interpretation of maternal effects in an ecological and evolutionary perspective is possible thanks to the flourishing literature concerning avian ecology and life-histories, as well as to the possibility to easily study birds in the wild. The eggs of birds provide an excellent opportunity to experimentally mimic variation during prenatal development and thus assessing the mechanisms underlying maternal effects on individual phenotype (e.g. Lipar and Ketterson, 2000; Eising and Groothuis, 2003). Moreover, after laying, the embryo develops outside the mother's body in a sealed environment, containing nutrients, water and minerals necessary for development, and where only gas exchanges (oxygen, carbon dioxide and water vapour) occur. Therefore, mothers can no longer have any direct influence on the development of their progeny, beyond incubation.

In birds, maternal effects are mainly mediated by the modulation of egg size and biochemical quality that are the major contributors to the fitness of the progeny. Egg size sets the amount of resources and the environment available for the embryo during development, thus determining the offspring size and survival soon after hatching (Sinervo, 1993; Mousseau and Fox, 1998b). The size of bird eggs can be affected by mothers' body condition and physiological constraints as well as by local environmental influences (Christians, 2002). Therefore, large variability in egg size exists at intraspecific level, which often can be dramatic, with the largest egg being up to 50% larger than the smallest (Williams, 1994; Bernardo, 1996a; Christians, 2002). Although ca. 70% of the variation in egg size is due to variation among clutches (Christians, 2002), large variability occurs also within clutches according to laying sequence, with the most marked differences expressed for the last-laid eggs, being smaller or larger than earlier ones (Williams, 1994; Bernardo, 1996b). A possible

interpretation is that mothers adopt maternal strategies, including ‘brood reduction’ and ‘brood survival’, depending on environmental conditions in order to maximize maternal fitness. The brood reduction strategy may entail the sacrifice of small last-hatched chick whilst the brood survival strategy may consist in allocating large amount of resources for growth of last-hatched chick to boost its competitive ability in food solicitation (Clark and Wilson, 1981; Slagsvold et al., 1984). Alternatively, mothers can adjust size of all eggs in a clutch in relation to the predictable environmental cues (Williams, 1994; Mousseau and Fox, 1998a,b; Christians, 2002). For instance, when the environment is hostile, mothers are expected to lay larger eggs, containing a greater amount of resources, from which usually emerge bigger chicks that grow faster and have higher survival compared to those hatching from small eggs (Williams, 1994; Bernardo, 1996b; Mousseau and Fox, 1998b; Remesˇ and Martin, 2002). Therefore, fitness differences between small and large offspring exist and they are more apparent under adverse environmental conditions (Mousseau and Fox, 1998a,b). This is in accordance with theoretical studies showing that maternal effects can be more valuable to the offspring under strict environmental regimes, including intense sibling competition (Uller, 2006) and/or seasonal decline in resource availability (Bernardo, 1996b).

Maternal effects mediated by egg quality

Evolutionary biologists have investigated, beside variation in quantitatively major compounds (e.g. lipid and albumen), variation in quality of maternal resources allocated to the eggs. The yolk of bird eggs is a main source of quantitatively minor components of maternal origin, including immune factors, antioxidants and steroid hormones, which mediate important forms of maternal effects influencing offspring developmental trajectories (Mousseau and Fox, 1998b; Surai, 2002; Groothuis et al., 2005). Mothers are thus expected to optimally equip their eggs with these components to promote offspring fitness, under the constraints imposed by their self-maintenance and environmental effects (Mousseau and Fox, 1998a,b; Surai, 2002). Indeed, these maternal compounds often vary in amount that can depend on mothers' quality and local environmental conditions (Nager et al., 1999; Blount et al., 2002; Rutkowska and Cichon, 2002; Royle et al., 2003; Saino et al., 2010). In addition, egg quality can also vary among and within clutches, in relation to the position in the laying sequence (Williams, 1994; Mousseau and Fox, 1998b; Saino et al., 2002; Groothuis et al., 2005; Krist, 2011), as well as covary with embryo sex. Mothers can adjust prenatal environment according to egg component requirements that differ between sexes (Nager et al., 1999; Müller et al., 2002; Rutkowska and Cichòn, 2002; Saino et al., 2003; Martinez-Padilla and Fargallo, 2007), or affect egg quality through changes in their physiology caused by sex allocation mechanisms (Pike and Petrie, 2003, 2006; Correa et al., 2005). These axes of variation, especially among sibling eggs, imply crucial mechanisms of differential allocation of maternal investment to the offspring that can generate a hierarchy of reproductive value among the members of the progeny (Mousseau and Fox, 1998a).

Among the mediators of maternal effects, antioxidants and steroid hormones are fundamental to interpret the evolution of maternal effects. Firstly, these maternal compounds are pivotal for the development of morphological, physiological and behavioural traits (e.g. Arnold, 2002; Rubolini et al., 2006; Groothuis and Schwabl, 2008; Pfannkuche et al., 2011; Selim et al., 2012; Parolini et al., 2015), secondly, their concentrations can vary according to laying order, suggesting the existence of allocation strategies and underlying trade-offs (Groothuis et al., 2005; Rubolini et al., 2011). Lastly, they have the potential to mechanistically and functionally link maternal environmental experience and offspring phenotype (Marshall and Uller, 2007).

Immune factors

Antibodies represent the main component of acquired immune system and they are maternally-transferred to the egg yolk in order to protect against pathogens (Hasselquist and Nilsson, 2009). The elements of innate immunity, such as lysozymes, are generally located into albumen and they mediate

an unspecific response enabling to reduce any bacterial infection (Wellman et al., 2007). Therefore, immune factors from both acquired and innate immunity are pivotal to contrast any pathogen attack, especially during the crucial early life stages thereby compensating for poor early offspring immune-competence.

Antioxidants

Antioxidants are a large class of compounds either endogenous, such as glutathione and uric acid, or exogenous, such as carotenoids and vitamins. Antioxidants are the main actors of the complex defence system in preventing and reducing oxidative damage to cellular macromolecules (i.e. DNA, lipids and proteins), caused by oxidizing substances that can originate from the external environment and internal milieu (Surai, 2002; Halliwell and Gutteridge, 1999, 2015). Antioxidant defence system relies on two major classes of mechanisms promoted by different effectors. Enzymatic defence pathways, mainly mediated by endogenous components, sequester pro-oxidant molecules or their intermediate derivatives, and induce their transformation into less active compounds (Surai, 2000; Halliwell and Gutteridge, 2007). Non-enzymatic antioxidants act as cofactors of antioxidant enzymes, eliminate metal ions, and oxidize radicals and other reactive species (Halliwell and Gutteridge, 2007). These two physiological pathways operate in an integrated or, in some cases, in a synergistic way, assembling the complex of antioxidant defences (Surai, 2002).

One of the main maternally-transferred exogenous antioxidants is vitamin E. In nature, only plants can synthetize this compound and its related isoforms, α- β- γ- δ-tocopherol and α- β- γ- δ-tocotrienol, which have different biological activity (Surai, 2002). Therefore, animals can acquire vitamin E only through food, and whether this resource is limited by dietary availability may depend on the species-specific diet and on contingent, local environmental conditions. In animal tissues, only α- and γ-tocopherol are retained in great amount (Rock et al., 1996) as it has been observed, for example, in chicken embryo, where more than 80% of vitamin E contained in tissues is represented by α-tocopherol whereas other isoforms are found in much lower concentrations (Surai et al., 1996). In bird species, vitamin E plays the most important role in the antioxidant defence system during embryogenesis and soon after hatching (Surai, 1999a). The importance of vitamin E during early life stages is demonstrated by the observation of its higher amount in the egg yolk compared to the concentration in maternal tissues of some wild birds (Surai, 2002). Moreover, while during embryonic development vitamin E is transferred from the residual yolk to the tissues and mostly accumulated in the liver, which is the main antioxidant storage organ (Surai, 2002), the concentration of α-tocopherol generally reaches its peak in all tissues soon after hatching (Surai, 1999b, 2002). Early life stages are characterized by rapid growth requiring high metabolic activities and the transition to pulmonary

respiration, which impose a notable production of oxidizing molecules (Kim et al., 2013; Costantini, 2014). Vitamin E acts as a free radicals scavenger, preventing damage to polyunsaturated fatty acids and DNA, and ultimately protecting and stabilising cell membranes and their organelles (Surai, 2002). Besides antioxidant activity, vitamin E is involved in the control of cell differentiation and proliferation, in the regulation of specific metabolic pathways, as well as in the transcription and expression of some genes involved in metabolic pathways (Surai, 2002). In addition, vitamin E can act as a modulator of some immune functions, such as cell-mediated and humoral responses, macrophage function, phagocytosis and antibody production (e.g. Sheffy and Schultz, 1979; Axelrod, 1980; Haq et al., 1996).

In an evolutionary context, because vitamin E is crucial for the antioxidant defence during early life stages, maternal allocation of this antioxidant to the eggs may be a strategy to minimize oxidative damage to developing embryos (Surai et al., 1996; Grether et al., 2001; Blount et al., 2002; Catoni et al., 2008; Costantini et al., 2010; Metcalfe and Monaghan, 2013; Costantini, 2014). Moreover, if vitamin E is a limiting resource, it may mediate a trade-off between allocation to maternal self-maintenance and to the eggs. It has been suggested that the allocation of vitamin E is a part of complex epigenetic 'maternal effects' playing an important role in the evolution of maternal reproductive strategies. Indeed, mothers may shape offspring phenotype and establish a hierarchy in the reproductive value of the progeny (Mousseau and Fox, 1998a), as suggested by the reduction of vitamin E concentration in egg yolk according to laying order (e.g. Rubolini et al., 2011).

Manipulative studies with dietary supplementation of vitamin E in young or adult birds have shown positive effects on hatching success (Muduuli et al., 1982), postnatal growth (de Ayala et al., 2006; Orledge et al., 2012; Parolini et al., 2015; see also Marri and Richner, 2014) and immune functions (e.g. Meydani and Hayek, 1995; Finch and Turner, 1996), as well as they elucidated its crucial role in the antioxidant defence (e.g. Giraudeau et al., 2013). On the contrary, deficiency of vitamin E has been found to reduce hatchability and growth, and to be associated with senescence and a large variety of diseases, which negatively affected the major body systems (Hvidsten and Herstad, 1973; Surai, 2002; Monaghan et al., 2009).

Steroid hormones

A large body of evidence has shown the existence of maternal plasticity in transferring hormones to the eggs according to diverse ecological factors, including interspecific competition, exposure to parasites, and male attractiveness and quality (e.g. Schwabl, 1997; Gil et al., 1999, 2006). Moreover, the transfer of hormones to the eggs can be affected by females' condition and strategically modulated

by mothers according to the position in the laying sequence, this pattern of variation can change across species (e.g. Groothuis et al., 2005; Rubolini et al., 2011).

Steroid hormones have both activational and organizational effects, acting as essential mediators of regulatory signals during embryo differentiation and are crucial for the development of physiological and behavioural traits, with 'pleiotropic' effects across multiple targets (Arnold, 2002; Groothuis et al., 2005). For example, androgens and in particular testosterone, are of pivotal importance to regulation of skeletal-muscular development, neural growth and are drivers of sexual phenotypic differentiation, thus participating in strategies of maternal sex allocation (e.g. Schlinger, 1997; Ketterson and Nolan, 1999; Navara and Mendonça, 2008; Adkins-Regan et al., 2013; Riedstra et al., 2013; Schweitzer et al., 2013). Moreover, a growing body of evidence is accumulating for the role of testosterone in sexual differentiation of several behavioural functions, suggesting its potential influence on the development of postnatal lateralization (e.g. Schwarz and Rogers, 1992; Rajendra and Rogers, 1993; Vallortigara et al., 1999; see review Pfannkuche et al., 2009). Finally, in some bird species, testosterone seems to directly induce oxidative stress in tissues and by upregulating the bioavailability of certain antioxidants (i.e. carotenoids) during adulthood (e.g. McGraw et al., 2006; Alonso-Alvarez et al., 2008) it can increase the susceptibility to oxidative stress (Alonso-Alvarez et al., 2008).

Ecological and evolutionary studies have focused the attention on the pattern of variation in testosterone deposition in relation to laying order, suggesting that its concentration can be strategically modulated by females (Groothuis et al., 2005; von Engelhardt and Groothuis, 2011). Because testosterone can promote growth, the differences in its concentration within a clutch may be interpreted in the context of sibling competition for food, which strongly depends on asynchronous hatching whose sequence is predicted by laying order. In the majority of bird species, the concentration of testosterone increases along the laying sequence, suggesting a maternal strategy to mitigate the disadvantage of being the last-hatched chick (Schwabl, 1993; Lipar et al., 1999). The opposite pattern of variation may favour the oldest chick when food is not sufficient for all hatchlings (Schwabl, 1997). However, high levels of testosterone in the offspring may entail a trade-off between immune system and growth. It may be speculated that mothers allocate large amounts of resources to boost growth of the last-hatched chick, which is encouraged in sibling competition but sacrifices the immune response and pays the cost of low survival rate (Eising and Groothuis, 2003; Eising et al., 2003; Metcalfe and Monaghan, 2003). Maternal testosterone appears to mediate trade-offs among offspring phenotypic traits but also between offspring fitness and maternal physiological conditions (Groothuis et al., 2005). On one hand, maternal testosterone transfer to the eggs can be an adaptation to increase offspring fitness, although it may involve direct or indirect costs (Groothuis et al., 2005).

On the other hand, deposition of high amount of testosterone into the eggs depends on high maternal circulating hormone levels, which have the potential to negatively interfere with the reproductive activities (Clotfelter et al., 2004; Ketterson et al., 2005). The optimal outcome of trade-off will be shaped by selection and may differ among individuals (Stearns, 1992).

Information from experimental studies, investigating whether variation in egg maternal compounds affected offspring phenotype, has indicated that androgens have short- and long-lasting effects. It has been observed that testosterone promoted somatic growth (e.g. Schwabl, 1996; Lipar and Ketterson, 2000), reduced oxidative damage during postnatal development (Noguera et al., 2011) and affected behavioural traits, reducing begging rate or increasing aggressiveness (Eising and Groothuis, 2003; Rubolini et al., 2006). However, the outcomes were inconsistent across species and phenotypic traits, probably due to ecological factors and the interaction with some other maternal compounds, as well as specie-specific trade-offs and differences in the sensitiveness to early androgen exposure (see Groothuis et al., 2005).

Corticosterone is another steroid hormone, contained in avian eggs, important for early developmental stages, although it received less attention than androgens. Corticosterone is the predominant adrenocorticoid hormone in birds, and it is secreted by the adrenal glands under stressful conditions by the stimulation of the hypothalamic-pituitary-adrenal axis (Wingfield and Romero, 2001; Henriksen et al., 2011; Costantini, 2014). Maternal corticosterone into the eggs can shape postnatal phenotype, including brain structure, cognitive and learning processes, physiological functions, and behavioural traits (Wingfield and Ramenofsky, 1997; Apanius, 1998; von Holst, 1998; Wingfield et al., 1998a,b; Sapolsky et al., 2000; Romero, 2004). This hormone is transferred by mothers to the egg yolk and albumen in amounts that can depend on females' quality and ecological conditions at the time of laying (Hayward and Wingfield, 2004; Saino et al., 2005; Love et al., 2008). Modulation of corticosterone, depending of environmental predictable cues experienced by mothers, may be an adaptive mechanism allowing offspring to better cope with stressful conditions that they will experience after hatching (Hayward and Wingfield, 2004; Groothuis et al., 2005). However, when a mismatch between pre- and postnatal environmental conditions occurs, the response can be inappropriate, leading to negative effects on offspring fitness (Henriksen et al., 2011). A rapid increase of corticosterone level, as a consequence of a stressful situation, is usually considered beneficial for the organisms but prolonged or high basal level may have negative effects (Cockrem, 2007). Indeed, experimentally elevated corticosterone level in the eggs has been found to reduce offspring growth (Eriksen et al., 2003; Saino et al., 2005; Janczak et al., 2006) and impair oxidative status promoting oxidative stress (Haussmann et al., 2012), although the outcomes often were inconsistent across species (Rubolini et al., 2005; Janczak et al., 2006; Davis et al., 2008; Love and

Williams, 2008; see review by Henriksen et al., 2011). Furthermore, high corticosterone concentration was observed to prevent the development of embryo visual asymmetry in response to light (Rogers and Deng, 2005), suggesting that corticosterone may have a pervasive effect on the development of lateralization in behavioural functions.

In conclusion, it appears that prenatal maternal effects mediated by egg quality represent a powerful tool through which mothers can adaptively shape offspring phenotype to increase their own fitness (Mousseau and Fox, 1998a,b), although in some cases maternal effects may be maladaptive due to ecological and physiological constraints to the expression of optimal maternal reproductive strategies.

Experimental method and focal endpoints

The present thesis aimed at investigating maternal effects mediated by egg quality on offspring phenotype in the yellow-legged gull (*Larus michahellis*). I focused on this gull as a model species because it is a very common bird with positive population trends and very large population size. The yellow-legged gull is a monogamous, mainly colonial, charadriiform bird, inhabiting the Mediterranean coasts. Body length is 52-58 cm with wingspan of 120-140 cm while body mass is about 1,010-1,390 g in males and 810-1,080 g in females. The breeding period usually starts in March and extends until the end of June. Females generally lay three eggs at 1-3 days interval, which decrease in size according to laying order and ranging in weight from 80 to 100 g. The incubation period lasts 27-32 days and hatching is asynchronous over 1-4 days. The chicks are semi-precocial, receive biparental care and fledge at 35-40 days of age (see Cramp, 1998).

As previously anticipated (see 'Maternal effects in bird'), avian eggs are a powerful tool to experimentally mimic variation of prenatal environment and thus to assess the mechanisms underlying maternal effects. The main literature concerning maternal effects in birds consists of experimental studies where maternal compounds were manipulated in laying females via food supplementation (for antioxidants; e.g. Surai et al., 2001; Blount et al., 2002) or thought subcutaneous implantation (for hormones; e.g. Love et al., 2005; Satterlee et al., 2007). However, these manipulative approaches are not immune from the potential indirect confounding effects of maternal physiology on egg quality that can affect offspring phenotype and are mediated by other aspects of mothers' physiology. Therefore, I relied on an experimental method by which I directly manipulated egg quality through the injection of a specific maternal compound into the yolk of unincubated eggs (Romano et al., 2008). Only few researches have adopted *in ovo* injection often increasing the concentration of the specific substance by administering a supra-physiological dose (e.g. Bhanja et al., 2012; Selim et al., 2012; Goel et al., 2013), leading to results that are not amenable to an ecological interpretation. Therefore, in designing experiments I paid special attention to increase the concentration of the specific maternal compound within the natural range of variation of the yolk concentration recorded for that focal substance in the same study colony (Rubolini et al., 2011). This crucial procedural device, along with the focus on a natural population, provides results that are suitable to an interpretation from an evolutionary perspective.

To assess maternal effects on offspring phenotype I manipulated the concentrations of an antioxidant (vitamin E) and two putative pro-oxidants (testosterone and corticosterone). I decided to focus on multiple phenotypic traits of gull embryos and hatchlings considering endpoints at different level of biological hierarchy. Firstly, I evaluated offspring oxidative status and its related oxidative damage.

It is widely known that organisms are exposed to oxidizing agents, originating from the environment and from their internal milieu (Halliwell and Gutteridge, 1999). The most sensitive early life stages, including embryonic development and the transition from chorioallantoic to pulmonary respiration, are characterized by rapid growth requiring intense metabolic processes and oxygen consumption, resulting in a massive production of oxidizing molecules (Surai, 2002). The overproduction of these radicals leads to the breakdown of the delicate equilibrium between the production of pro-oxidants and the complex of antioxidant defence in favour of the former (Finkel and Holbrook, 2000; Costantini, 2014). This imbalance determines an oxidative stress condition, often resulting in oxidative damage that can impair structures and functions of biological macromolecules like DNA, lipids or proteins with long-lasting, negative fitness consequences (Surai, 2002; Monaghan et al., 2009). Organisms thus have evolved a complex protection system, where the main actors are the maternally-allocated antioxidants operating in an integrated way, to prevent oxidative stress and damage. Therefore, I analysed the biochemical markers related to oxidative status like total non-enzymatic antioxidant capacity (TAC), total amount of pro-oxidant molecules (TOS), and lipid peroxidation and protein carbonylation as markers of oxidative damage.

Recently it has been shown that oxidative stress interferes with telomere dynamics (Boonekamp et al., 2014; Herborn et al., 2014). Telomeres are non-coding DNA sequences conserved along vertebrate lineages that cap the ends of chromosomes, which shorten at each cellular division, and thus protect genomic integrity (Blackburn, 2000). Evidence suggests that oxidative stress is involved in telomere attrition during early life, hastening cell senescence and leading to rapid telomere loss that negatively affect offspring phenotype (Boonekamp et al., 2014; Herborn et al., 2014). I thus tested whether variation in the concentration of the focal maternal substance increased or reduced oxidative stress and, consequently, hastened or decelerated telomere shortening in gull offspring.

Moreover, being maternal compounds crucial in promoting pre- and postnatal development, I also measured morphological traits, including body mass and tarsus length of offspring as proxies of somatic growth and body size.

Finally, a novel topic of this thesis was the focus on behavioural lateralization, which is rarely considered as an endpoint in the study of maternal effects. The focus of this novel topic stems from evidence showing the involvement of steroid hormones, especially androgens and corticosterone, in the development of brain asymmetry and functions and suggesting their putative role in the ontogeny of behavioural lateralization. I hence decided to assess the effects of maternal hormones on the direction (i.e. lateral preference) and the consistency (i.e. the extent to which an individual prefers either side or shows no specific lateral preference) of lateralization. I focused on three behavioural

tasks of functional importance to yellow-legged gull hatchlings: begging behaviour that is a key stimulus consisting in pecking at the red patch on the parental bill to solicit food provisioning, until parents regurgitate a food bolus; escape behaviour; the reverting to the prone position response.

Outline of the study

The present thesis deals with maternal effects mediated by egg quality in the yellow-legged gull (*Larus michahellis*). I mainly adopted a manipulative approach, whereby I experimentally increased, by an *in ovo* injection method, the concentration of a specific maternal compound within the physiological range of variation, in order to mimic large maternal transfer into the eggs. The experimental procedures entailed a within-clutch design, whereby both control (i.e. eggs injected with corn oil as a vehicle) and treated-eggs (i.e. eggs injected with the specific maternal compound) were established in the same clutch in order to minimize the consequences of both environmental and parental effects, because chicks from the same brood experience more similar micro-ecological conditions and interactions with adults compared to chicks from different broods. The strength of these studies lied in the possibility to operate in the wild under natural selection regime and to interpret the outcomes in an ecological and evolutionary perspective. The focal endpoints involved biochemical, morphological and behavioural traits of the progeny, which were assessed at different ontogenetic stages, including pre- and postnatal period.

Antioxidant-mediated maternal effects

This **first part**, including **Chapters 2** to **4**, concerns maternal effects mediated by the allocation of antioxidants to the eggs. Early life stages are characterized by an overproduction of oxidizing molecules stemming from the intense metabolic processes and oxygen consumption (Halliwell and Gutteridge, 2007); organisms have thus evolved an antioxidant defence system to efficiently counteract the detrimental effects of pro-oxidants. In birds, vitamin E is one of the most important maternal exogenous antioxidants that protects embryonic tissues and newly hatched chicks (Surai, 2002). I therefore analysed the consequences of elevated level of vitamin E on oxidative status, telomere length and morphological traits in both embryos and hatchlings (Chapters 2-3). In Chapter 4, I scrutinized the relationships occurring among different egg antioxidants, and the consequences of the experimental increase of vitamin E concentration on the other antioxidant distributions as well as their effects on embryo oxidative status and morphology.

The vast majority of the researches investigating vitamin E effects has been performed by maternal dietary supplementation or injecting eggs of captive species (e.g. Selim et al., 2012; Goel et al., 2013) whereas information on the consequences of egg quality manipulation under natural selection regime and on prenatal period is scant. For this reason, I assessed the effects of the supplementation of vitamin E on somatic growth and oxidative status of embryos shortly before hatching (**Chapter 2**) whereas on oxidative status and telomere length in newly hatched chicks (**Chapter 3**). I expected that

a physiological increase in yolk vitamin E promoted embryonic growth, positively affected oxidative status reducing oxidative damage, and resulted in longer hatchling telomere length. In addition, because a previous study has shown that maternal vitamin E was sub-optimal for the growth of chicks from the third-laid eggs, which have the smallest concentration of vitamin E in the yolk (Rubolini et al., 2011; Parolini et al., 2015), I predicted a more marked positive effect on embryos/chicks from last-laid eggs.

In the study presented in **Chapter 4**, I examined antioxidant defence in a broader perspective. Because the complex antioxidant system consists in two main mechanisms, namely enzymatic and non-enzymatic defences (Surai, 2002), which are thought to operate in an integrated way, it would be interesting to evaluate the functional relationships across different maternal antioxidants and their physiological pathways, which are rarely explored. Moreover, no study to date has tested the effects of experimental increase in yolk concentration of one antioxidant on the distribution of other antioxidants, and their additive or synergistic effects on embryonic phenotypic traits. Hence, to achieve these goals, I capitalized on a previous study (Chapter 2), considering the main antioxidant compounds stored in the egg yolk, embryo liver and brain. I thus focused on vitamin E (α- and γ-tocopherol and the corresponding tocotrienols), carotenoids, retinol, coenzyme Q10 (in the yolk only) and ascorbic acid (in the brain only). Aiming at determining the consequences on embryo phenotypic traits, I analysed oxidative status and damage in the liver and the brain, and mophology of the embryos. I expected that embryo growth was positively predicted by antioxidant concentrations and total antioxidant capacity (TAC), and negatively predicted by the total amount of pro-oxidant molecules (TOS) and by markers of oxidative damage. Moreover, based on the evidence showing that antioxidants are most often available in limiting amount in the diet, I also expected that antioxidant concentrations and oxidative status markers positively reciprocally correlated within and between organs (or the yolk). In addition, higher concentrations of antioxidants were expected to be associate with better oxidative status. However, because of limited information on the combined effects of different antioxidants on other embryo traits and their reciprocally interactions, I had no *a priori* expectation on synergistic effects of antioxidants and of experimental vitamin E supplementation.

Hormone-mediated maternal effects

This **second part** of the thesis, including **Chapters 5** to **8**, deals with maternal effects mediated by egg steroid hormones, especially testosterone and corticosterone, in the yellow-legged gull. As for antioxidants, hormone deposition to the eggs can be influenced by maternal and environmental conditions during the breeding period (e.g. Gil et al., 1999; 2006, Marshall and Uller, 2007), as well as vary within clutches in relation to the laying sequence (e.g. Schwabl, 1997;Groothuis et al., 2005; Rubolini et al., 2011), corroborating the idea that maternal allocation strategies exist. High amounts of maternal hormones into the eggs can lead to maladaptive effects on offspring phenotype, including an increase in susceptibility to oxidative stress, costs for growth and alterations of postnatal behaviour (e.g. Rubolini et al., 2006; Groothuis et al., 2006; Alonso-Alvarez et al., 2008), although these effects can vary among species and in relation to ontogenetic stages. To explore any alterations due to an increase in yolk hormone levels on offspring phenotype, I manipulated the concentration of testosterone and corticosterone into the eggs. The effects of testosterone were assessed on oxidative status and morphological traits of embryos (**Chapter 5**), and on morphology and lateralization of hatchlings (**Chapter 6**), whereas the effect of corticosterone supplementation was analysed on behavioural lateralization of newly hatched chicks (**Chapter 7**). While in the previous studies I relied on injection method, which mimic variation in prenatal egg milieu, in the work of **Chapter 8** I experimentally increased predation risk perceived by the breeding adults and I investigated non-consumptive effects of predation in terms of variation in egg biochemical composition and clutch and egg size.

Despite previous researches have disclosed that testosterone affected postnatal growth (e.g. Navara et al., 2005; Podlas et al., 2013) and the expression of several behavioural traits (e.g. Eising and Groothuis, 2003; Rubolini et al., 2006), information on oxidative status (e.g. Noguera et al., 2011; Treidel et al., 2013) or on prenatal effects (Hegyi and Schwabl, 2010; Muriel et al., 2013) is scant. Moreover, these studies have provided results that can vary in intensity, in age-dependent or sex-dependent ways and were inconsistent on different phenotypic traits. Therefore, the study presented in **Chapter 5** aimed at scrutinizing morphology and oxidative status of embryos in order to fill the gap of knowledge on the effects of testosterone on prenatal development and elucidate its effect on physiology. Because of the heterogeneity of testosterone effects and of very few studies on pre-hatching stages, I had no directional predictions on the outcomes of testosterone manipulation on both morphology and oxidative status.

As a related work, the study in **Chapter 6** focused on the consequences of testosterone injection on morphology and behavioural lateralization of hatchlings. The broad interest on the potential

organizational and activational effects of hormones on brain structure and functions (e.g. Groothuis et al., 2005; Adkins-Regan, 2007; Rogers, 2008) and the observation of sex-dependent lateralization in diverse functions lead to hypothesize the involvement of testosterone in lateralization development (e.g. Schwarz and Rogers, 1992; Rajendra and Rogers, 1993; and see Pfannkuche et al., 2009). I thus tested this hypothesis on begging and escape behaviours, analysing the direction (i.e. lateral preference) and the consistency (i.e. the extent to which an individual prefers either side or shows no specific lateral preference) of chicks. The consistency of lateralization was considered because testosterone is known to have positive effect on attention during stimulation, possibly increasing consistency of behavioural lateralization (Andrew, 1972; Andrew and Rogers, 1972; Klein and Andrew, 1986; Andrew and Jones, 1992).

Similarly, corticosterone is believed to have a pervasive effect on brain structure and functions, suggesting that it may influence post-hatching lateralization. Very few studies have focused on the consequences of elevated corticosterone concentration on lateralization during prenatal stage (Rogers and Deng, 2005; Freire et al., 2006), showing an impairment of visual asymmetry development (Rogers and Deng, 2005) with a reduction of the ability of chicks to perform more than one task simultaneously (Rogers et al., 2004; Dharmaretnam and Rogers, 2005; Freire et al., 2006). However, despite the potential role of corticosterone in the development of visual asymmetry and the related functions, its effect on the direction of lateralization has not been tested directly. Hence, in the study in **Chapter 7** I analyzed the direction of lateralization of hatchlings focusing on two behavioural tasks: begging behaviour and the reverting to prone position response.

The study in **Chapter 8** can be considered as an 'outlier' in this study of maternal effects. I analysed non-consumptive effects of predation on egg composition and on clutch and egg size. Since maternal hormone transfer can be influenced by ecological influences experienced by mothers, I manipulated environmental conditions experimentally increasing predation risk. To achieve this goal I presented stuffed predators (fox, *Vulpes vulpes*, and buzzard, *Buteo buteo*) to breeding adults before egg laying. I then assessed the variation in testosterone and corticosterone concentrations in egg yolk and in clutch and egg size. Experimental studies of non-consumptive effects of predation on egg biochemical composition are rare (Cockrem and Silverin, 2002; Saino et al., 2005; Coslovsky and Richner, 2011; Pitk et al., 2012), whereas the studies on clutch and egg size showed negative effects, although with some exceptions depending on the focal species (Slagsvold ,1984; Safriel, 1975; Martin, 1995; Doligez and Clobert, 2003; Eggers et al., 2006; Fontaine and Martin, 2006; Massaro et al., 2008; Cassey et al., 2010; Lima, 2009; Coslovsky and Richner, 2011). Therefore, I expected an increase in corticosterone concentration as an inevitable consequence of environmental factors on maternal physiology and/or of an active modulation of maternal corticosterone in the eggs. However, I had no

directional prediction on the effect on testosterone concentration because of the lack of theoretical background on the effect of predation on maternal transfer of androgens. According to life-history theory and evidence from previous experimental studies on different species, a reduction in clutch size was expected. Finally, the predictions on the effect of predators on egg mass were ancipital. On one hand, an increase in egg size can be expected because large eggs, containing large reserves, can promote postnatal development and reduce food solicitation activity by the hatchlings to the attending parents. On the other hand, a reduction in egg size can be expected if exposure to predators caused a decline in reproductive value of the offspring and/or impaired egg laying performance via a negative effect on maternal physiology.

To conclude, in **Chapter 9** I presented an overview of the main findings emerging from the studies introduced in the earlier chapters, together with concluding remarks.

PART I

ANTIOXIDANT-MEDIATED MATERNAL EFFECTS

Chapter 2

Yolk vitamin E positively affects prenatal growth but not oxidative status in yellow-legged gull embryos

Current Zoology (2017): 1-8

Current Zoology, 2017, 1–8
doi: 10.1093/cz/zox037
Advance Access Publication Date: 23 May 2017
Accepted Article

OXFORD

Accepted Article

Yolk vitamin E positively affects prenatal growth but not oxidative status in yellow-legged gull embryos

Marco Parolini[a,*], Cristina Daniela Possenti[a], Filiz Karadas[b], Graziano Colombo[c], Maria Romano[a], Manuela Caprioli[a], Isabella Dalle-Donne[c], Diego Rubolini[a], Aldo Milzani[c], and Nicola Saino[a]

[a]Department of Environmental Science and Policy, University of Milan, via Celoria 26, 20133 Milano, Italy, [b]Department of Animal Science, University of Yüzüncü Yil, 65090 Van, Turkey and [c]Department of Biosciences, University of Milan, via Celoria 26, 20133 Milan, Italy

*Address correspondence to Marco Parolini. E-mail: marco.parolini@unimi.it

Received on 10 December 2016; accepted on 12 May 2017

Abstract

Parental effects occur whenever the phenotype of parents or the environment that they experience influences the phenotype and fitness of their offspring. In birds, parental effects are often mediated by the size and biochemical quality of the eggs in terms of maternally transferred components. Exogenous antioxidants are key egg components that accomplish crucial physiological functions during early life. Among these, vitamin E plays a vital role during prenatal development when the intense metabolism accompanying rapid embryo growth results in overproduction of pro-oxidant molecules. Studies of captive birds have demonstrated the positive effect of vitamin E supplementation on diverse phenotypic traits of hatchling and adult individuals, but its effects on embryo phenotype has never been investigated neither in captivity nor under a natural selection regime. In the present study, we experimentally tested the effect of the *in ovo* supplementation of vitamin E on morphological traits and oxidative status of yellow-legged gull (*Larus michahellis*) embryos. The supplementation of vitamin E promoted somatic growth in embryos soon before hatching, but did not affect their oxidative status. Our results suggest that maternally transferred vitamin E concentrations are optimized to prevent imbalances of oxidative status and the consequent raise of oxidative damage in yellow-legged gull embryos during prenatal development.

Key words: *Larus michahellis*, maternal effects, morphological traits, oxidative status, prenatal period, vitamin E.

Parents can maximize their Darwinian fitness by modulating the allocation of care to individual offspring according to their reproductive value. In oviparous organisms, mothers can adjust offspring phenotype via the modulation of the size and biochemical quality of their eggs, which can widely vary not only among mothers but also among sibling eggs (Mousseau and Fox 1998; Saino et al. 2002; Groothuis et al. 2005). In fact, the size and the concentration of quantitatively major (e.g., lipids, albumen) and minor (e.g., steroid hormones, vitamins) maternally transferred components often vary within-clutch according to laying order (Royle et al. 2001; Badyaev et al. 2006; Groothuis et al. 2006; Rubolini et al. 2011; von Engelhardt and Groothuis 2011). Mothers may adaptively tune their investment, including prenatal maternal effects *via* eggs to individual offspring.

Exogenous antioxidants (e.g., vitamins and carotenoids) are acquired with food, and may be available in limiting amounts, implying that mothers may tune the amount of antioxidants they transfer to their eggs according to the reproductive value of their individual offspring as determined, for example, by hatching order (Grether et al. 2001; Catoni et al. 2008). The existence of reproductive trade-offs and the major role that antioxidants have in early-life

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offspring physiology have attracted considerable attention to the study of ecology and evolution of maternal effects mediated by egg antioxidants. Egg antioxidants of maternal origin provide protection to the developing embryo against the detrimental effects of free radicals produced during early-life growth (Surai et al. 1996). Low levels of maternally transferred yolk antioxidants impair embryonic development (Wilson 1997), suggesting that they play a crucial role in counteracting oxidative stress (Surai and Speake 1998; Blount et al. 2000; McGraw et al. 2005). In fact, intense metabolic activity during early developmental stages exposes the organism to oxidative stress, resulting from the breakdown of the equilibrium between the production of pro-oxidants (reactive oxygen and nitrogen species, ROS and RNS, respectively), and antioxidant defense and repair mechanisms in favor of the former (Finkel and Holbrook 2000). The prenatal period is crucial to redox homeostasis because high metabolic rates during rapid growth stages can induce ROS overproduction (Rollo 2002), leading to oxidative damage to cellular macromolecules (i.e., DNA, lipids, and proteins) and providing a potential mechanism for negative effects on fitness-related traits (Costantini 2014). Because of the adverse consequences of oxidative stress on phenotype, selection is expected to favor the evolution of mechanisms for antioxidant defense and repair of oxidative damage (Costantini et al. 2010; Metcalfe and Alonso-Alvarez 2010; Isaksson et al. 2011; Metcalfe and Monaghan 2013; Costantini 2014). Variation in oxidative stress (Monaghan et al. 2009, Metcalfe and Alonso-Alvarez 2010) and in maternal transfer of antioxidants depending on environmental conditions experienced by the mother (Blount et al. 2002; Royle et al. 2003) suggests that the response to oxidative stress may be modulated by maternal effects. Therefore, maternal allocation of exogenous antioxidants to egg yolk may constitute a strategy to minimize oxidative damage to developing embryos (Blount et al. 2002).

In birds, vitamin E is one of the most important yolk antioxidants (Surai et al. 2016). Vitamin E is transported from the yolk to the embryonic tissues during development (Surai et al. 1996; Cherian and Sim 2003) and protects embryos against the toxicity of free radicals (Khan et al. 2011). Vitamin E acts as chain-breaking lipid antioxidant and free radical scavenger in the membranes of cells and subcellular organelles (Young et al. 2003), maintaining the integrity and functioning of the reproductive, muscular, circulatory, nervous, and immune systems of vertebrates (Leshchinsky and Klasing 2001). The effects of egg vitamin E have been mostly investigated by means of maternal dietary supplementation in captivity. These studies have shown that vitamin E supplementation positively affects growth, immune function, performance, and antioxidant capacity of poultry (Gore and Qureshi 1997; Surai et al. 2001; Bhanja et al. 2012; Selim et al. 2012; Goel et al. 2013), as well as the transcription and the expression of specific genes involved in diverse metabolic pathways (Surai 2002). Experiments in captivity where egg vitamin E has been manipulated by injection have partly clarified its direct effects on offspring phenotype. Direct manipulation of yolk vitamin E levels improved hatchability, immune status, and both embryonic and post-hatch growth of Muscovy ducks *Cairina moschata* (Selim et al. 2012), and reduced the production of ROS in tissues of hen chicks (Cherian and Sim 1997; Surai et al. 1999a). Although these experiments are valuable to identify the effects and mechanisms behind the allocation of antioxidants to eggs, the most insightful perspective for the interpretation of the evolution of maternal effects rests on the experimental analysis of the consequences of egg quality manipulation under a natural selection regime in the wild. However, information on yolk vitamin E effects derived from yolk manipulation in free-ranging populations under natural selection regimes is scanty and to date no study has investigated the effects on embryonic growth or oxidative status in important organs that are likely to be the target of the antioxidant activity of vitamin E.

In a recent study, we have shown that a physiological increase of vitamin E concentration in yellow-legged gull *Larus michahellis* eggs enhanced postnatal body size of chicks from the last-laid eggs in a clutch (Parolini et al. 2015). However, information on the effects of vitamin E during the prenatal period in free-living species is largely unavailable. For this reason, here we investigate the effect of a physiological increase in yolk vitamin E concentration on phenotypic traits of embryos shortly before hatching. We expected that the supplementation of vitamin E would promote growth, positively affect oxidative status, and reduce embryo oxidative damage. In addition, because vitamin E concentration declines with laying order (Rubolini et al. 2011) and in our previous study we showed that it limits postnatal growth of chicks from third-laid eggs, we expected a decrease of pro-oxidant molecules accompanied by an increase of total antioxidant capacity (TAC) mainly in embryos from last-laid vitamin E-injected eggs. Lastly, although the concentration of vitamin E in the yolks of yellow-legged gull eggs does not vary according to the sex of developing embryos (Rubolini et al. 2011), we also tested if the effect of egg treatment depended on the sex of the embryo because embryos of either sex may differ in their susceptibility to yolk antioxidants (Romano et al. 2008). Thus, we studied the effects of vitamin E on embryo morphology (body mass and tarsus length) and oxidative status by measuring TAC, amount of pro-oxidant molecules (called as 'TOS' 128 according to the terminology by Erel 2005) lipid peroxidation (LPO) and protein carbonylation (PCO) in brain and liver explanted from the embryos. We focused on brain for 3 reasons; it is particularly sensitive to LPO because the phospholipids of the neuronal membranes contain large amounts of highly polyunsaturated fatty acids, it generates free radicals at a greater extent than other tissues as a consequence of high rates of energy metabolism and oxygen consumption, and the amount of many exogenous antioxidants is lower compared to other tissues (Surai et al. 1999b). Liver was chosen because it is the main repository of antioxidants, including vitamin E (Surai 2002), and it has a crucial role in antioxidant defense.

Materials and Methods

Field and experimental procedures

The yellow-legged gull is a monogamous species that breeds mostly colonially (Cramp 1998). Clutch size ranges between 1 and 3 eggs (modal size = 3), which are laid at 1–4 (most frequently 2) days intervals and hatch 27–31 days after laying. Hatching is asynchronous and spans over 1–4 days. The chicks are semi-precocial and are fed by both parents and fledge at 35–40 days of age (Cramp 1998). We studied a large colony (>400 pairs) breeding on an island in the Comacchio lagoon (NE Italy, 44°20′ N–12°11′ E) in March–May 2014. The colony was monitored every other day and when a new nest was found the newly laid egg was temporarily removed and replaced with an egg collected from a nest outside of the study colony (i.e., "dummy" egg) to avoid interference with parental incubation behavior. Nests that were found with more than 1 egg were considered, but egg order was estimated based on previously described differences in egg mass for the species. The removed egg was marked and taken to a nearby tent for experimental manipulation.

The experimental design has been described in details by Parolini et al. (2015) and in the Supplementary material, therefore it is only

briefly summarized here. Our objective was to increase the concentration of vitamin E by 1 standard deviation (SD) of the concentrations measured in the egg yolk of individuals from the same colony in a previous study (Rubolini et al. 2011). Since the concentration of vitamin E in the yolk of yellow-legged gull eggs varied according to egg size and position in the laying sequence (Rubolini et al. 2011), we adjusted the injection dose according to these factors. We estimated yolk mass based on total egg mass for each of the eggs in laying sequence based on a Linear Mixed Model from previously collected yellow-legged gull eggs (yolk mass = 0.227 (0.039 SE) egg mass + 1.815 (3.461 SE); $F_{1,88} = 34.38$, $P < 0.001$). Then, we grouped first (a-), second (b-), or third (c-) laid eggs into 3 classes (tertiles) of size according to egg mass and calculated the standard deviation of vitamin E concentration in the yolk for each tertile. The injection amount of vitamin E was computed as the product of the SD (in μg g^{-1}) of vitamin E concentration for each tertile and position in the laying sequence and the estimated yolk mass (see Supplementary material). Corn oil was used as the carrier solvent of vitamin E and it was used as a control treatment in the control group of eggs. We adopted a within-clutch design, whereby both sham-(control) and vitamin E-injected eggs were established within each clutch to minimize the confounding effects of environmental and parental effects. The following treatment schemes were assigned sequentially to the clutches as follows: (nest, a-, b-, c-egg): nest 1, vitamin E injection (E), control injection (C), E; nest 2, C-E-C; nest 3, E-C-C; nest 4, C-E-E and so forth with the following nests. The injection procedure was performed according to a previously validated method on eggs from the same species (Romano et al. 2008).

After the *in ovo* vitamin E supplementation and 5 days before the earliest expected hatching date, all the nests were visited daily to check for any sign of imminent hatching such as eggshell fractures (i.e., "cracking stage"). When eggshells were fractured, eggs were weighed (to the nearest g), collected and frozen at −20 °C within 3 h from sampling.

Field collected eggs ($n = 76$ eggs) were transferred to the lab where they were dissected. We focused on 26 clutches, 15 of which had 3 eggs, while the remaining 11 clutches had 2 eggs only. We first removed and weighed the residual yolk sac from each egg, which was frozen at −80 °C until the analysis of total vitamin E concentration and TAC that we performed as a validation of the experimental treatment. We expected that vitamin E injection would result in a measurable increase in the yolk concentrations into late developmental stages, as well as in an increase in TAC. Then, the embryos were weighed (to the nearest g) and tarsus length was measured by calipers before the dissection of liver and brain, which were immediately weighed (to the nearest mg) and frozen at −80 °C until biochemical analyses. All the measurements were taken by the same person to ensure consistency. Molecular sexing of embryos and chicks was performed according to Saino et al. (2008).

The study was carried out under permission of the Parco Regionale del Delta del Po (#657, 4 February 2014), which allowed both the manipulation and the collection of eggs when the eggshell showed signs of imminent hatch (eggshell fractures). According to the Guidelines for the Euthanasia of Animals by the American Veterinary Medical Association, physical methods of euthanasia may be necessary in some field situations if other methods are impractical or impossible to implement. We performed a field experiment in which we could not euthanize embryos by methods such as carbon dioxide (CO_2), anesthetic agents, or decapitation. Thus, we euthanized embryos by placing eggs into a −20 °C freezer within 3 h from the collection.

Analysis of vitamin E content in residual yolk sac, brain, and liver of embryos

The concentration of vitamin E in residual yolk sac, brain, and liver from embryos was determined according to Karadas et al. (2006) using a high-performance liquid chromatography system (Shimadzu Liquid Chromatography, LC-10AD, Japan Spectroscopic Co. Ltd.). Briefly, 100–150 mg of yolk and organs were homogenized with 1 mL of ethanol plus 0.7 mL NaCl 5% and extracted twice by centrifugation with 2 mL of hexane each. Then, hexane extracts were pooled and evaporated at 60–65 °C under nitrogen flow and the residual was dissolved in 500 μL of a dichloromethane:methanol mixture (50:50 v/v). Vitamin E (α-, and γ-tocopherol) concentrations were detected with a Hypersil GOLD type 3 μm C18 reverse-phase column (150 × 4.6 mm Phase Separation, Thermo Fisher Scientific 81, Wyman, Street Waltham, MA USA) with a mobile phase of methanol:distilled water (97:3 v/v) at a flow rate of 1.05 mL min^{-1} using fluorescence detection by excitation and emission wavelength of 295 nm and 330 nm, respectively. Peaks of α-, and γ-tocopherol were identified and quantified by comparison with the retention time of standards of tocopherols at renown concentration (Sigma, Poole, UK). According to Karadas et al. (2006), standard solutions α-tocopherol in methanol were used for instrument calibration, while tocol was used as an internal standard to check for the reliability of analytical process.

Oxidative stress methods

TAC, TOS, PCO and LPO were measured in liver and brain homogenates. In addition, TAC was also measured in the residual yolk from sampled eggs.

An appropriate amount of yolk (∼0.15 g), brain, and liver (∼0.1 g) was homogenized in 100 mM phosphate buffer pH 7.4, with 1 mM EDTA and 100 mM KCl, by an automatic homogenizer. After 10 min centrifugation at 13,000 rpm, an aliquot of the supernatant was immediately processed for the determination of protein content according to the Bradford method (Bradford 1976) using bovine serum albumin (BSA) as a standard, while the remainder was used for oxidative stress assays. A detailed description of applied methods is reported in Supplementary material. Briefly, TAC was measured according to a colorimetric method based on the discoloration of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{*+}$), adapted from Erel (2004). TOS was measured according to a colorimetric method developed by Erel (2005), adapted to tissue homogenates. Carbonylated proteins were measured with 2,4-dinitrophenylhydrazine (DNPH). Protein carbonylation (PCO) was measured by Western immunoblotting and immunostained protein bands were visualized with enhanced chemiluminescence detection. Carbonylated proteins were quantified by densitometric analysis using Image J 1.40d software (Schneider et al. 2012). LPO was measured according to the thiobarbituric acid reactive substances (TBARS) method (Ohkawa et al. 1979), adapted to tissue homogenates of embryos and were expressed as nmol TBARS g^{-1} wet weight.

Statistical analyses

The effect of vitamin E treatment on its concentration in residual yolk sac and embryo focal organs, embryo morphological traits and oxidative status markers, was analyzed in Linear Mixed Models (LMM; Normal as the distribution and Identity as the link function), including clutch identity as a random intercept effect. Egg mass at the time of laying was included as a covariate in all the models.

Egg treatment (vitamin E versus sham-injection), embryo sex, and egg-laying order were included as fixed-effect factors along with their two-way interactions. All non-significant ($P > 0.05$) interaction terms were removed from the model in a single step. In all the models, the effect of clutch identity was tested by a likelihood ratio test, by comparing the log-likelihood value of the model including or excluding the random effect of clutch identity. Mixed models with the same design, but assuming a binomial error distribution, were run to investigate the effects of vitamin E treatment on the proportion of eggs that reached the "cracking stage", as well as on the sex ratio of embryos. A single embryo could not be dissected because of sample deterioration. All the statistical analyses were performed by using SAS 9.3 PROC MIXED and PROC GLIMMIX. Group statistics are presented as estimated marginal means (± SE).

Results

Vitamin E concentration in residual yolk sac, embryo brain, and liver

To assess the reliability of the injection procedure in causing an increase in vitamin E yolk concentration, and consequently on yolk TAC, we first analyzed whether the concentration of vitamin E and TAC in the residual yolk sac differed between sham- and vitamin E-injected eggs. We used the yolk sac samples from 66 embryos ($n = 26$ nests). As expected, vitamin E concentration was significantly higher in vitamin E treated eggs compared to controls ($F_{1,44.5} = 4.314$; $P = 0.044$) (Figure 1A). Neither sex ($F_{1,38} = 1.162$; $P = 0.286$) nor laying sequence ($F_{1,36} = 0.841$; $P = 0.438$) affected yolk sac vitamin E concentrations. In addition, we estimated the total amount of vitamin E in the yolk as the product of vitamin E concentration (expressed in µg/g) and the yolk mass estimated according to the relationship described above (see Materials and Methods Section). The total amount of yolk vitamin E was significantly higher in vitamin E treated eggs compared to controls ($F_{1,44.3} = 4.623$; $P = 0.037$), while neither sex ($F_{1,50.7} = 0.905$; $P = 0.346$) nor laying sequence ($F_{1,48.7} = 0.899$; $P = 0.413$) affected mass of vitamin E in the yolk. Accordingly, vitamin E supplementation caused a significant increase of TAC in residual yolk sac (Figure 1B; $F_{1,36} = 4.298$; $P = 0.045$), while no significant effect of embryo sex ($F_{1,38} = 0.01$; $P = 0.929$) or laying order ($F_{1,36} = 1.25$; $P = 0.298$) was found. Since vitamin E is efficiently transferred from yolk to developing embryos, we also measured its concentration in brain and liver. The concentrations of vitamin E measured in focal organs from vitamin E-treated embryos soon before hatching were not significantly higher than controls in the brain ($F_{1,59} = 1.707$; $P = 0.196$) or in the liver ($F_{1,35} = 0.305$; $P = 0.584$), and did not vary according to sex (brain: $F_{1,59} = 0.083$; $P = 0.775$; liver: $F_{1,38} = 0.107$; $P = 0.746$ for liver), laying order (brain: $F_{1,35} = 0.066$; $P = 0.936$; liver: $F_{2,42} = 2.561$; $P = 0.089$) or their interactions (all $P > 0.05$), which were removed from the LMM.

Effect of vitamin E on embryo morphology and oxidative status

The sample included 26 clutches, 15 of which had 3 eggs while the remaining 11 clutches had 2 eggs. The proportion of eggs that reached the cracking stage did not differ significantly between the control (proportion of eggs at cracking = 30/66 = 0.455; 95% confidence interval = 0.335–0.575) and the vitamin E-injected eggs (36/66 = 0.545; 95% confidence interval = 0.424–0.575; $\chi^2_1 = 0.76$, $P = 0.384$). In a LMM where clutch identity was included as a random effect, egg mass did not differ between the experimental groups ($F_{1,36} = 0.40$, $P = 0.530$) but significantly declined with laying order ($F_{2,36} = 36.41$, $P < 0.001$; estimated marginal means (SE): a-eggs: 91.8 (1.25); b-eggs: 90.1 (1.21); c-eggs: 83.8 (1.28)). The sex ratio (proportion of males) did not differ significantly between the experimental groups (controls: 10/30 = 0.333; 95% confidence interval = 0.164–0.502 and vitamin E: 19/36 = 0.528; 95% confidence interval = 0.365–0.691; $\chi^2_1 = 1.78$, $P = 0.182$).

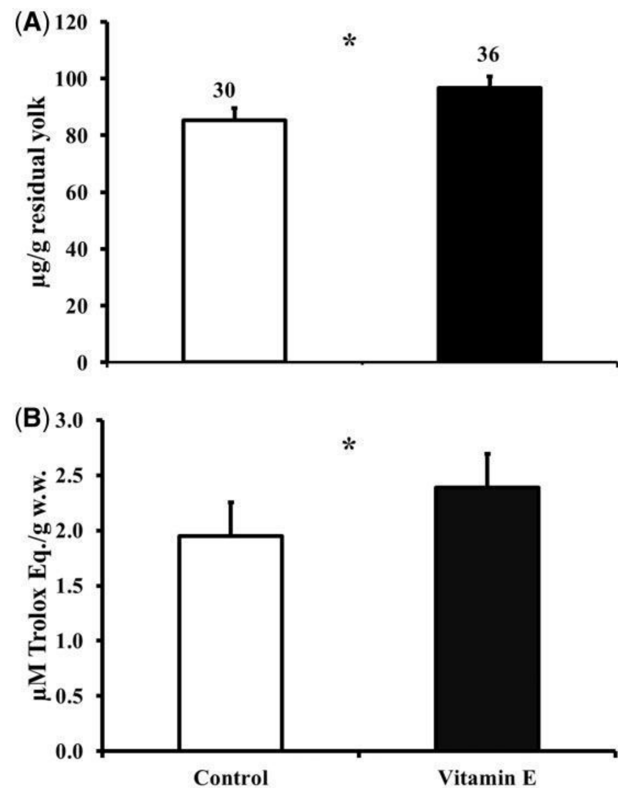

Figure 1. Marginal means (+ SE) of (A) concentration of total vitamin E (µg g^{-1} wet weight) and (B) total antioxidant capacity (TAC - µM Trolox Eq. g^{-1} wet weight) in the residual yolk sac from the embryos at the cracking stage. Sample sizes are reported. Significant differences between vitamin E and control embryos are indicated by the asterisk (*$P < 0.05$).

A LMM of embryo body mass showed no significant effect of the interactions among fixed-effect factors (Table 1; Figure 2). The reduced model, retaining the main effects of sex, treatment and laying order, showed a statistically significant difference in body mass of embryos between the experimental groups ($F_{1,38} = 4.19$, $P = 0.048$; control embryos: 43.2 (1.03) and vitamin E embryos 45.0 (0.99)). There was large among-clutch variation in body mass (Likelihood ratio test; $\chi^2_1 = 21.00$, $P < 0.001$). No significant effect on tarsus length was found (Table 1). LMM of embryo liver and brain mass revealed no significant effect of vitamin E treatment (liver: $F_{1,38} = 0.17$; $P = 0.678$; brain: $F_{1,39} = 0.08$; $P = 0.784$).

No significant effect of vitamin E treatment, embryo sex, laying order, and their interactions was found for all the considered oxidative stress endpoints in both the target organs, with the exception for a significant effect of laying order on TOS in the liver, and of sex on TOS in the brain (Table 2).

Discussion

We experimentally increased vitamin E concentrations within physiological limits in yolks of yellow-legged gull eggs and found

Table 1. LMM of morphological traits of embryos at the cracking stage in relation to vitamin E treatment, sex of the embryo, and laying order. Clutch identity was included in the model as a random intercept effect. We controlled for egg mass at the time of laying by including it as covariate in the models. The non-significant effects of the 2-way interactions were excluded from the final model. Significant effects are reported in bold

Morphological traits	Body mass			Tarsus length		
	F	*df*	*P*	*F*	*df*	*P*
Final model						
Treatment	4.19	1, 38	**0.048**	1.54	1, 38	0.222
Sex	0.34	1, 41	0.565	0.64	1, 42	0.430
Laying order	1.68	2, 47	0.198	2.01	2, 46	0.145
Excluded terms						
Treatment × sex	0.08	1, 48	0.777	0.37	1, 47	0.545
Treatment × laying order	1.14	2, 51	0.329	0.49	2, 50	0.613
Sex × laying order	2.83	2, 49	0.069	1.61	2, 50	0.210

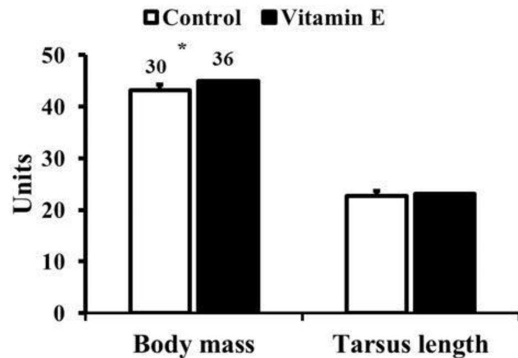

Figure 2. Marginal means (+ SE) of body mass (g) and tarsus length (mm) at the cracking stage of embryos from control or vitamin E injected eggs. Sample sizes are reported. Significant differences between vitamin E and control embryos are indicated by the asterisk (*$P < 0.05$).

that the supplementation of this exogenous antioxidant promoted growth of embryos at late prenatal stages, while it did not affect oxidative status of their brains or livers.

A number of studies of captive and free-living birds have shown that vitamin E supplementation via the maternal diet increased the concentration of this antioxidant in the egg yolk and in embryonic tissues, promoting somatic growth at hatching (Surai et al. 1999b; Larcombe et al. 2010; Noguera et al. 2011; Surai and Fisinin 2013; Surai et al. 2016). However, these studies manipulated vitamin E availability to mothers and tested the effect of dietary vitamin E supplementation on the offspring. This approach integrates information on the direct effect of maternal vitamin E on progeny with indirect effects mediated by the consequences of increased availability of dietary vitamin E on maternal physiology. In contrast, our *in ovo* injection approach reveals the direct effects of vitamin E on the offspring, independently of maternal physiology (Surai et al. 1998; Blount 2004). In addition, several experiments (e.g., Cherian and Sim 1997; Surai et al. 1999a; Selim et al. 2012; Goel et al. 2013), mainly in captivity, applied supra-physiological vitamin E doses, hampering the ecological and evolutionary interpretation of maternal effects mediated by egg vitamin E content. In designing our experiment we, therefore, paid special attention to scale the injection amount of vitamin E to natural variation, as well as according to estimated yolk size and to position in the laying sequence. Thus, we are confident that our vitamin E supplementation caused a post-manipulation concentration that did not exceed the upper limit of the natural range of variation, at least in the vast majority of the eggs.

The injection of a physiological dose of vitamin E into the yolk caused an increase in embryo body mass around hatching, independently of egg laying order and mass of the original egg. Since the concentration of vitamin E in yellow-legged gull eggs from the same colony declines with laying order, showing a 1.6-fold difference between the second- and third-laid eggs (Rubolini et al. 2011), a more marked positive effect of vitamin E supplementation on somatic growth of embryos from third-laid eggs was expected. In fact, previous evidence showed that chicks hatched from third-laid eggs injected with vitamin E were heavier and had significantly longer tarsi than controls, whereas vitamin E treatment had no effect on the size of chicks from first- or second-laid eggs (Parolini et al. 2015). These positive effects on morphological traits of chicks from the third-laid eggs suggest that the concentration of vitamin E in first- and second-laid eggs at hatchling is close to optimal, whereas in the third-laid eggs is sub-optimal. In contrast, the results from embryos suggest that during pre-hatching development the concentration of vitamin E in the yolk might be sub-optimal for somatic growth, and the administration of an additional dose is beneficial to growth independent of position in the laying sequence.

From a functional perspective, maternal allocation of vitamin E to the eggs may serve to increase body size in late prenatal stages and to enhance post-hatching growth. Yet, the mechanisms underlying the positive effect of vitamin E supplementation on embryo (and chick) body size remains to be elucidated. Although no information is available for embryos of any bird species, vitamin E may increase the efficiency of conversion of egg materials into somatic tissues, as suggested for commercial Muscovy ducks during the first 2 weeks after hatching (Selim et al. 2012). Alternatively, vitamin E supplementation may reduce the production of pro-oxidant molecules, preventing oxidative stress. During early developmental stages, embryos and chicks are particularly prone to suffering oxidative stress because of high metabolic rates and the onset of aerobic respiration at hatching, implying that they need efficient antioxidant protection particularly during the late embryo and the early post-hatching stages (Panda and Cherian 2014). The overproduction of pro-oxidants and the consequent oxidative imbalance should be detrimental to developmental and growth processes (Smith et al. 2016). The latter hypothesis is supported by a number of studies showing that vitamin E supplementation improves antioxidant defense increasing superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity, preventing negative effects of LPO in broiler chicks (Sodhi et al. 2008; Tsai et al. 2008). Since oxidative stress has been suggested to limit growth rates (Alonso-Alvarez et al. 2007), enhanced body mass in vitamin E-treated embryos (and hatchlings) likely reflects the antioxidant properties of tocopherols (Marri and Richner 2015).Thus, vitamin E may protect lipid membranes from the harmful effects of ROS, allowing increased lipid utilization for energy production (Schaal 2008) to be used in somatic growth. However, the present results on oxidative status markers do not support this interpretation. While TAC was found to be larger in residual yolk from vitamin E-injected eggs (Figure 1B), TAC, TOS, and oxidative damage to lipids and proteins in the brain and in the liver were not affected by vitamin E supplementation (Table 2). The lack of significant effects on oxidative status markers may depend on the amount of residual yolk in eggs at the cracking stage. Thus,

Table 2. LMM of oxidative status markers in the liver and brain of embryos at the cracking stage in relation to vitamin E treatment, sex of the embryo, and laying order. Clutch identity was included in the model as a random intercept effect. We controlled for egg mass at the time of laying by including it as covariate in the models. The non-significant effects of the 2-way interactions were excluded from the final model. Significant effects are reported in bold

Oxidative status markers	TAC			TOS			PCO			LPO		
	F	*df*	*P*	*F*	*df*	*P*	*F*	*df*	*P*	*F*	*df*	*P*
Liver												
Final model												
Treatment	1.33	1, 37	0.257	0.16	1, 40	0.694	0.10	1, 38	0.750	1.45	1, 31	0.237
Sex	0.03	1, 40	0.861	0.15	1, 45	0.701	0.29	1, 41	0.595	0.68	1, 34	0.417
Laying order	1.07	2, 46	0.350	3.98	2, 48	**0.025**	0.25	2, 43	0.780	0.20	2, 39	0.816
Excluded terms												
Treatment × sex	0.01	1, 45	0.938	0.75	1, 49	0.391	0.22	1, 42	0.642	0.62	1, 39	0.435
Treatment × laying order	0.13	2, 49	0.880	0.14	2, 51	0.872	2.98	2, 46	0.061	0.36	2, 43	0.697
Sex × laying order	0.65	2, 46	0.526	1.88	2, 52	0.163	1.98	2, 46	0.150	0.32	2, 38	0.729
Brain												
Final model												
Treatment	1.03	1, 37	0.316	0.26	1, 40	0.616	1.65	1, 41	0.206	2.30	1, 38	0.138
Sex	0.20	1, 41	0.657	6.24	1, 44	**0.016**	2.13	1, 42	0.152	0.20	1, 41	0.655
Laying order	0.01	2, 46	0.987	0.07	2, 48	0.932	0.73	2, 44	0.486	0.46	2, 47	0.636
Excluded terms												
Treatment × sex	1.79	1, 47	0.187	0.96	1, 50	0.332	0.02	1, 49	0.902	0.32	1, 46	0.576
Treatment × laying order	1.81	2, 50	0.174	1.91	2, 52	0.158	0.77	2, 46	0.468	0.12	2, 50	0.889
Sex × laying order	2.11	2, 48	0.133	0.96	2, 53	0.390	0.24	2, 50	0.790	0.43	2, 48	0.650

the small amount of yolk adsorbed by the embryos up to the cracking stage may have limited the transfer of an "effective" dose of vitamin E, able to affect the oxidative status and to reduce oxidative damage of developing embryos. In fact, although the amount of vitamin E was higher in the residual yolk sac of injected eggs compared to controls (Figure 1), no significant differences were measured in brains or livers dissected from embryos. Indeed, our results may suggest that maternally transferred vitamin E concentration up to late prenatal stages seems to be optimal in preventing the occurrence of oxidative damage, and embryos may use the supplemental vitamin E dose to promote somatic growth rather than to limit the detrimental consequences of oxidative stress. These findings are consistent with those reported in a study of red-winged blackbird *Agelaius phoeniceus* nestlings treated with an antioxidant-enriched diet (Hall et al. 2010). The lack of positive consequences of increased vitamin E concentration on oxidative status markers do not lessen the role of vitamin E in protecting embryo by oxidative stress during prenatal development. It simply suggests that vitamin E concentrations transferred from yolk to embryo tissues during pre-hatching development could show its beneficial effects after hatching. In fact, yolk vitamin E is effectively transferred to the embryo and its initial concentration determines the reserve of the chick at least for the first week post-hatch (Surai et al. 1997). For instance, the highest concentrations of vitamin E in the liver occur at hatching and protect chicks from the adverse effects of oxidative stress for up to 2 weeks post-hatching (Surai et al. 1998). Thus, since newly hatched chicks are not able to effectively assimilate vitamin E from the diet and are dependent on their reserve built during embryonic development (Surai 2002), its accumulation in the embryo tissues, mainly in liver, is considered an adaptive mechanism providing antioxidant defense in the critical time of hatching (Surai et al. 1996).

In conclusion, our findings suggest that physiological variation in maternally transmitted vitamin E has no major effect on embryo oxidative status in two major target organs, that is, the liver and brain. In addition, they show that a physiological increase in yolk vitamin E concentration boosts embryonic somatic growth, consistent with previous findings on hatchlings. The conspicuous differences in the effects of maternal vitamin E on offspring phenotype occurring between the prenatal and the early postnatal life stages, which may differ according to hatching order, should suggest that vitamin E is of primary importance mainly during post-hatching periods. However, the partial inconsistency of the present results compared to some previous experimental studies of birds suggests that further studies are required to assess the role of this maternally transferred antioxidant during early life periods under a natural selection regime.

Supplementary material

Supplementary material can be found at http://www.cz.oxfordjournals.org/.

Author contribution

M. P. participated in field activity, performed biochemical analyses and wrote the article; C. D. P. and G. C. performed biochemical analyses; F. K. performed analyses to assess vitamin E concentration in yolk eggs; M. R. and M. C. performed the field experiment; I. D. D. and A. M. supervised biochemical analyses and contributed writing the article; D. R. participated in field activity and helped to write the article; N. S. designed the experiment, helped to perform statistical analyses, and to write the article and supervised both field and laboratory work.

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Supplementary Material

Yolk vitamin E positively affects prenatal growth but not oxidative status in yellow-legged gull embryos

Marco PAROLINI[a,*], Cristina Daniela POSSENTI[a], Filiz KARADAS[b], Graziano COLOMBO[a], Maria ROMANO[a], Manuela CAPRIOLI[a], Isabella DALLE-DONNE[a], Diego RUBOLINI[a], Aldo MILZANI[a], Nicola SAINO[a]

[a]Department of Biosciences, University of Milan, via Celoria 26, I-20133 Milan, Italy; [b]Department of Animal Science, University of Yüzüncü Yil, 65090 Van, Turkey

*Address correspondence to Marco PAROLINI. E-mail: marco.parolini@unimi.it

Received on 10 December 2016; accepted on 12 May, 2017

Field procedures

The yellow-legged gull *Larus michahellis* is a monogamous species that breeds mostly colonially (Cramp, 1998). Clutch size ranges between 1 and 3 eggs (modal size = 3), which are laid at 1–4 (most frequently 2) days intervals and hatch 27-31 days after laying. Hatching is asynchronous and spans over 1-4 days. The chicks are semi-precocial, are fed by both parents and fledge at 35-40 days of age (Cramp, 1998). We studied a large colony (> 400 pairs) breeding on an island in the Comacchio lagoon (NE Italy, 44°20' N–12°11' E) in March-May 2014. We visited the colony every second day starting from March 23. When a new nest was found, it was marked with a stake and the newly laid egg was temporarily removed. It was then replaced with a 'dummy' egg to avoid interference with parental incubation behaviour. The 'dummy' egg is a yellow-legged gull egg we collected from nests located in an island in the proximity of our study area at the beginning of the breeding season. We collected 15 eggs from 15 nests to be used as 'dummy' eggs. We discriminated 'dummy' eggs from experimental eggs because they were marked with a small red circle on the top of the eggshell using a waterproof red marker. The removed egg was marked with a waterproof marker indicating the number of the nest and the position in the laying sequence of the egg (first-, second- or third-laid), and taken to a nearby tent for experimental manipulation. Parents did not reject neither 'dummy' nor marked eggs.

The experiment was performed as described in detail by Parolini et al. (2015). We aimed at increasing the concentration of vitamin E (mixture of α- and γ-tocopherol; 93:7 ratio) by 1 standard deviation (SD) of that measured in the yolk of yellow-legged gull eggs from the same colony (Rubolini et al. 2011), by *in ovo* injection. After injection, the final concentration of vitamin E was within the natural range of variation. Since the concentration of vitamin E in the yolk varied according to egg size and position in the laying sequence, we adjusted the injection dose according to these factors. Therefore, based on Rubolini et al. (2011), we grouped first (a-), second (b-) or third (c-) laid eggs into three classes (tertiles) of size according to egg mass and we calculated the standard deviation of vitamin E concentration in the yolk for each tertile within each position in the laying sequence (Table S1).

Then, we estimated yolk mass based on total egg mass for each class of position in laying sequence as follows: yolk mass = 0.227 (0.039 SE) egg mass + 1.815 (3.461 SE); $R^2 = 0.252$; $F_{1,88} = 34.38$, $P < 0.001$). The injection amount of vitamin E was computed as the product of the SD (in ng g^{-1}) of vitamin E concentration for each tertile and position in the laying sequence and the estimated yolk mass. Vitamin E in the yolk of yellow-legged gull eggs from the same colony was mostly present in two forms, namely α- tocopherol and γ-tocopherol (93:7 ratio). Since the α-tocopherol : γ-tocopherol ratio did not vary according to the laying order, we set the same proportion in vitamin E that was dosed to all eggs. We adopted a within-clutch design, whereby both control and vitamin E-injected groups were established within each clutch, to minimize the confounding effects of environmental and parental effects. The following treatment schemes were assigned sequentially to the clutches, according to the order in which the first egg was found (nest, a-, b-, c-egg): nest 1, vitamin E injection (E), control injection (C), E; nest 2, C-E-C; nest 3, E-C-C; nest 4, C-E-E and so forth with the following nests. The amount of vitamin E injected in the three classes of egg mass for the three positions in the laying sequence is reported in Table S2.

Vitamin E solutions were prepared in sterile vials by dissolving α- and γ-tocopherol in corn oil to the final dilution required. Each vial contained the desired concentration of vitamin E to be injected in egg yolk depending on egg mass and laying order. Treated eggs were injected with 30 µl of the appropriate concentration of vitamin E, while control eggs were injected only with 30 µl of corn oil.

Vitamin E was injected in the yolk with the same procedure reported in Romano et al. (2008). Before being injected, the egg was weighed (to the nearest g) and placed with the longitudinal axis vertical. After disinfecting the eggshell, a hole was drilled using a sterile pin close to the acute pole. *In ovo* injection was performed by means of 1-mL sterile syringe mounting a 0.6 × 30 mm needle while the egg was held firmly with its longitudinal axis vertical. Immediately after

extracting the needle from the egg, the hole was sealed with a drop of epoxidic glue and a small piece of eggshell superimposed to the hole. After the *in ovo* vitamin E supplementation and five days before the earliest expected hatching date, all the nests were visited daily to check for any sign of imminent hatching such as eggshell fractures (i.e. 'cracking stage'). When eggshells were fractured, eggs were weighed (to the nearest g), collected and frozen at -20 °C within three hours from sampling. We inoculated eggs from 26 nests (n = 76 eggs; in two nests were laid only two eggs) and the 88% of the injected eggs reached the cracking stage.

Field collected eggs (*n* = 76 eggs) were transferred to the lab where they were dissected. We focused only on eggs from complete clutched (nests with three eggs; *n* = 22 nests for a total of *n* = 66 eggs). We first removed and weighed the residual yolk sac from each egg, which was frozen at -80 °C until the analysis of total vitamin E concentration and total antioxidant capacity that we performed as a validation of the experimental treatment. Then, the embryos were weighed (to the nearest g) and tarsus length was measured by calipers prior to the dissection of liver and brain, which were immediately weighed (to the nearest mg) and frozen at -80 °C until biochemical analyses. All the measurements were taken by the same person to ensure consistency. Molecular sexing of embryos and chicks was performed according to Saino et al. (2008) basing on methods developed by Griffiths et al. (1998). DNA extracted from a small piece of liver was amplificated by polymerase Chain Reaction (PCR) using a T1 Thermocycler (Biometra, Goettingen, Germany) under the following conditions: an initial denaturing step at 94 °C for 7 min was followed by 30 cycles at 48 °C for 30 s, 72 °C for 30 s and 94 °C for 1 min. The program concluded with a final cycle at 48 °C for 30 s and 72 °C for 5 min. Reactions were performed in a total volume of 20 µl including 50-100 ng of genomic DNA as template, 50 mM KCl, 10 mM Tris–HCl pH 9 (25 °C), 0.1 % Triton X100, 2 mM $MgCl_2$, 0.2 mM of dNTPs; 200 ng of each primer and 1.25 units of Taq Polymerase (Promega, Madison, WI, USA). PCR products were separated by electrophoresis for 20 min at 7–10 V/cm in a 1.5% agarose gel stained with ethidium bromide and visualized under UV light. A single band identified a male, while two different bands a female. A 100 bp ladder (100–2642 bp range) was used as size marker.

Analysis of vitamin E content in residual yolk sac

The concentration of vitamin E in residual yolk sac was determined according to Karadas et al. (2006) using high-performance liquid chromatography system (Shimadzu Liquid Chromatography, LC-10AD, Japan Spectroscopic Co. Ltd.). Briefly, 100–150 mg of yolk were homogenized with 1 mL of ethanol plus 0.7 mL NaCl 5% and extracted twice by centrifugation with 2 mL of hexane each. Then, hexane extracts were pooled and evaporated at 60–65 °C under nitrogen flow and the residual was dissolved in 500 μL of dichloromethane:methanol mixture (50:50 v/v). Vitamin E (α- and γ-tocopherol) concentrations were detected with a Hypersil GOLD type 3μm C18 reverse-phase column (150 × 4.6 mm Phase Separation, Thermo Fisher Scientific 81, Wyman, Street Waltham, MA USA) with a mobile phase of methanol:distilled water (97:3 v/v) at a flow rate of 1.05 mL min^{-1} using fluorescence detection by excitation and emission wavelength of 295 nm and 330 nm, respectively. Peaks of α-, and γ-tocopherol were identified by comparison with the retention time of standards of tocopherols (Sigma, Poole, UK).

Oxidative stress assay methods

Total antioxidant capacity (TAC), Total Oxidative Status (TOS), Protein carbonyl content (PCO) and Lipid peroxidation (LPO) were measured in the brain and the liver dissected from embryos. In addition, TAC was also measured in the residual yolk.

An appropriate amount of yolk (~ 0.15 g), brain and liver (~ 0.1 g) was homogenized in 100 mM phosphate buffer pH 7.4, with 1 mM EDTA and 100 mM KCl, by an automatic homogenizer. After 10 min centrifugation at 13,000 rpm, a small aliquot of the supernatant was immediately processed for the determination of protein content according to the Bradford method using bovine serum albumin (BSA) as a standard, while the remainder was used for oxidative stress assays.

TAC was measured according to a colorimetric method developed by Erel (2004), with modifications. The color of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{*+}$) bleaches depending on the concentration of antioxidants in the sample. The reaction is monitored spectrophotometrically and the final absorbance is inversely related to TAC of the sample. The assay was calibrated by a standard curve with serial dilutions of Trolox and the results were expressed as μM Trolox equivalent g^{-1} wet weight. Mean TAC intra-assay coefficient of variation (CV) was 2.8 ± 0.5 % (n = 3 replicates), while the mean inter-assay CV was 6.9 ± 0.5 % (n = 3 assay plates).

TOS was measured according to a colorimetric method adapted from Erel (2005). The oxidants in the samples oxidize the ferrous ion-o-dianisidine complex to the ferric ion, which reacting with xylenol orange gives a blue complex. Coloration was measured by a spectrophotometer at $\lambda = 535$ nm and is proportional to the oxidants in the plasma. The assay was calibrated by using a standard curve with serial dilution of hydrogen peroxide (H_2O_2). The results were expressed as nM H_2O_2 equivalents g^{-1} wet weight. The mean TOS intra-assay CV was 2.4 ± 1.2 % ($n = 3$ replicates) and the inter-assay CV was 4.1 ± 2.2 % ($n = 3$ assay plates). The oxidative status index (OSI) was calculated as the TOS / TAC ratio for each individual; high ratios reflected high oxidative stress situation.

Carbonylated proteins were derivatized with 2,4-dinitrophenylhydrazine (DNPH). Briefly, 400 μg proteins in 50 mM Tris-HCl pH 7.4 (final concentration 1 mg mL^{-1}) were mixed with 80 μL of 10 mM DNPH in 2N HCl and incubated for 60 min in the dark with frequent vortexing. After derivatization, protein samples were mixed with 480 μL of 20% trichloroacetic acid (TCA) 20% and incubated for 10 min in ice. After centrifugation at 20,000 g for 15 min at 4 °C, protein pellets were washed three times with 1:1 ethanol:ethylacetate to remove free DNPH. After air drying, pellets were resuspended in 2× reducing Laemmli sample buffer. Proteins were separated by SDS-PAGE (10% Tris-HCl resolving gel) and transferred to polyvinylidene difluoride (PVDF) membrane. Derivatized proteins were detected by Western immunoblotting with anti-dinitrophenyl-KLH (anti-DNP) antibody. In particular, PVDF membrane was washed in PBST (10mM Na phosphate, pH 7.2, 0.9% (w/vol) NaCl, 0.1% (vol/vol) Tween-20) and blocked for 1 h in 5% (w/vol) nonfat dry milk in PBST. After washing three times with PBST for 5 min each, carbonyl formation was probed by 2 h incubation with 5% milk/PBST containing anti-DNP antibodies (1:40,000 dilution). After three 5-mins washes with PBST, the membrane was incubated in a 1:80,000 dilution of the secondary antibody linked to horseradish peroxidase in 5% milk/PBST for 1 h. After washing three times with PBST for 5 min each, immunostained protein bands were visualized with enhanced chemiluminescence detection. Densitometric analysis was performed after scanning the chemiluminescence films by using Image J 1.40d software (National Institutes of Health). A single assay for each sample was performed, so no intra- or inter-assay variation can be calculated.

Lipid peroxidation was measured according to the method developed by (Ohkawa et al., 1978) and adapted to tissue homogenates of embryos. Two hundreds μL of tissue homogenates were added to a solution composed by TCA 12%, thiobarbituric acid (TBA 0.37%) and Tris-HCl (0.6 M), and boiled for 1 hour. After a centrifugation at 11,500 rpm for 15 min at 4 °C, the absorbance of the obtained supernatant was measured at 535 nm and the amount of thiobarbituric acid reactive

substances (TBARS) formed was calculated and expressed as nmol TBARS g^{-1} wet weight. The levels of lipid peroxidation were measured in duplicate in both brain and liver homogenates.

Table S1. Mean concentrations (± SD) of vitamin E (µg/g) measured in yolk from yellow-legged gull eggs (data from Rubolini et al., 2011). First (a-), second (b-) and third (c-) laid eggs were grouped into three classes (tertiles) of size according to egg mass.

Mean vitamin E concentration ± SD (µg/g)			
		Tertile	
Laying order	1st	2nd	3rd
a-egg	115.1 ± 21.3	132.0 ± 22.1	124.0 ± 24.6
b-egg	102.3 ± 21.2	115.7 ± 22.5	102.2 ± 23.1
c-egg	73.5 ± 19.5	92.9 ± 21.6	88.9 ± 21.5

Table S2. Amount (μg) of vitamin E (α : γ – tocopherol ratio *per* egg) injected into the yolk of yellow-legged gull eggs depending on egg mass at the time of deposition and laying order (first, second or third egg is a-, b-, or c-egg, respectively). The doses were designed to increase the post-manipulation vitamin E concentration of 1 standard deviation compared to that previously recorded in the same population for each class of egg mass and position in the laying sequence.

Laying order	***Egg mass (g)***	***Vitamin E (μg) (α : γ - tocopherol)***
a-egg	84-91	670 (623:47)
	92-95	748 (696:52)
	96-108	697 (648:49)
b-egg	80-88	509 (473:36)
	89-92	699 (650:49)
	93-99	688 (640:48)
c-egg	75-82	305 (283:22)
	82-87	616 (573:43)
	88-98	643 (596:45)

Chapter 3

Yolk vitamin E prevents oxidative damage in gull hatchlings

Royal Society Open Science ***4*** *(2017): 170098*

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Cite this article: Parolini M, Khoriauli L, Possenti CD, Colombo G, Caprioli M, Santagostino M, Nergadze SG, Milzani A, Giulotto E, Saino N. 2017 Yolk vitamin E prevents oxidative damage in gull hatchlings. *R. Soc. open sci.* **4**: 170098. http://dx.doi.org/10.1098/rsos.170098

Received: 3 February 2017
Accepted: 4 May 2017

Subject Category:
Biology (whole organism)

Subject Areas:
ecology/evolution

Keywords:
maternal effects, vitamin E, oxidative stress, telomere, yellow-legged gull

Author for correspondence:
Marco Parolini
e-mail: marco.parolini@unimi.it

Electronic supplementary material is available online at https://dx.doi.org/10.6084/m9.figshare.c.3784661.

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Yolk vitamin E prevents oxidative damage in gull hatchlings

Marco Parolini[1], Lela Khoriauli[3], Cristina Daniela Possenti[1], Graziano Colombo[2], Manuela Caprioli[1], Marco Santagostino[3], Solomon G. Nergadze[3], Aldo Milzani[2], Elena Giulotto[3] and Nicola Saino[1]

[1]Department of Environmental Science and Policy, and [2]Department of Biosciences, University of Milan, via Celoria 26, I-20133 Milan, Italy
[3]Department of Biology and Biotechnology, University of Pavia, via Ferrata 1, I-27100 Pavia, Italy

MP, 0000-0003-0226-1709

Oxidative stress experienced during early development can negatively affect diverse life-history traits, and organisms have evolved complex defence systems against its detrimental effects. Bird eggs contain maternally derived exogenous antioxidants that play a major role in embryo protection from oxidative damage, including the negative effects on telomere dynamics. In this study on the yellow-legged gull (*Larus michahellis*), we manipulated the concentration of vitamin E (VE) in the egg yolk and analysed the consequences on oxidative status markers and telomere length in the hatchlings. This study provides the first experimental evidence that, contrary to the expectation, a physiological increase in yolk VE concentration boosted total antioxidant capacity and reduced the concentration of pro-oxidant molecules in the plasma, but did not reduce telomere attrition or ameliorate oxidative damage to proteins and lipids in the early postnatal period.

1. Introduction

Early-life development is characterized by rapid growth requiring high metabolic activity and oxygen consumption, which imposes notable reactive oxygen species (ROS) production [1], even if there is still ambiguity regarding the relationship between oxygen consumption and ROS production [2]. To efficiently counteract the detrimental effects of oxidizing molecules, organisms have evolved a complex antioxidant machinery, which relies on enzymatic and non-enzymatic defence. In oviparous species, embryo protection against ROS largely depends on egg exogenous non-enzymatic antioxidants of maternal origin [3]. However, a

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small amount of ROS may escape from the protective shield of antioxidants, causing an oxidative stress situation to organisms, which can lead to oxidative damage to cellular macromolecules, including lipids, protein and DNA [1]. Oxidative stress-related adverse effects can occur throughout an individual's life and influence diverse life-history traits, representing a constraint in many biological processes [4]. Only recently, oxidative stress has been shown to interfere with telomere dynamics [5,6]. In vertebrates, telomeres are conserved non-coding sequences of the repeated TTAGGG motif that cap the ends of chromosomes and protect genomic integrity [7]. Telomeres shorten with age, and short telomeres at birth or rapid telomere loss are associated with reduced performance at several fitness traits and survival [5,6]. Oxidative stress has been suggested to provide a potential mechanism for telomere attrition in early life, hastening cell senescence and leading to negative consequences on survival and fitness-related traits of the offspring [5,6]. As antioxidants can decelerate telomere shortening [8], maternal allocation of exogenous antioxidants to the egg yolk may contribute to the maintenance of telomere length (TL) during early development.

Maternal egg antioxidants can modulate offspring performance and phenotype according to complex 'maternal effects' pathways. Low levels of maternal yolk antioxidants impair embryo development, suggesting their pivotal role in the early defence against ROS [3]. Vitamin E (VE) is one of the most important maternally transferred yolk antioxidants and plays a fundamental role in ROS scavenging [3]. Experimental dietary administration of VE has been shown to have beneficial effects on diverse offspring traits and in the prevention of deleterious effects caused by ROS in chicks of captive and wild species [1]. Differently, the beneficial effect of VE supplementation on telomere dynamics in birds, mainly during the early-life period, is still largely unexplored, albeit expected. As oxidative stress accelerates telomere shortening [8], VE supplementation may prevent telomere shortening because of its antioxidant capacity [9]. Studies of humans and other vertebrates, but not in birds, have demonstrated the beneficial effects and the underlying mechanism of action of VE supplementation on telomeres. Shortening of telomeres was slowed down in human cells supplemented with physiological doses of VE, which reduced ROS production and limited oxidative damage to telomeric DNA [10], although Guan and coauthors [11] showed that VE supplementation did not positively affect TL in peripheral blood mononuclear cells from Alzheimer's disease patients. Larger dietary intake of VE has been found to be associated with longer telomeres in humans [12], and *in vitro* experiments on skin fibroblasts have demonstrated that VE restores telomerase activity and protects against telomere erosion [13], suggesting that the protective role of VE against ROS-induced DNA damage is mediated by the up-regulation of c-fos expression and AP-1-binding activity [13].

In this study of the yellow-legged gull (*Larus michahellis*), we assessed the effect of a physiological increase in yolk VE concentration on oxidative status markers (i.e. total antioxidant capacity, amount of pro-oxidant molecules, lipid peroxidation and protein carbonylation) and TL of the newly hatched chicks. We expect that VE supplementation positively affects oxidative status, reduces oxidative damage and results in longer TL in VE-treated chicks as compared to controls. As VE concentration declines with laying order [14] and limits the postnatal growth of hatchlings from the last-laid (typically third) eggs [15], we also expect a differentially larger positive effect of VE on chicks from third-laid eggs. Because no difference in the concentration of yolk VE in the yellow-legged gull according to the sex of the embryo occurs [14] but embryos of either sex may show different susceptibility to yolk antioxidants, we also tested if the effect of VE injection depended on the sex of the chicks.

2. Material and methods

The experiment was performed during March–May 2014 in a large breeding colony in the Comacchio lagoon (NE Italy, 44°20′ N–12°11′ E). Full details of the experiment are reported in [15] and in the electronic supplementary material. We aimed at increasing the yolk VE concentration (α- and γ-tocopherol mixture) by 1 standard deviation of that measured in eggs of gulls from the same colony [14] through a previously validated injection method. We adopted a within-clutch design whereby the VE dose due to be injected was tuned according to egg size at laying and position in the laying sequence. After VE injection, the nests were visited every day. At hatching a blood sample was collected for molecular sexing, oxidative status markers and TL analyses. Total antioxidant capacity (TAC) and the amount of pro-oxidant molecules (i.e. TOS) were measured according to colorimetric methods [16]. Protein carbonylation was assessed by western immunoblotting [16], while lipid peroxidation through the thiobarbituric acid reactive substances (TBARS) method [17]. It should be noted that the TBARS method may not measure oxidative damage to lipids accurately because TBA reacts with other

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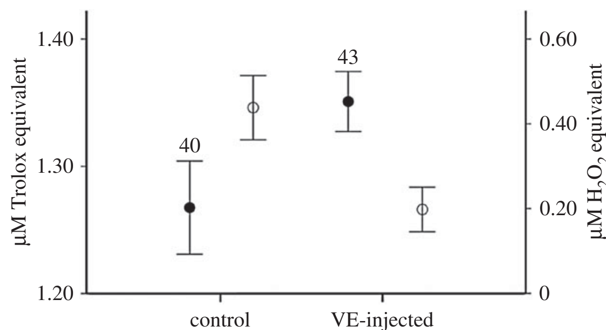

Figure 1. Estimated marginal mean (±95% confidence intervals) of TAC (µM Trolox equivalent; black circles) and TOS (µM H_2O_2 equivalent; white circles) measured in the plasma of hatchlings from control and VE-injected eggs. Sample sizes are reported.

compounds, apart from the main lipid peroxidation by-product malondialdehyde (MDA). Thus, TBARS results should be interpreted with caution because they may overestimate lipid peroxidation (LPO). TL was measured using the monochrome multiplex quantitative PCR method (MMQPCR) [18] and expressed as the ratio between the amount of telomeric repeats in the sample (T) and that of a single copy gene (S), relative to a reference sample (relative telomere length, RTL). All methods are fully described in the electronic supplementary material. We also verified the effectiveness of VE injection by measuring VE concentration in the yolk of some VE-injected eggs, which was always higher than that of sham-injected eggs (see electronic supplementary material for details). The effect of VE on investigated endpoints was analysed in linear mixed models (LMMs), including clutch identity as a random intercept effect. Egg treatment, embryo sex and egg-laying order were included as fixed-effect factors along with their two-way interactions. Egg mass at laying was included as a covariate in all models. All non-significant ($p > 0.05$) interaction terms were removed from the models in a single step. The effect of clutch identity was tested by the likelihood ratio test. Five chicks could not be sexed and were therefore excluded from all the analyses. Oxidative damage and TL analyses could not be assessed in some (2–9) hatchlings. In all the analyses, we always used the largest sample available. Statistical analyses were performed by using SAS 9.3 PROC MIXED. Statistics are presented as estimated marginal means (EMMs) ± standard error (SE).

3. Results

An LMM showed that VE treatment caused a statistically significant increase in TAC in the hatchlings from VE-injected eggs compared to controls (EMM: controls: 1.27 (0.02); VE-treated: 1.36 (0.02); figure 1). Sex and laying order did not significantly predict TAC of the hatchlings (table 1). TOS levels were significantly lower in the plasma of chicks hatched from VE-injected eggs with respect to controls (control eggs: 0.429 (0.03); VE-injected eggs: 0.192 (0.03); figure 1). Both TAC and TOS significantly varied among broods (likelihood ratio test; $\chi^2_1 > 5.6$, $p < 0.017$). No effect of VE treatment, sex, laying order and their interactions was found for protein carbonylation and lipid peroxidation in blood samples from hatchlings (table 1). Finally, VE supplementation did not significantly affect RTL, after controlling for the potentially confounding effects of sex and laying order (table 1). Separate LMMs of RTL where we included the markers of oxidative status as covariates did not reveal any significant effect ($F < 2.04$, $p > 0.157$ in all cases).

4. Discussion

The experimental increase in yolk VE concentration within physiological limits ameliorated plasma TAC and TOS, but this was not mirrored in a reduction in oxidative damage to proteins and lipids. In addition, VE supplementation did not affect TL, contrary to the expectation, stemming from the hypothesis of a negative effect of pro-oxidants on TL.

VE supplementation significantly increased plasma TAC and reduced TOS, confirming its crucial antioxidant role (figure 1). Similar effects were found in the plasma of hen chicks supplemented via the diet with supra-physiological VE doses [19], but are not consistent with those found in great tit nestlings, where neither plasma TAC nor TOS differed between experimental groups after administration of VE-enriched food [20]. Although our previous studies showed that VE supplementation exerted

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Table 1. Linear mixed models of total antioxidant capacity (TAC), amount of pro-oxidant molecules (TOS), lipid peroxidation (LPO), protein carbonylation (PCO) and relative telomere length (RTL) in the blood of yellow-legged gull hatchlings in relation to VE treatment, sex and laying order. Clutch identity was included in the model as a random intercept effect. The non-significant effects of the two-way interactions between fixed factors were excluded from the final model. C, control; VE, vitamin E-injected. Significant effects are reported in italics.

	TAC (C = 40; VE = 43)			TOS (C = 40; VE = 43)			PCO (C = 38; VE = 42)			LPO (C = 36 ; VE = 38)			RTL (C = 38; VE = 42)		
sample size	*F*	d.f.	*p*	*F*	d.f.	*p*	*F*	d.f.	*p*	*F*	d.f.	*p*	*F*	d.f.	*p*
final model															
treatment	25.06	1, 57	*<0.001*	36.14	1, 59	*<0.001*	2.24	1, 55	0.140	0.01	1, 50	0.927	0.13	1, 74	0.723
sex	0.39	1, 63	0.536	0.24	1, 65	0.629	0.31	1, 60	0.581	1.18	1, 46	0.282	1.88	1, 74	0.174
laying order	4.79	2, 59	*0.012*	1.69	2, 61	0.193	1.63	2, 59	0.205	0.43	2, 45	0.651	0.40	2, 74	0.670
excluded terms															
treatment × sex	0.46	1, 70	0.502	0.16	1, 71	0.692	0.05	1, 67	0.815	0.59	1, 46	0.445	0.02	1, 69	0.883
treatment × laying order	0.66	2, 67	0.522	1.14	2, 68	0.326	0.90	2, 64	0.413	0.13	2, 52	0.875	0.42	2, 69	0.656
sex × laying order	0.58	2, 66	0.565	1.28	2, 68	0.284	0.19	2, 62	0.831	0.17	2, 45	0.846	0.21	2, 69	0.810

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positive effects on morphological traits of chicks hatched from third-laid VE-injected eggs [15], the significant effect on TAC and TOS was independent of egg laying order, suggesting that all chicks benefited from VE supplementation. However, we did not detect any effect of yolk VE increase on oxidative damage to proteins and lipids according to previous studies of wild birds [1,21]. Contrary to the expectation, VE treatment had no effect on TL in red blood cells, despite having positive effects on oxidative status. Oxidative stress has been often invoked as a determinant of telomere attrition, but no experimental study to date has capitalized on the advantages of the avian eggs as a cleidoic environment amenable to controlled manipulation of the level of antioxidants in the prenatal environment. While *in ovo* corticosterone injection caused ROS overproduction and telomere shortening in domestic chickens at 21 days [22], no experimental study of birds has tested for the effect of prenatal antioxidants on TL at the end of the embryonic stage, when telomere attrition is believed to have already progressed. These findings are the first experimental evidence that VE egg supplementation, mimicking physiological variation in maternal transfer to the egg, does not affect TL at hatching. Postnatal dietary supplementation of VE and vitamin C in the yellow-legged gull has also been shown to have no effect on TL of 7-day-old chicks [23]. These results combined suggest that availability of egg maternal and dietary VE has little influence on telomere dynamics in early life stages. However, such effects may become apparent at a later life stage, as shown for blue tit nestlings where the positive effect of a one-shot treatment with VE and methionine via subcutaneous injection on TL could be recorded 1 year after treatment [24].

Our study shows that a physiological increase in VE yolk concentration has positive effects in terms of plasma TAC and reduction in TOS but has no effect on oxidative damage or TL at hatching. This suggests that maternal allocation of VE to the egg is not limiting to protection from oxidative damage and any reduction of TL during prenatal life. However, we cannot exclude that the improvement of oxidative status of hatchlings due to the increase of VE concentration may result in positive effects in later life stages. Although TL at birth is considered an important predictor of fitness-related traits, telomere attrition can be more intense during postnatal growth. Thus, the availability of maternally transferred dietary antioxidants during early life may have long-term consequences by alleviating the costs of stressful conditions experienced during growth and preventing telomere attrition and the subsequent age-related risk factors for disease and increased risk of mortality.

Ethics. This study was conducted under permission of the Parco Regionale del Delta del Po (#657, 4 February 2014), which allowed both the manipulation and the withdrawal of hatchling blood.

Data accessibility. The data supporting this article are in the electronic supplementary material.

Authors' contributions. M.P. and N.S. conceived the study. M.P. and N.S. performed field experiments. C.D.P., G.C., M.C. and A.M. performed analysis of data on oxidative status markers. L.K., M.S., S.G.N. and E.G. performed data analysis on telomere length. M.P. and N.S. performed statistical analyses and wrote the article. All the authors gave their final approval for publication.

Competing interests. The authors declare to have no competing interests.

Funding. The authors declare that no specific funding was obtained to perform this research.

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Electronic Supplementary Material (ESM)

Yolk vitamin E prevents oxidative damage in gull hatchlings

Field procedures

The present study was carried out on a large colony (> 400 breeding pairs) of yellow-legged gull (*Larus michahellis*) in the Comacchio lagoon (NE Italy) during March-May 2014. The colony was visited every second day to check for any new nests and newly laid eggs, which were marked to monitor the progress of laying and to identify laying sequence. When a new egg was found, it was temporarily removed from the nest for experimental manipulation while temporarily replacing it with a 'dummy' egg.

The experiment was performed as described in detail by Parolini et al. (2015). We aimed at increasing the concentration of vitamin E (VE; mixture of α- and γ-tocopherol; 93:7 ratio) by 1 standard deviation (SD) of that measured in the yolk of yellow-legged gull eggs from the same colony (Rubolini *et al.* 2011), by *in ovo* injection. After injection, the final concentration of VE was within the natural range of variation. Since the concentration of VE in the yolk varied according to egg size and position in the laying sequence, we tuned the dose due to be injected according to these factors. Therefore, based on Rubolini *et al.* (2011), we grouped first (a-), second (b-) or third (c-) laid eggs into three classes (tertiles) of size according to egg mass and we calculated the standard deviation of VE concentration in the yolk for each tertile within each position in the laying sequence (Table S1).

Table S1. Mean concentrations (± SD) of VE (µg/g) measured in yolk from yellow-legged gull eggs (data from Rubolini et al., 2011). First (a-), second (b-) and third (c-) laid eggs were grouped into three classes (tertiles) of size according to egg mass.

Mean VE concentration ± SD (µg/g)			
	Tertile		
Laying order	1st	2nd	3rd
a-egg	115.1 ± 21.3	132.0 ± 22.1	124.0 ± 24.6
b-egg	102.3 ± 21.2	115.7 ± 22.5	102.2 ± 23.1
c-egg	73.5 ± 19.5	92.9 ± 21.6	88.9 ± 21.5

Then, we estimated yolk mass based on total egg mass for each class of position in laying sequence as follows: yolk mass = 0.227 (0.039 SE) egg mass + 1.815 (3.461 SE); $R^2 = 0.252$; $F_{1,88} = 34.38$, $P < 0.001$). The amount of VE due to be injected was then calculated as the product of the relevant standard deviation value and yolk mass thus estimated. We adopted a within-clutch design, whereby both control and VE-injected groups were established within each clutch, to minimize the confounding effects of environmental and parental effects. The following treatment schemes were assigned sequentially to the clutches, according to the order in which the first egg was found (nest, a-, b-, c-egg): nest 1, VE injection (E), control injection (C), E; nest 2, C-E-C; nest 3, E-C-C; nest 4, C-E-E and so forth with the following nests. The amount of VE injected in the three classes of egg mass for the three positions in the laying sequence is reported in Table S2.

Table S2. Amount (μg) of VE (α : γ – tocopherol ratio *per* egg) injected into the yolk of yellow-legged gull eggs depending on egg mass at the time of deposition and laying order (first, second or third egg is a-, b-, or c-egg, respectively). The doses were designed to increase the post-manipulation vitamin E concentration of 1 standard deviation compared to that previously recorded in the same population for each class of egg mass and position in the laying sequence.

Laying order	*Egg mass (g)*	*Vitamin E (μg) (α : γ - tocopherol)*
a-egg	84-91	670 (623:47)
	92-95	748 (696:52)
	96-108	697 (648:49)
b-egg	80-88	509 (473:36)
	89-92	699 (650:49)
	93-99	688 (640:48)
c-egg	75-82	305 (283:22)
	82-87	616 (573:43)
	88-98	643 (596:45)

VE solutions were prepared in sterile vials by dissolving α- and γ-tocopherol in corn oil to the final dilution required. Each vial contained the desired concentration of VE to be injected in egg yolk depending on egg mass and laying order. Treated eggs were injected with 30 μl of the appropriate concentration of VE, while control eggs were injected only with 30 μl of corn oil.

VE was injected in the yolk with the same procedure reported in Romano et al. (2008). Before being injected, the egg was weighed (to the nearest g) and placed with the longitudinal axis vertical. After disinfecting the eggshell, a hole was drilled using a sterile pin close to the acute pole. *In ovo* injection was performed by means of 1-mL sterile syringe mounting a 0.6 × 30 mm needle while the egg was held firmly with its longitudinal axis vertical. Immediately after extracting the needle from the egg, the hole was sealed with a drop of epoxidic glue and a small piece of eggshell superimposed to the hole.

We verified the effectiveness of VE injection by measuring VE concentration in yolk of some VE-injected eggs, which was always higher than that of sham-injected eggs (see details below).

After the *in ovo* VE injection, all the nests were visited every day and eggs were monitored until hatching. Because normally up to two days elapse between the time when the egg reaches the pipping stage and hatching, we assigned chicks to their original egg by injecting in the pipping egg a small drop of food dye (either blue or green; Bonisoli-Alquati et al., 2007). Upon the first daily visit to the nest when any individual chick was found to have hatched, the chick was weighed (to the nearest g) and its tarsus was measured (to the nearest 0.1 mm) (see Parolini et al. 2015). Finally, a blood sample (about 100 μl) was collected in capillary tubes after puncturing the ulnar vein. Blood samples were centrifuged at 11,500 rpm for 10 min to separate red blood cells from plasma, which were stored at – 20 °C until biochemical analyses. All the measurements were taken by the same person for consistency. Molecular sexing of embryos and chicks was performed according to Rubolini et al. (2006).

As reported by Parolini et al. (2015), mean clutch size was 2.86 (0.41 SD) eggs, with 39 (89%) of the clutches containing 3 eggs. Hatching success was very similar between the control, sham-injected (proportion of hatched eggs = 43/61 = 0.705) and the VE-injected (45/63 = 0.714; χ^2_1 = 0.01, P = 0.93) eggs. The sex ratio among the chicks that successfully hatched was also similar between experimental groups (proportion of males: sham-injected: 18/40 = 0.450; VE: 20/43 = 0.465; χ^2_1 = 0.01, P = 0.91). In a LMM on the eggs that successfully hatched, where clutch identity was included as a random effect, egg mass did not differ between the two experimental groups ($F_{1,49.5}$ = 0.02, P = 0.894). In the same model, egg mass significantly declined with laying order (estimated marginal means (SE): first eggs: 91.4 (0.90); second eggs: 89.1 (0.90); third eggs: 83.8 (0.97)), with significant pairwise differences among all laying order groups (LSD test: P < 0.012 in all cases).

Methods of Oxidative status markers

Oxidative stress assays were performed in blood samples of chicks hatched from both control and VE-injected eggs. Total antioxidant capacity (TAC), amount of pro-oxidant molecules (TOS) and protein carbonyl content (PCO) were measure in plasma, while lipid peroxidation (LPO) was evaluated in red blood cells.

TAC was measured according to a colorimetric method developed by Erel (2004), with modifications. The color of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS*+) bleaches depending on the concentration of antioxidants in the sample. The reaction is monitored spectrophotometrically and the final absorbance is inversely related to TAC of the sample. The assay was calibrated by drawing a standard curve with serial dilutions of Trolox and

the results were expressed as μM Trolox equivalent. Mean TAC intra-assay coefficient of variation (CV) was 2.8 ± 0.5 % (n = 3 replicates), while the mean inter-assay CV was 6.9 ± 0.5 % (n = 3 assay plates).

TOS was measured according to a colorimetric method adapted from Erel (2005). The oxidants in the plasma oxidize the ferrous ion-*o*-dianisidine complex to the ferric ion, which reacting with xylenol orange gives a blue complex. Coloration was measured by a spectrophotometer at λ = 535 nm and is proportional to the oxidants in the plasma. The assay was calibrated by using a standard curve with serial dilution of hydrogen peroxide (H_2O_2). The results were expressed as μM H_2O_2 equivalents. The mean TOS intra-assay CV was 2.4 ± 1.2 % (n = 3 replicates) and the inter-assay CV was 4.1 ± 2.2 % (n = 3 assay plates).

Carbonylated proteins were derivatized with 2,4-dinitrophenylhydrazine (DNPH). Briefly, 400 μg proteins in 50 mM Tris-HCl pH 7.4 (final concentration 1 mg mL^{-1}) were mixed with 80 μL of 10 mM DNPH in 2N HCl and incubated for 60 min in the dark with frequent vortexing. After derivatization, protein samples were mixed with 480 μL of 20% trichloroacetic acid (TCA) 20% and incubated for 10 min in ice. After centrifugation at 20,000 g for 15 min at 4 °C, protein pellets were washed three times with 1:1 ethanol:ethylacetate to remove free DNPH. After air drying, pellets were resuspended in 2× reducing Laemmli sample buffer. Proteins were separated by SDS-PAGE (10% Tris-HCl resolving gel) and transferred to polyvinylidene difluoride (PVDF) membrane. Derivatized proteins were detected by Western immunoblotting with anti-dinitrophenyl-KLH (anti-DNP) antibody. In particular, PVDF membrane was washed in PBST (10mM Na phosphate, pH 7.2, 0.9% (w/vol) NaCl, 0.1% (vol/vol) Tween-20) and blocked for 1 h in 5% (w/vol) nonfat dry milk in PBST. After washing three times with PBST for 5 min each, carbonyl formation was probed by 2 h incubation with 5% milk/PBST containing anti-DNP antibodies (1:40,000 dilution). After three 5-mins washes with PBST, the membrane was incubated in a 1:80,000 dilution of the secondary antibody linked to horseradish peroxidase in 5% milk/PBST for 1 h. After washing three times with PBST for 5 min each, immunostained protein bands were visualized with enhanced chemiluminescence detection. Densitometric analysis was performed after scanning the chemiluminescence films by using Image J 1.40d software (National Institutes of Health). A single assay for each sample was performed, so no intra- or inter-assay variation can be calculated.

Lipid peroxidation was measured according to the method developed by Ohkawa et al. (1979) and modified by Cinar et al. (2014) for blood samples. About 100 μL of blood were added to a solution composed by TCA 12%, thiobarbituric acid (TBA 0.37%) and Tris-HCl (0.6 M), and boiled for 1

hour. After a centrifugation at 11,500 rpm for 15 min at 4 °C, the absorbance of the obtained supernatant was measured at 535 nm and the amount of thiobarbituric acid reactive substances (TBARS) formed was calculated and expressed as nmol TBARS/µL. A single assay for each sample was performed, so no intra- or inter-assay variation can be calculated.

Telomere length analysis

Telomere length analysis was performed according to the method described by Parolini et al. (2015). Genomic DNA was extracted from 10-20 µl of red blood cells using 1 ml TNSE buffer (10 mM Tris HCl, 400 mM NaCl, 100 mM EDTA and 0.6% SDS) and a standard phenol/chloroform method. DNA samples of nestlings from the same nest were extracted in the same batch. We measured the quantity and purity of the extracted genomic DNA using a Nanophotometer (IMPLEN). Telomere length was measured by the monochrome multiplex quantitative PCR method (MMQPCR; Cawthon, 2009) on a PikoReal 96 thermal cycler (Thermo Scientific): telomere length was measured as the ratio (T/S) of the amount of telomeric repeats (T) to the amount of a single copy gene (S), relative to a reference sample. By this method, telomere length is evaluated indirectly by measuring the relative number of telomeric repeats in a genome and it is indicated from now as relative telomere length (RTL). The sequences of telomeric primers for MMQPCR were (telg 5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3'; telc 5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3'), while the single copy sequence used as control was a fragment from the 12th exon of the swallow CTCF gene (CCCTC-binding factor zinc finger protein). The CTCF primers used were: forward (5'-<u>CCCGCGGCGGGCGGCGCGGGCTGGGCG</u>GCTCCCAATGGAGACCTCAC-3') and reverse (5'-<u>CGCCGCGGCCCGCCGCGCCCGTCCCGC</u>CCATCACCGGTCCATCATGC-3'); these primers are composed of a swallow genomic sequence and a GC-clamp at the 5' end (underlined) to increase the melting temperature of the PCR product. Since the melting temperature of telomeric and CTCF PCR products are different, both primer pairs could be used in the same reaction. PCR reactions were prepared using 20 ng of genomic DNA as template, 1x DyNAmo ColorFlash SYBR Green qPCR Master Mix (Thermo Scientific), telomeric and CTCF primers at a final concentration of 1,000 nM and 500 nM each, respectively. Three-fold serial dilutions of a barn swallow reference sample (from 5.5 to 150 ng) were included in each plate to produce a standard curve to measure reaction efficiency and quantify the amount of telomeric repeats and single copy gene in each sample. We used the same reference sample per plate. All reactions were run in triplicate and six plates containing 25 samples each were performed. Cycling parameters for the PCR reactions were: Stage 1: 15 min at 95 °C; Stage 2: 2 cycles of 15s at 94 °C, 15s at 49 °C; and Stage 3: 35 cycles of

15s at 94 °C, 10s at 62 °C, 15s at 74 °C with signal acquisition, 10s at 84 °C, 15s at 88 °C with signal acquisition. The PikoReal Software (Thermo Scientific) was used to calculate the amount of telomeric repeats (T) for each sample by interpolation of the quantification Cycle (Cq) into the linear function $y = ax + b$ of the standard curve of the telomeric primers. Similarly, the software calculates the amount of the single copy gene (S) for each sample. Mean values for T and S for each sample were used to calculate the T/S ratios relative to a reference sample, so telomere length was indicated as relative telomere length (RTL).

All reactions were run in triplicate and five plates containing on average 25 samples each were performed. Ten samples were run in each plate. The mean reaction efficiencies for both telomere and CTCF amplifications were greater than 84%. The intra- and inter-plate repeatability of RTL measures, expressed as intra-class correlation coefficient, was 0.56 and 0.59, respectively. The mean intra-and inter-plate coefficient of variation (± SD) of RTL measures was 10.9 ± 8.6 % and 15.5 ± 10.7 %, respectively.

Since the MMQPCR method evaluates the number of telomeric repeats, it cannot be used when large amounts of telomeric-like repeats at non-terminal sites (Interstial Telomeric Sequences, ITSs) are present in the genome under study. ITSs have been described in all vertebrate species analyzed so far and can be classified, according to sequence organization, into short-ITSs (s-ITSs), composed by short stretches of TTAGGG repeats (up to a few hundreds bp), and heterochromatic-ITSs (het-ITSs), composed by extended blocks of repeats spanning several kilobases and located mainly at pericentromeric regions (Ruiz-Herrera et al., 2008). Since the sequence of the yellow-legged gull genome was not available, a preliminary analysis aimed at determining the possible presence of het-ITSs was carried out by the standard Terminal Restriction Fragment (TRF) method (Figure S1). and by a *Bal*31 assay (Figure S2) as previously described (Smirnova et al., 2013; Faravelli et al., 2002; Parolini et al., 2015).

For TRF analysis, genomic DNA was digested with the restriction enzymes HinfI and RsaI (Thermo Scientific) separated by electrophoresis, denatured and transferred to a nylon membrane (Amersham Hybond-N, GE Healthcare). The DNA was then hybridized with a ^{32}P-α[dCTP]-labeled telomeric probe and exposed to an autoradiographic film. As in barn swallows (Parolini et al 2015), no intense bands corresponding to het-ITSs were detected in yellow-legged gulls. As expected (Faravelli et al. 2002), in CHO cells several intense and discrete bands, corresponding to extended blocks of interstitial telomeric sequences, were observed (Figure S1).

For the Bal31 assay, genomic DNA was digested with either 0.05 (chicken DT40) or 0.005 (yellow-legged gull and barn swallow) units of *Bal*31 (Takara) per µg of DNA. Aliquots containing 3 µg of digested DNA were withdrawn from all reactions after 0, 5, 10 and 30 minutes. Additional aliquots

of digested DT40 and barn swallow genomic DNAs were withdrawn after 60 (chicken DT40 and barn swallow) and 120 minutes (chicken DT40). Reactions were blocked by the addition of EGTA (final concentration 20 mM) and incubation at 65°C for 10 minutes. After phenol-chloroform extraction, DNAs were ethanol-precipitated, resuspended in water and digested for 12 hours with 10 units of *Hinf*I (Thermo Scientific) per µg of DNA. Digested DNA was electrophorased in 1% agarose gel, denatured and transferred to a nylon membrane (Amersham Hybond-N, GE Healthcare). Membranes were then hybridized with a ^{32}P-α[dCTP]-labeled telomeric probe and exposed to autoradiography films. In DT40 we observed intense bands, resistant to *Bal*31 digestion, hybridizing with the telomeric repeat probe and corresponding to extended blocks of het-ITSs were located at internal chromosome sites, as expected. On the contrary, when we digested yellow-legged gull DNA with the *Bal*31 exonuclease, we detected a clear reduction in intensity and molecular weight of the smear, similar to that observed for barn swallow DNA, indicating that the majority of telomeric repeats detected in this sample were located at chromosome ends. This experiment suggests that, if present, ITSs in the yellow-legged gull genome are composed by a small number of repeats, as in human and barn swallow.

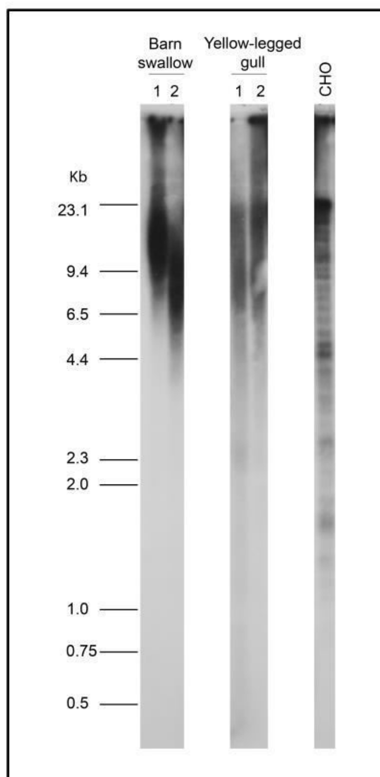

Figure S1. Terminal Restriction Fragment (TRF) analysis by Southern blotting in 2 barn swallows, 2 yellow-legged gulls and Chinese Hamster Ovary (CHO).

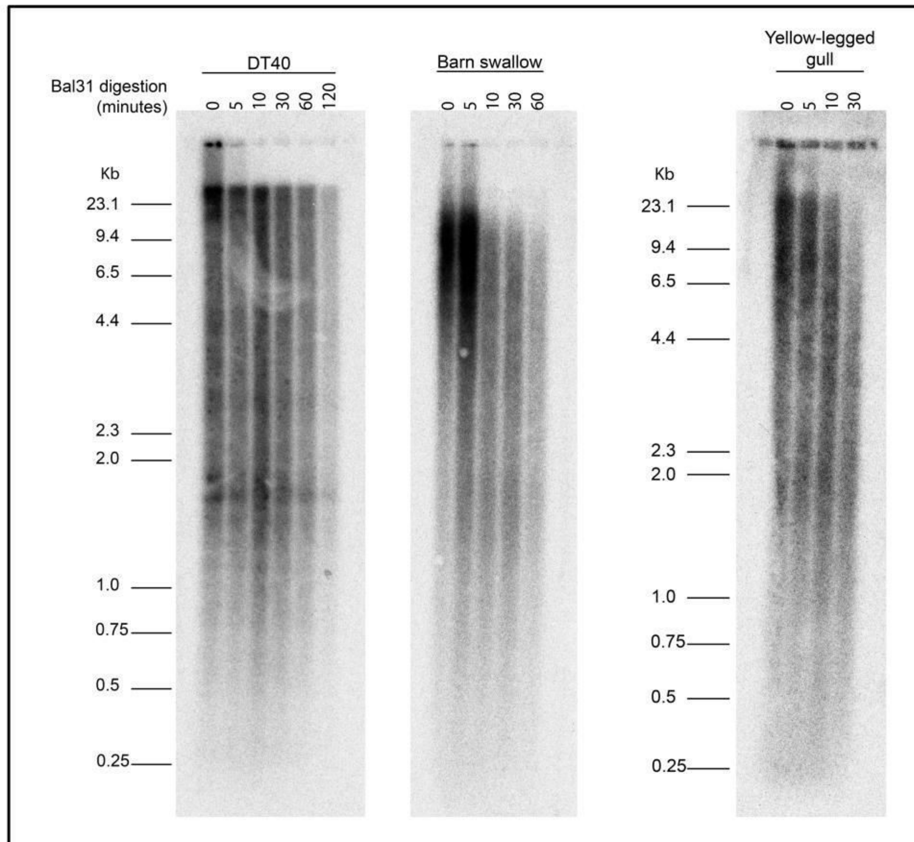

Figure S2. *Bal*31 exonuclease assay in chicken (DT40), barn swallow and yellow-legged gull.

Analysis of Vitamin E content in yolk

To assess the reliability of the injection procedure we first analyzed whether the concentration of VE in the residual yolk sac differed between sham- and VE-injected eggs. We relied on the yolk sac samples of 66 embryos (n = 26 nests) from a companion study in which we investigated potential effects of the injection of a physiological VE level on embryo traits. We highlight that the eggs were injected with the same VE concentrations described in the present study following the same experimental design. The concentration of VE in residual yolk sac was determined according to Karadas et al. (2006) using high-performance liquid chromatography system (Shimadzu Liquid Chromatography, LC-10AD, Japan Spectroscopic Co. Ltd.). Briefly, 100-150 mg of yolk were homogenized with 1 mL of ethanol plus 0.7 mL NaCl 5% and extracted twice by centrifugation with 2 mL of hexane each. Then, hexane extracts were pooled and evaporated at 60-65 °C under

nitrogen flow and the residual was dissolved in 500 μL of dichloromethane:methanol mixture (50:50 v/v). VE (α- and γ-tocopherol) concentrations were detected with a Hypersil GOLD type 3μm C18 reverse-phase column (150 × 4.6 mm Phase Separation, Thermo Fisher Scientific 81, Wyman, Street Waltham, MA USA) with a mobile phase of methanol:distilled water (97:3 v/v) at a flow rate of 1.05 mL min^{-1} using fluorescence detection by excitation and emission wavelength of 295 nm and 330 nm, respectively. Peaks of α-, and γ-tocopherol were identified by comparison with the retention time of standards of tocopherols (Sigma, Poole, UK).

As expected, vitamin E concentration was significantly larger in vitamin E treated eggs compared to controls ($F_{1,44.5} = 4.314$; $P = 0.044$). Even if the effect of the laying sequence per treatment interaction on yolk sac vitamin E concentration was statically non-significant ($F_{2,47.8} = 0.795$; $P = 0.457$), the concentration of vitamin E in the residual yolk from VE-injected eggs was always higher compared to that measured in controls (Figure S3).

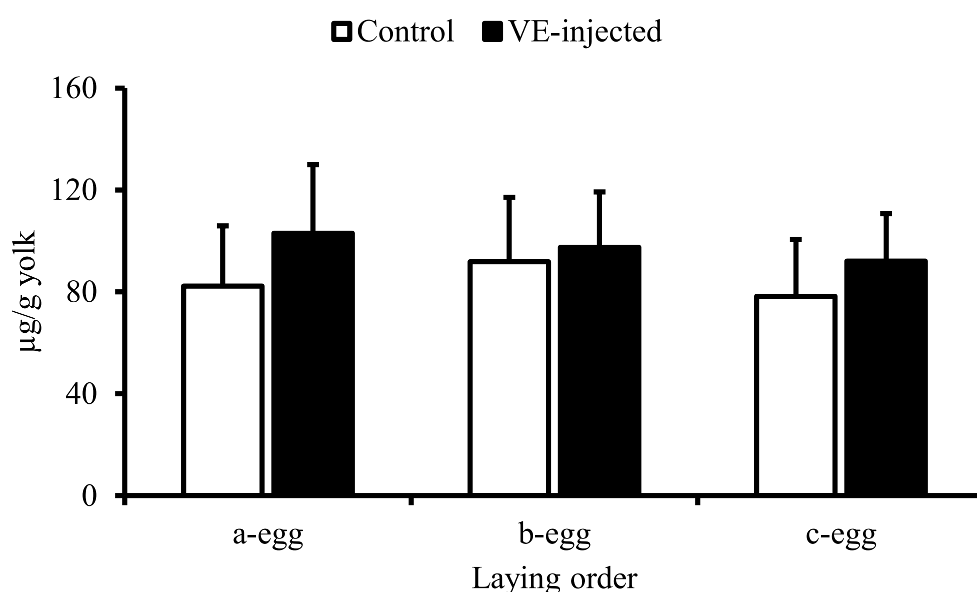

Figure S3: Mean of VE concentration (+SD) in residual yolk sac of yellow-legged gull eggs shortly before hatching (i.e. 'cracking stage', when eggshell fractures appear).

In detail, basing on data reported in Rubolini et al. (2011) in which levels of VE in eggs of yellow-legged gulls from the same colony have been measured, we expected a post-injection mean VE concentration in a-, b- and c-eggs as 145.6 μg/g (mean ± SD; 119.2 ± 26.4), 133 μg/g (mean ± SD; 104.9 ± 28.1) and 103.8 μg/g (mean ± SD; 81.4 ± 22.4), respectively, which corresponds on average to a 25% increase of VE concentration compared to the physiological levels of the species. Our analyses showed that VE concentration in residual yolk sac from VE-injected in a-, b- and c-eggs soon before hatching was 103.0 ± 26.8 μg/g, 97.5 ± 21.7 μg/g and 92.1 ± 18.6 μg/g (mean ± SD),

respectively, showing on average a 25%, 6% and 18% increase (mean 16%) with respect to the corresponding sham-injected eggs. In addition, as we weighted the residual yolk sac in eggs at the cracking stage, we estimated the total yolk weight at the time of laying for each egg and, consequently the percentage of the residual yolk that each embryo has to adsorb before hatching. Then, we estimated the possible amount of VE at the deposition for each egg according to the percentage of the residual yolk at the cracking stage. Mean estimated total VE concentration in VE-injected a-, b- and c-eggs should 130 µg/g, 121 µg/g and 113 µg/g, which on average correspond to a 22% increase compared to the corresponding sham-injected eggs. Thus, the estimated VE concentrations based on our analysis are very close to the expected values by the study of Rubolini et al. (2011) and our data definitively confirm the effectiveness of VE-injection in the yolk of yellow-legged gull eggs.

Unfortunately, we cannot measure the concentration of VE in blood of hatchlings because of sample scarceness. However, since VE is efficiently transferred from yolk to developing embryos, we relied on the embryos developed into the eggs used to certify the injection methods (see above) and we certified VE transfer to the embryos by measuring the concentration of this antioxidant in two organs, namely the brain and the liver. Even if the concentrations of VE measured in VE–treated embryos soon before hatching were not significantly larger than controls, neither in the brain ($F_{1,59} = 1.707$; $P = 0.196$) nor in the liver ($F_{1,35} = 0.305$; $P = 0.584$), overall the measured concentrations were higher in chick organs from VE-injected eggs compared to sham-injected ones (Figure S4), revealing a higher transfer from yolk to organs in embryos from VE-injected eggs compared to controls.

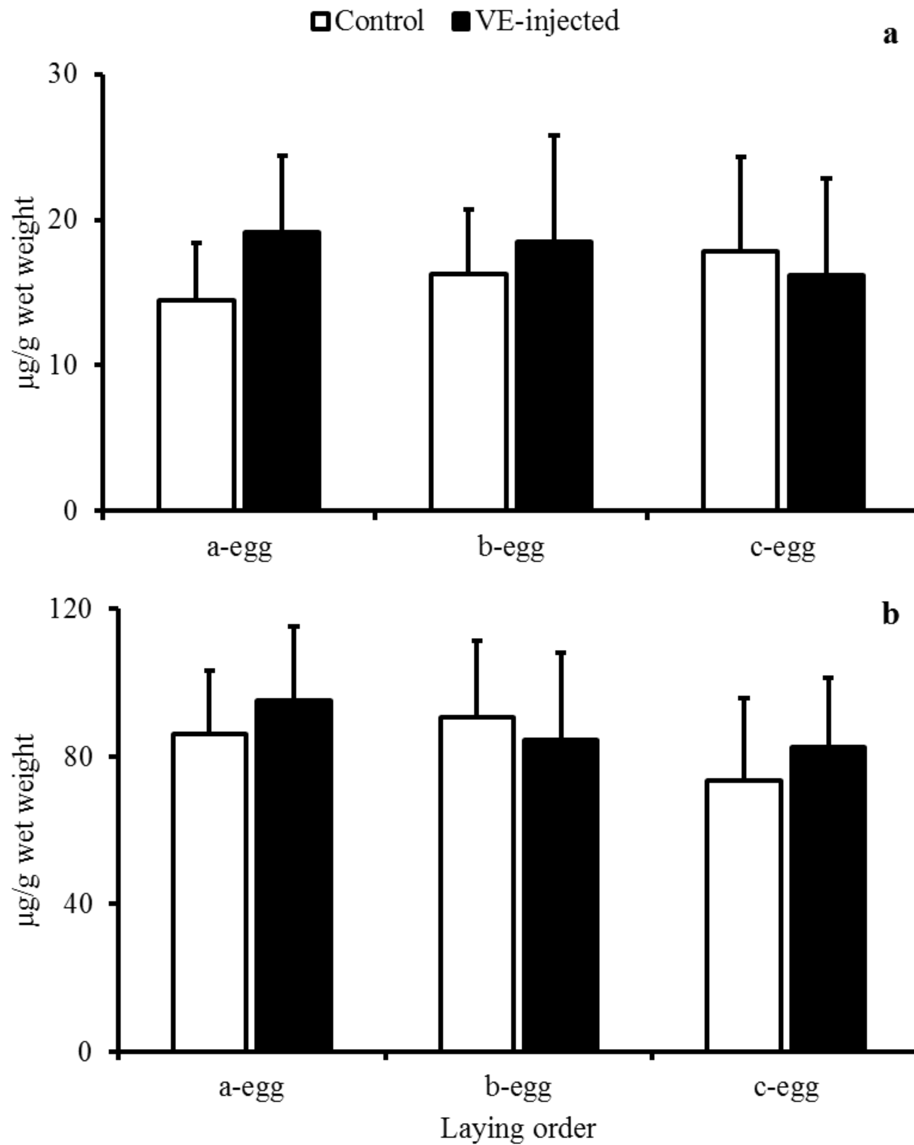

Figure S4: Mean of VE concentration (+SD) in brain (a) and liver (b) of yellow-legged gull embryos shortly before hatching (i.e. 'cracking stage', when eggshell fractures appear).

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Chapter 4

Antioxidants and embryo phenotype: is there experimental evidence for strong integration of the antioxidant system?

Journal of Experimental Biology ***220*** *(2017): 615-624*

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RESEARCH ARTICLE

Antioxidants and embryo phenotype: is there experimental evidence for strong integration of the antioxidant system?

Cristina Daniela Possenti[1], Filiz Karadas[2], Graziano Colombo[1], Manuela Caprioli[1], Diego Rubolini[1], Aldo Milzani[1], Isabella Dalle Donne[1], Nicola Saino[1] and Marco Parolini[1,*]

ABSTRACT

Organisms have evolved complex defense systems against oxidative stress. Bird eggs contain maternally derived antioxidants that protect embryos from oxidative damage. The antioxidant system components are thought to be integrated, but few studies have analyzed the covariation between antioxidant concentrations, embryo 'oxidative status' and morphology. In addition, no study has tested the effects of experimental change in yolk antioxidant concentration on other antioxidants, on their reciprocal relationships and on their relationships with embryo oxidative status or growth, which are expected if antioxidants defenses are integrated. In yellow-legged gull (*Larus michahellis*) embryos, we analyzed the covariation between several antioxidants, markers of 'oxidative status' [total antioxidant capacity (TAC), concentration of pro-oxidants (TOS), lipid peroxidation (LPO) and protein carbonylation (PC)] in the yolk, liver and brain, and morphology. Yolk and liver antioxidant concentrations were positively correlated reciprocally and with embryo size, and positively predicted TAC but not oxidative status. TOS and LPO were positively correlated in the liver, while TAC and LPO were negatively correlated in the brain. Weak relationships existed between antioxidants and TOS, PC and LPO. The effects of antioxidants on oxidative status and morphology were non-synergistic. An experimental physiological increase in yolk vitamin E had very weak effects on the relationships between other antioxidants or oxidative status and vitamin E concentration, the concentration of other antioxidants or oxidative status; the covariation between other antioxidants and oxidative status, and relationships between morphology or oxidative status and other antioxidants, challenging the common wisdom of strong functional relationships among antioxidants, at least for embryos in the wild.

KEY WORDS: Bivariate mixed models, *Larus michahellis*, Maternal effects, Morphological traits, Oxidative status, Vitamin E

INTRODUCTION

Organisms are exposed to oxidizing agents originating from the external environment and also from their internal physiological milieu (Halliwell and Gutteridge, 1999). Because oxidation of biological molecules can result in loss of biological function, selection has promoted the evolution of complex physiological adaptations to prevent or reduce propagation, or repair oxidative damage (Costantini, 2014).

[1]Department of Biosciences, University of Milan, via Celoria 26, Milan I-20133, Italy. [2]Department of Animal Science, University of Yüzüncü Yil, Van 65090, Turkey.

*Author for correspondence (marco.parolini@unimi.it)

M.P., 0000-0003-0226-1709

Received 20 July 2016; Accepted 25 November 2016

Antioxidant defenses of vertebrates consist of two major classes of mechanisms and the associated effector molecules. First, enzymatic defense pathways, mainly mediated by endogenous substances, remove reactive molecular species or their intermediate derivatives, or catalyze their transformation into less active compounds (Halliwell and Gutteridge, 2007; Surai, 2000). Second, non-enzymatic antioxidants act as cofactors of antioxidant enzymes, remove metal ions, or undergo oxidation to quench free radicals and other reactive species. Several non-enzymatic antioxidants cannot be synthesized by animals and are therefore acquired either via the food or, before hatching, from the maternal egg materials (Møller et al., 2000; Surai, 2002).

While antioxidant defense is thought to be important throughout an organism's life, this is especially the case during embryo development and growth because intense embryonic metabolism entails massive production of oxidizing molecules, and inefficient defense from oxidative damage can have long-lasting, negative fitness consequences (Surai, 2002). The eggs of vertebrates contain large amounts of antioxidants of maternal origin (Surai, 2002). Mothers are expected to tend to optimally equip their eggs with exogenous antioxidants, under the constraints set by trade-offs with their self-maintenance requirements, dietary limitation of antioxidants and other environmental effects (Mousseau and Fox, 1998; Müller et al., 2012; Surai, 2002). Transfer of antioxidants to the eggs is therefore part of complex epigenetic 'maternal effects' whereby mothers modulate offspring performance and phenotype.

The developmental and growth consequences of variation in the concentration of egg yolk antioxidants have been at the focus of increasing interest in ecological evolutionary studies and in animal production disciplines (Ebrahimi et al., 2012; Müller et al., 2012; Romano et al., 2008; Saino et al., 2002, 2003; Selim et al., 2012; Surai, 2002). Some studies have investigated the consequences of variation in maternal dietary antioxidants on egg composition and subsequent offspring performance (Blount et al., 2002). Other studies have manipulated the concentration of specific antioxidants in the yolk and recorded the behavioral, growth or physiological consequences in the offspring (de Ayala et al., 2006; Gao et al., 2013; Romano et al., 2008; Saino et al., 2003).

The antioxidant system is thought to operate in a highly integrated way, meaning that functional relationships occur among antioxidants and their physiological pathways (Surai, 2002). For example, vitamin E can be recycled to its non-oxidized form by other antioxidants (e.g. ascorbic acid, carotenoids; Palozza and Krinsky, 1992; Surai, 2002). Functional integration also implies that different antioxidant pathways may operate in a synergistic way, if the effect of one antioxidant depends on the concentration of other antioxidants.

Functional relationships among exogenous antioxidants lead to the expectation that mothers should tune not only the absolute amount of antioxidants that they allocate to the eggs, but also their

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List of abbreviations

BLMM	bivariate linear mixed model
LMM	linear mixed model
LPO	lipid peroxidation
PC	protein carbonylation
TAC	total antioxidant capacity
TBARS	thiobarbituric acid reactive substances (method)
TOS	total concentration of pro-oxidant molecules

relative concentrations, so as to achieve an optimal balance. A corollary expectation is therefore that variation in the concentration of individual antioxidants alters the functional relationships between other interacting antioxidants. Moreover, the patterns of covariation among the concentrations of different antioxidants can vary according to embryo sex (Berthouly et al., 2008; Martínez-Padilla and Fargallo, 2007; McGraw et al., 2005) and laying order (Rubolini et al., 2011). This is expected because maternal physiology limits the ability to transfer antioxidants to eggs that are laid in rapid sequence, and/or because mothers adopt adaptive strategies of allocation of critical resources to eggs with different expected reproductive value (see Rubolini et al., 2011). Finally, variation in the concentration of a specific antioxidant can affect the distribution of other antioxidants across bodily districts, and also their covariation with embryo traits (such as growth) and oxidative status.

Despite evidence that optimal functioning of antioxidant defenses depends on the relative concentration of the individual antioxidants, studies of the patterns of correlation among functionally related egg components are rare (Rubolini et al., 2011). Moreover, the consequences of experimental supplementation of antioxidants on the distribution of other antioxidants and on their effects on markers of oxidative status are largely unknown. Indeed, notwithstanding considerable interest in the analysis of variation in pre-natal exposure to antioxidants and oxidative status, several important issues still need to be tackled.

Here, we therefore strived to answer the general questions that are detailed below and are graphically, qualitatively illustrated in Fig. 1. We capitalize on an experiment on the yellow-legged gull (*Larus michahellis* Naumann 1840) where we injected the egg yolk with physiological vitamin E (α- and γ-tocopherol) doses. We collected tocopherol-supplemented and control eggs shortly before hatching and dissected them to measure embryo morphology. The residual yolk in the yolk sac (ca. 70% of the estimated original yolk mass), the liver and brain were dissected to measure the concentration of vitamin E (α- and γ-tocopherols and the corresponding tocotrienols), carotenoids (mainly lutein, zeaxanthin and β-carotene; see Rubolini et al., 2011), retinol (vitamin A), coenzyme Q10 and ascorbic acid (vitamin C) were measured only in yolk and brain, respectively. Oxidative status was assessed by measuring total antioxidant capacity (TAC), the concentration of total pro-oxidant molecules (TOS, according to the terminology by Erel, 2005), protein carbonylation (PC) and lipid peroxidation (LPO), in the liver and in the brain. TAC was also measured in residual yolk. We focused on liver because it is the main organ where antioxidants are stored, and on brain, because it is believed to be particularly sensitive to lipid peroxidation (Surai, 2002).

The following questions were addressed.

(Q1) Do embryo morphological traits covary with the concentration of egg antioxidants or oxidative status? We expected embryo growth to be positively predicted by the antioxidant concentrations and TAC, and negatively predicted by TOS and markers of oxidative damage (Fig. 1A; see also Rubolini et al., 2011).

(Q2) Do antioxidants and oxidative status markers covary within or between organs? We predicted that antioxidant concentrations and oxidative status markers would positively reciprocally correlate within and between organs (or the yolk) (Fig. 1B,C). This was expected because antioxidants are believed to be most often limiting in the diet, thereby causing mothers with larger access to dietary antioxidants to allocate more of them to all bodily districts.

(Q3) Does the concentration of antioxidants predict oxidative status? Higher concentrations of antioxidants were expected to be associated with better oxidative status (Fig. 1D), i.e. lower concentration of total pro-oxidant molecules and oxidative damage.

(Q4) Do antioxidants synergistically affect embryo growth and oxidative status? Because of limited information on the combined effects of different antioxidants on other embryo traits, we had no specific prediction on synergistic (i.e. interaction) effects of antioxidants (Fig. 1E).

(Q5) Does the concentration of one antioxidant affect the relationship between that particular antioxidant and other antioxidants? The increase in the concentration of one antioxidant could affect the distribution and use of other antioxidants, and thus the relationship of embryo traits with other antioxidants. However, we have no directional expectation on these relationships (Fig. 1F).

(Q6) Does egg supplementation with one antioxidant affect the concentration of other antioxidants or oxidative status, and do the effects depend on sex or laying order?

(Q7) Does egg supplementation with one antioxidant affect the covariation between other embryo traits?

(Q8) Does egg supplementation with one antioxidant affect the relationship between morphology or oxidative status and other antioxidants? An increase in the concentration of a focal antioxidant may differentially affect the use of other antioxidants (Fig. 1G,H), variation in other antioxidant or oxidative status (Fig. 1I), and thus the relationships between embryo traits and other antioxidants (Fig. 1J).

As for the consequences of experimental manipulation of egg vitamin E levels (Q6–Q8), because of limited information on interactions among antioxidants, we had no explicit directional predictions. However, the paradigm of the integration of the antioxidant system led us to expect functional interactions between components. We therefore tested for any such effects and decided to interpret any emerging pattern *a posteriori*.

We emphasize that the present study is conceived as an exploratory exercise of the relationships between antioxidants, oxidative status markers and morphology of the embryos also after manipulation of antioxidant concentrations to contribute filling a remarkable gap of studies asking the very general questions listed above.

The analyses that specifically refer to antioxidants in the yolk rest on the assumption that the antioxidant concentrations in the residual yolk at the stage when the eggs were collected, which is on average 70% of the original yolk mass, are proportional to the concentrations in the yolk at earlier times of embryo development. Because we are aware of no study where this has been tested and we have no reason to speculate that differential absorption of antioxidants from the yolk produced the relationships that we observed, this will be considered as an assumption.

MATERIALS AND METHODS

Field and experimental procedures

We studied a large colony of yellow-legged gull (*Larus michahellis*) in the Comacchio lagoon (NE Italy; 44°20′ N–12°11′ E) during March–May 2014. The colony was visited every second day and when a new egg was found, it was temporarily removed from the

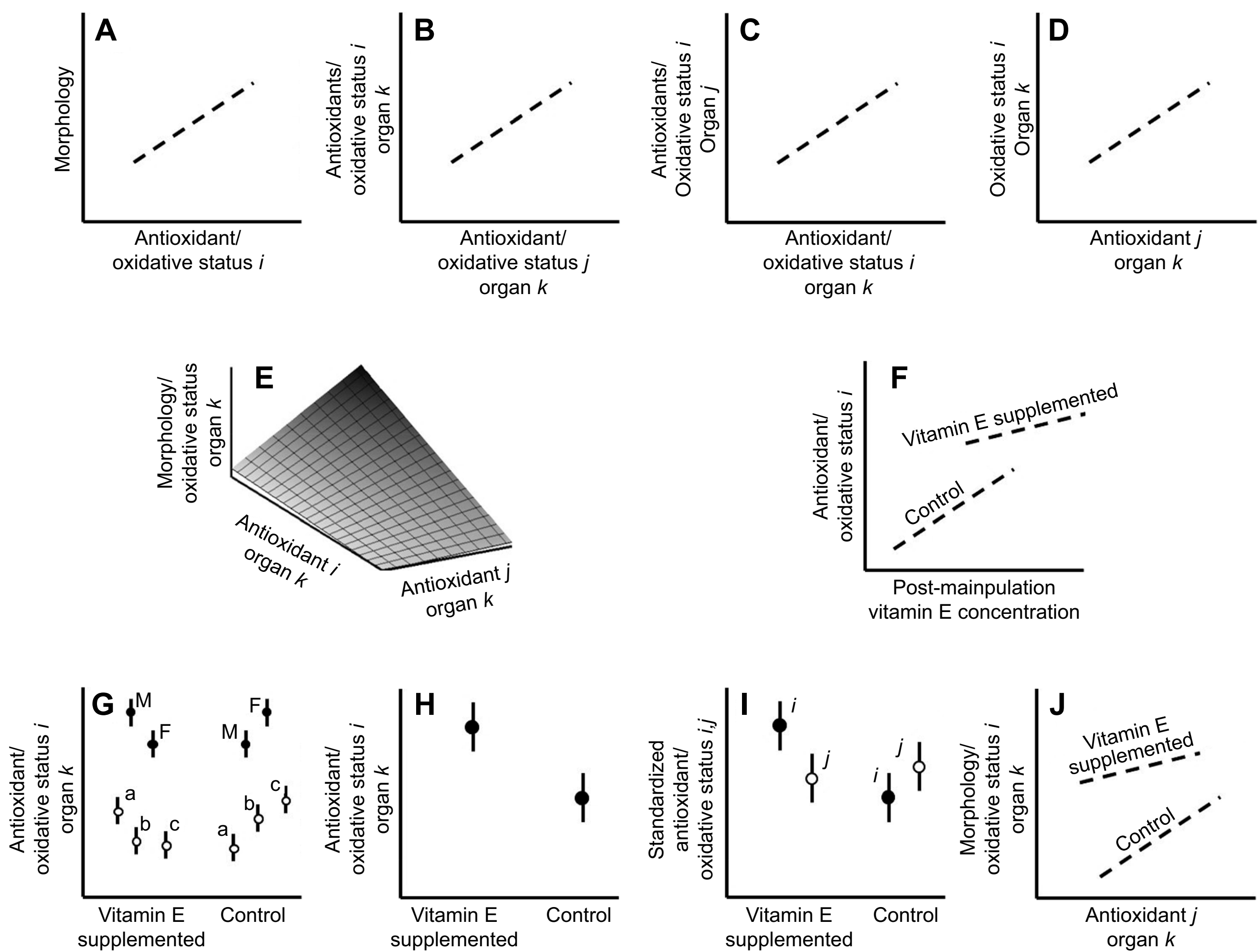

Fig. 1. Investigated relationships among antioxidant concentrations in the yolk, embryo morphological traits and oxidative status. The graphs are merely illustrative examples. M: males; F: females; a, b, c: first-, second-, third-laid eggs.

nest for experimental manipulation. The experiment was performed as described in Parolini et al. (2015). We aimed at increasing the concentration of vitamin E (α- and γ-tocopherol; 93:7 ratio) by 1 standard deviation of that measured in the eggs from the same colony (Rubolini et al., 2011), by *in ovo* injection, so that the final concentration of vitamin E was within the natural range of variation. The dose due to be injected was scaled depending on egg mass and laying order (Parolini et al., 2015) (Table S1).

We adopted a within-clutch design, whereby both control and vitamin E-injected groups were established within each clutch, to minimize the confounding effects of environmental and parental effects. The following treatment schemes were assigned sequentially to the clutches (nest, a-, b-, c-egg): nest 1, vitamin E injection (E), control injection (C), E; nest 2, C-E-C; nest 3, E-C-C; nest 4, C-E-E and so forth with the following nests.

Egg collection and embryo dissection and measurement

When eggshell fractures appeared (ca. 24 days after laying), the eggs were collected and stored frozen (−20°C) until dissection. In the laboratory, we removed the eggshell and the residual yolk sac was detached from the embryo. Before dissection, the embryo was weighed and tarsus and skull lengths were measured using calipers. The liver and brain were explanted from the embryo, weighed and frozen at −80°C until biochemical analyses. All measurements were performed blind of embryo treatment, sex and laying order by a single operator to ensure consistency. Embryo sex was determined molecularly (Saino et al., 2008).

The study was performed under permission of the Parco Regionale del Delta del Po (no. 657, 4 February 2014). Although the Guideline on The Use and Euthanasia Procedures of Chicken/ Avian Embryos draft by the Animal Care and Use Committee discourages hypothermia for euthanasia of avian embryos, we had to perform this procedure, placing the eggs into a −20°C freezer within 2 h of collection owing to facility constraints. As confirmed by the Guidelines for the Euthanasia of Animals by the American Veterinary Medical Association, physical methods of euthanasia may be necessary in some field situations if other methods are impractical. This was the case here because we performed a field experiment in which we could not euthanize embryos by methods such as carbon dioxide (CO_2), anesthetic agents or decapitation. Dissection occurred within 1 month of collection.

Antioxidant concentrations

As antioxidants, we measured vitamin E (α- and γ-tocopherol and -tocotrienols), retinol and carotenoid concentrations in liver, brain and residual yolk sac, while coenzyme Q10 and ascorbic acid were measured only in yolk and in brain, respectively. All the analyses were performed using high-performance liquid chromatography (HPLC), as described by Karadas et al. (2016) and Mitić et al. (2011; for ascorbic acid).

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Markers of oxidative status

As markers of oxidative status, we measured TAC, TOS, PC and LPO. These assays were performed on liver and brain homogenates, while only TAC was measured in residual yolk sac. Briefly, TAC and TOS were measured according to colorimetric methods developed by Erel (2004, 2005, respectively), with slight modifications. Carbonylated proteins were measured as described by Parolini et al. (2016), while lipid peroxidation was measured using the thiobarbituric acid reactive substances (TBARS) method (Ohkawa et al., 1979). However, it should be noted that the TBARS method may not measure oxidative damage to lipids accurately because TBA reacts with other compounds, apart from the main LPO byproduct malondialdehyde. Thus, TBARS results should be interpreted with caution because they may overestimate LPO (Halliwell and Gutteridge, 2007).

Statistical analyses

Given the complexity of the statistical analyses performed to answer the focal questions of the study (see Introduction), we present the analyses for each question separately. However, as we run the same correlation analyses to answer Q1, Q2 and Q3 (see above), these were grouped under a single heading.

Questions 1–3

The correlation between morphological traits, antioxidant concentrations and oxidative status markers (Fig. 1A–D) was analyzed using bivariate linear mixed models (BLMMs) where egg treatment, sex, laying order (factors) and egg mass (covariate) were included as independent effects and brood was included as a grouping factor, according to the procedure outlined in Dingemanse and Dochtermann (2013). Restricted maximum likelihood was adopted to estimate model parameters. The within-brood correlations were computed using the variance and covariance estimates according to eqn 7d in Dingemanse and Dochtermann (2013). The significance of the within-brood correlation coefficients was estimated by likelihood ratio tests (maximum likelihood estimation) (Dingemanse and Dochtermann, 2013). When BLMMs failed to converge (23% of the cases), we relied on correlation analyses.

We did not test all the possible 780 bivariate relationships and focused on the relationships between embryo morphology and antioxidants or oxidative status markers in all ‘organs’ (including the yolk); antioxidants and oxidative status markers within organs; and individual antioxidants or oxidative status markers between organs, yielding a total of 497 relationships (Fig. S1A–F). However, in calculating the number of relationships that were consistent or, conversely, opposite to the expectation, we did not consider total tocopherols or total tocotrienols but only the α and γ isoforms of these compounds separately, in order to avoid pseudo-replication of the information.

For easier visualization of the results of BLMM analyses, we graphically represented the relationships as ellipses in synoptic graphs included in Fig. S1. We refrained from estimating the between-brood correlations from BLMMs (Dingemanse and Dochtermann, 2013) because of the small size of the clutches (maximum three eggs) and the unbalanced sample of either sex or egg treatment within broods.

Question 4

Individual embryo morphological traits or oxidative status markers were analyzed in linear mixed models (LMMs) including vitamin E egg treatment, sex and laying order as factors and egg mass as a covariate. In the model we also included one pair at a time of antioxidant concentration variables together with their interaction (Fig. 1E). Clutch was included as a random effect. In these analyses we only considered total tocotrienols or tocopherols, because we assume that the effects of α and γ isoforms of either class of compounds are additive.

Question 5

We tested whether an experimental increase in yolk vitamin E concentration affected the relationship between embryo traits and (post-manipulation) vitamin E concentration (i.e. if the slope of the relationship between embryo traits and post-manipulation vitamin E concentration differed between control and vitamin E supplemented eggs; Fig. 1F) in LMMs where we included the effects of sex, laying order (factors), original egg mass (covariate) as well as vitamin E treatment, vitamin E post-manipulation concentration (covariate; total tocopherols only) and their two-way interaction. In the model we also included the random effect of clutch.

Question 6

We tested whether vitamin E treatment differentially affected the concentration of the focal substances and oxidative status markers depending on sex and laying order (Fig. 1G,H) in LMMs where we included the effects of vitamin E egg treatment, sex, laying order (factors), original egg mass (covariate) as well as the two-way interaction effects between factors. Clutch was included as a random effect. We also ran these models excluding the two-way interaction between factors.

Question 7

To test whether vitamin E treatment had a differential effect on the concentration of antioxidants or markers of oxidative status (Fig. 1I), we designed LMMs with treatment, sex, laying order (factors) and original egg mass (covariate) as predictors. In addition, we included a trait (factor) and its two-way interaction between treatment and trait. This analysis posed the problem that different traits can be incommensurable (being measured in different units) or have different means and/or variances. The values of each of the two focal traits were therefore standardized to a mean of 0 and variance of 1. These analyses were performed considering each pair of variables within organs. Thus, in these models, the trait-by-vitamin E treatment term tests whether an increase in vitamin E concentration caused a differential variation, expressed in standard deviation units, in the concentration of different antioxidants or oxidative status markers.

Question 8

We tested whether the relationship between morphological traits or oxidative status markers and antioxidants differed between vitamin E treatment groups (Fig. 1J) in LMMs where vitamin E egg treatment, sex, laying order (factors) and original egg mass (covariate) were included as predictors. In addition, in the model we included the effect of the specific antioxidant under scrutiny as well as its interaction with vitamin E treatment.

Multiple testing issues

Throughout this study, we performed a large number of tests. This was the case because this was admittedly an exploratory exercise where we described the relationships among many variables. Performing multiple tests can inflate the risk of incurring type I statistical errors. In contrast, lowering of the α-level of the tests according to commonly used procedures (e.g. Bonferroni correction) would cause excessive reduction of statistical power. We therefore adopted the approach taken, for example, by Cohen

et al. (2008): we present the results of the tests and qualify those with *P*-values <0.05 as 'significant'. However, we warn the readers that part of these 'significant' tests could be due to type I statistical errors. In addition, we focus on the general patterns of association among the variables and qualitatively check whether the relationships are consistent in sign with the expectation. To qualitatively summarize the information from so many tests, in analyses relevant to Q1–Q4 we report the number of tests that were statistically significant (see above), whose associated *r* was >0.15, or was such that $-0.15 \leq r \leq 0.15$, while distinguishing between the relationships that were in the direction predicted or, respectively, opposite to the expectation. Because very limited evidence for significant relationships emerged from the analyses used to answer Q5–Q8, the results of these analyses are only briefly summarized in the Results. All analyses were run in SAS 9.3 (see Dingemanse and Dochtermann, 2013 and references therein).

Meta-analyses of the relationships among morphological traits, antioxidants and oxidative status markers

We computed unsigned Fisher *z*-transformed correlation coefficients (*Zr*) for each relationship. When the direction of the observed correlation was consistent with the expectation, we assigned *Zr* a positive sign, whereas we assigned a negative sign when the direction was opposite to the expectation. We then tested whether mean *Zr*, weighted by *n*–3 (*n*=number of individuals in the correlation analysis), significantly deviated from 0 (Borenstein et al., 2009) within each set of correlations (see Results). Significance was implied when the confidence interval (CI) of the estimated mean *Zr* did not encompass 0.

RESULTS

Overall, the sample included 66 late-stage embryos from the eggs of 26 clutches (30 controls, 36 vitamin E-treated; 29 males, 37 females; 21 a-, 26 b-, 19 c-eggs). In some analyses, information for up to 10 eggs was not available; sample size thus ranged between 56 and 66 eggs depending on the analysis.

Question 1

Embryo morphological measures were generally positively correlated (relationship in Fig. 1A and Fig. S1A), as expected (*n*=10 relationships; positive: 90%; significantly positive: 60%; null: 10%), with mean *Zr* (0.372; CI: 0.249–0.495) being significantly larger than 0 (Fig. 2).

The concentration of carotenoids, tocopherols and γ-tocotrienols in the yolk were generally positively correlated with the morphological measures (relationship in Fig. 1A and Fig. S1B), consistent with expectations (*n*=45; expected positive direction: 58%; expected and significant: 33%; null: 36%; opposite: 7%; significantly opposite: 0%). Morphological measures also positively covaried with the concentration of retinol (body size and tarsus length only) and α- and γ-tocopherol in the liver. Also for the liver, the pattern of association of the morphological measures with the concentrations of antioxidants was mostly consistent with expectations (*n*=40; expected positive direction: 53%; expected and significant: 20%; null: 45%; opposite: 3%; significantly opposite: 0%). However, there were generally weak, inconsistent relationships between morphological traits and antioxidant concentrations in the brain (*n*=45; expected positive direction: 24%; expected and significant: 0%; null: 64%; opposite: 11%; significantly opposite: 4%). The mean *Zr* (0.141; CI: 0.105–0.177) for the relationships between antioxidants and morphological traits was significantly larger than 0 and thus consistent with the expectation (Fig. 2).

The correlations between embryo morphology and markers of oxidative status are shown in Fig. S1C (relationship in Fig. 1A). Embryo morphological traits showed a weak positive relationship with yolk TAC. As expected, TOS in the liver significantly negatively covaried with body mass, skull length and liver mass, and non-significantly negatively covaried with tarsus length, whereas the association with brain size was 'null'. LPO and PC in the liver did not show any clear pattern of association with morphological traits. TOS and LPO in the brain were negatively associated with brain size and also with liver size, as expected. Finally, body mass was also negatively associated with LPO in the brain. The mean *Zr* (0.070; CI: 0.010–0.131) for the relationships between morphological traits and markers of oxidative status was significantly larger than 0, again consistent with the expectation (Fig. 2).

Question 2

The correlations that we observed between pairs of antioxidants are summarized in Fig. S1D. Within (relationship in Fig. 1B) the yolk or the liver, the correlations between the concentrations of carotenoids, retinol, and α- or γ-tocopherols or -tocotrienols were mostly positive and consistent with expectations (yolk: *n*=15; expected direction: 60%; expected and significant: 53%; null: 40%; opposite: 0%; liver: *n*=15; expected direction: 60%; expected and significant: 20%; null: 40%; opposite: 0%) (total tocopherols and tocotrienols are not considered here as they are the sum of α and γ isoforms). In addition, the concentration of coenzyme Q10 in the yolk was positively correlated with yolk antioxidants (*n*=6; expected direction: 83%; expected and significant: 67%; null: 17%; opposite: 0%). Within the brain, the relationships were also mostly positive, as expected (*n*=15; expected direction: 60%; expected and significant: 27%; null: 20%), while some were in the direction opposite to the expectation (opposite: 20%; significantly opposite: 13%).

Between (relationship in Fig. 1C) yolk and liver or brain, the correlations of antioxidants (we considered concentrations of carotenoids, retinol and α- and γ-tocopherols or -tocotrienols isoforms separately) were also generally positive and consistent with the expectation, or null, but were never negative and in the direction opposite to the expectation (yolk–liver: *n*=18; expected direction: 44%; expected and significant: 22%; null: 56%; opposite: 0%; yolk–brain: *n*=18; expected direction: 39%; expected and significant: 17%; null: 61%; opposite: 0%). The correlations between liver and brain were generally weak (*n*=18; expected direction: 28%; expected and significant: 6%; null: 61%; opposite: 11%; opposite and significant: 0%). The mean *Zr* (0.266; CI: 0.189–0.343) for the relationships between pairs of antioxidants traits was significantly larger than 0, as expected (Fig. 2).

The correlations that we observed between pairs of oxidative status markers are summarized in Fig. S1F. There were generally weak relationships within organs between markers of oxidative status, with the exception of the expected positive relationship between TOS and LPO in the liver and the negative relationship between TAC and LPO in the brain. However, the relationship between TAC and TOS in the brain was statistically significantly positive, contrary to our expectation. No correlations for oxidative status markers emerged between organs. The mean *Zr* (0.003; CI: –0.092 to 0.099) for the relationships between oxidative status markers did not significantly deviate from 0, contrary to the expectation (Fig. 2).

Question 3

The observed correlations (relationship in Fig. 1D) are summarized in Fig. S1E. Antioxidant capacity in the yolk

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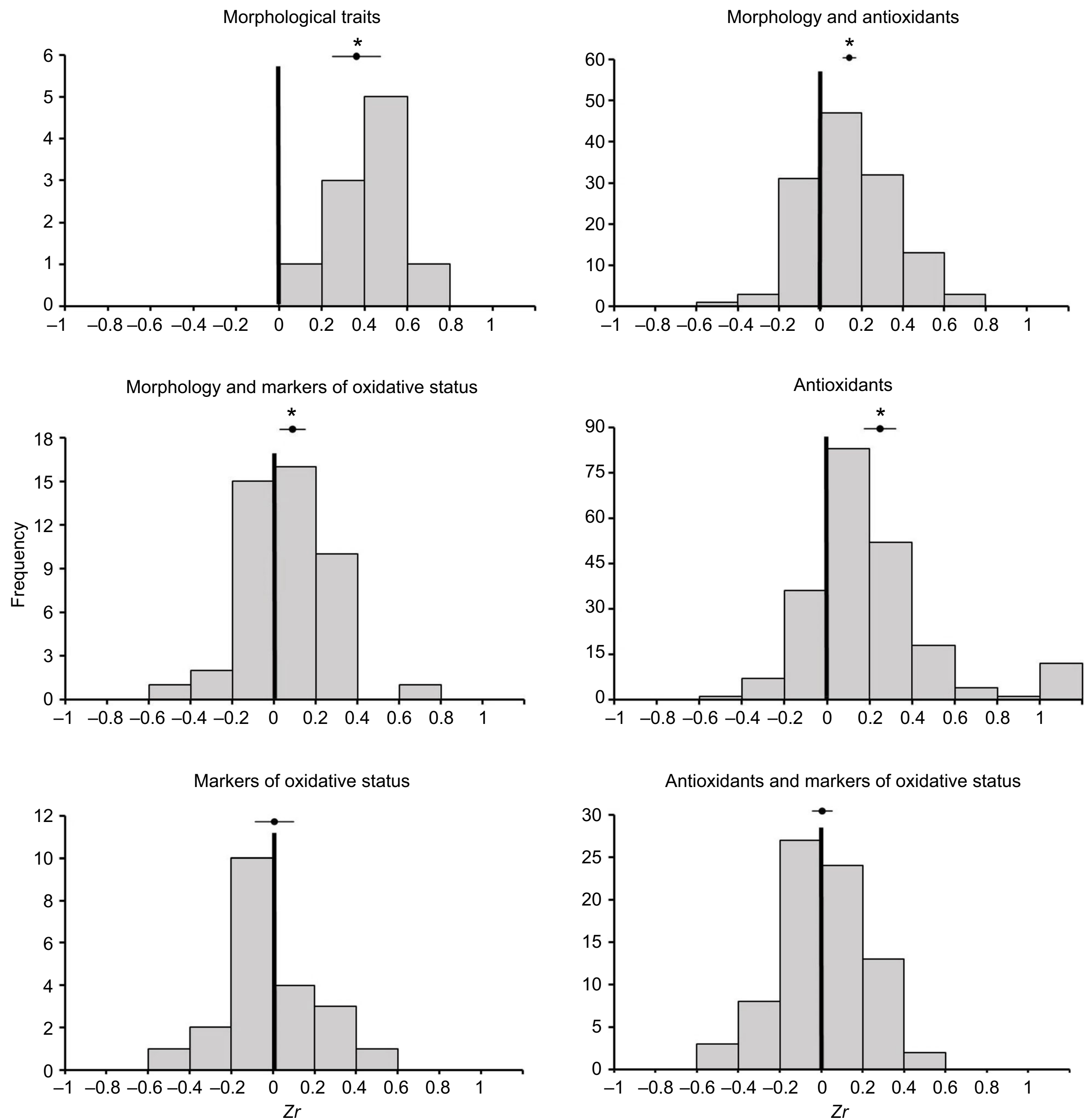

Fig. 2. Frequency of Fisher *z*-transformed correlation coefficients (*Zr*) calculated for the relationships among morphological traits, between morphology and antioxidants, between morphology and markers of oxidative status, among antioxidants, among markers of oxidative status, and between antioxidants and markers of oxidative status. For the relationship among antioxidants, we pooled *Zr* values >1 into a single class. When the direction of the observed correlation was consistent with the expectation (see Introduction), we assigned *Zr* a positive sign, whereas we assigned a negative sign when direction was opposite to the expectation. The mean *Zr* value (black circle) is reported with its 95% confidence interval (CI). Significance (*) is implied when the estimated CI did not encompass zero.

positively covaried with the concentration of most of the focal antioxidants (total tocopherols and tocotrienols are not considered here as they are the sum of α and γ isoforms), although statistical significance was attained in just one case (n=7; expected direction: 57%; expected and significant: 14%; null: 43%). Similarly, in the liver, TAC was positively predicted by the concentration of all antioxidants but in no test was statistical significance attained (n=6; expected direction: 100%; expected and significant: 0%). A positive, expected association between TAC in the brain and antioxidants was observed for half of the relationships (n=7; expected direction: 43%; expected and significant: 14%; null: 29%; opposite: 29%; significantly opposite: 0%).

There were generally weak, inconsistent relationships between antioxidants and markers of oxidative status in the liver (n=18; expected direction: 6%; expected and significant: 0%; null: 67%; opposite: 28%; significantly opposite: 17%) or in the brain (n=21; expected direction: 5%; expected and significant: 0%; null: 57%; opposite: 38%; significantly opposite: 5%; Fig. S1E). The mean z (0.015; CI: −0.033 to 0.063) for the relationships between

antioxidants and markers of oxidative status did not significantly differ from 0, contrary to the expectation (Fig. 2F).

Question 4

There was only very weak evidence that the statistical effects of antioxidants on embryo traits were synergistic (relationship in Fig. 1E). In fact, out of 135 LMMs testing the interaction between pairs of antioxidants on morphological traits or oxidative status markers, only in three cases did the interaction effect between antioxidants attain statistical significance (see Table S2).

Question 5

Experimental increase in vitamin E yolk concentration could affect the relationship between post-manipulation vitamin E concentration and the concentration of the other antioxidants or oxidative status markers (relationship in Fig. 1F). In LMMs, we found generally no statistically significant effects of treatment by vitamin E concentration in the yolk, with the exception of a significant interaction effect on α-tocotrienol or total tocotrienol concentration in the yolk (Table S3). The relationships were marginally non-significantly positive in control eggs and non-significantly negative in vitamin E eggs (Table S3).

Question 6

We tested whether egg treatment differentially affected the concentration of antioxidants and the oxidative status markers depending on sex and position in the laying sequence (relationship in Fig. 1G). In no case did we find a significant effect of the egg treatment by sex or the egg treatment by laying order interactions in LMMs (P>0.05).

LMMs testing the main effect of egg vitamin E treatment on the concentrations of the focal substances in the yolk (excluding tocopherols) and in the organs (relationship in Fig. 1H) generally did not disclose significant effects (P>0.05), with the exception of the concentration of α-tocotrienol ($F_{1,35}$=4.83, P=0.035) and total tocotrienols ($F_{1,35}$=4.46, P=0.042) in the liver, which was significantly larger in vitamin E eggs.

Hence, vitamin E treatment did not generally affect the relationship between vitamin E concentration post-treatment and the concentration of the focal antioxidants or oxidative status markers in the yolk and organs. In addition, vitamin E treatment did not affect the concentration of the other compounds in the yolk or in the liver and brain.

Question 7

In general, there was only weak evidence for a differential variation of pairs of antioxidants/oxidative status markers (values standardized to a mean of 0 and variance of 1) between the control and the vitamin E supplemented eggs (relationship in Fig. 1I). γ-tocotrienol concentration and LPO in the liver declined after vitamin E supplementation, while concentration of α-tocotrienol increased. In addition, the total antioxidant capacity in the brain declined in vitamin E-treated embryos, while TAC increased in the yolk. It must be emphasized that these differential patterns of variation among the endpoints following vitamin E treatment with respect to controls emerged out of a large number (n=497) of tests and should therefore be considered with caution.

Question 8

Out of 122 LMMs of morphological or oxidative status traits, the two-way interaction between vitamin E treatment and antioxidant concentrations (the interactions were tested for all the possible pairs of antioxidants; relationship in Fig. 1J), had a significant effect in only 12 cases (Table S4). Thus, there was weak overall evidence for differential effects of individual antioxidants depending on the experimental manipulation of vitamin E in the egg.

DISCUSSION

In this study of the yellow-legged gull, we explored the patterns of covariation among late-stage embryo morphological traits, concentration of antioxidants and oxidative status markers in focal embryo organs and in the yolk. In addition, we manipulated the concentration of yolk vitamin E to test for the consequences of variation in a major antioxidant on the distribution and use of the other antioxidants and on oxidative status markers in the yolk and focal organs. We found evidence that the embryo growth was positively associated with the antioxidant concentrations, and weaker evidence that it was negatively predicted by markers of oxidative status (Q1). Consistent with the expectations, the antioxidant concentrations were positively correlated both within organs and between the yolk and the other organs (Q2). However, markers of oxidative status were only partly correlated within organs and not correlated between organs (Q2). TAC was positively associated with the concentration of antioxidants in the yolk, liver and also in the brain, but there was no clear pattern of association between TOS, LPO or PC and antioxidant concentration (Q3). Antioxidants did not synergistically predict embryo growth or oxidative status (Q4). Finally, experimental increase in egg vitamin E level did not affect the relationship between other antioxidants or markers of oxidative status and vitamin E concentration (Q5); the concentration of other antioxidants or the markers of oxidative status, also depending on sex and laying order (Q6); the covariation between other antioxidants or oxidative status markers (Q7); or the relationship between morphological traits or oxidative status and other antioxidants (Q8).

The main methodological novelty of our study is that we coupled information on traits (morphology, antioxidant concentration and oxidative status markers) of late-stage embryos with information on the concentration of antioxidants in the residual yolk, thereby investigating how late-embryo traits covary with the quality of the original egg environment. Below, we discuss the general findings of our study, but we do not go into the specific details of the individual relationships because part of the statistically significant correlations could have arisen because of an inflation of type I statistical errors.

Question 1

Consistent with the general expectation that antioxidants promote condition and thus embryonic growth (Bhanja et al., 2012; de Ayala et al., 2006; Noguera et al., 2011; Parolini et al., 2015; Selim et al., 2012), there were generally positive bivariate relationships between antioxidant (carotenoids, tocopherols) concentrations in the yolk and in the liver (retinol, tocopherols) and embryo morphological traits, while controlling for the effects of sex, laying order and egg mass. This relationship could be causal, as suggested by experimental studies (Deeming and Pike, 2013; Marri and Richner, 2014; Parolini et al., 2015; Romano et al., 2008; Saino et al., 2011). Alternatively, the size of an embryo may also reflect its degree of maturation, with larger/more mature embryos showing stronger antioxidant defenses. The observations that embryo size was negatively associated with TOS in the liver and that markers of oxidative status (PC in the liver, TOS and LPO in the brain) negatively predicted brain size are also consistent with expectations, because overproduction of pro-oxidants and the consequent oxidative imbalance should be detrimental to developmental and growth processes (Smith et al., 2016).

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Question 2

Eggs with relatively large concentrations of one antioxidant also tended to have relatively high concentrations of other antioxidants. In addition, the concentrations of antioxidants in the yolk tended to be positively correlated with those in the liver and brain. These results are consistent with those of a previous study where we considered yolk but not liver and brain composition because we relied on eggs at a very early incubation stage (Rubolini et al., 2011). Present findings suggest that some mothers have access to relatively large amounts of all antioxidants, and this results in large concentrations in the yolk and, concomitantly, in embryo organs (Costantini and Verhulst, 2009). Alternatively, in order to optimally accomplish their functions, the amounts of antioxidants that mothers allocate to the eggs must be balanced. Thus, dietary antioxidants may not be limiting, and mothers may decide to allocate different antioxidants to the eggs in amounts that are reciprocally positively correlated.

The relationships between markers of oxidative status were consistent with expectations, with, for example, markers of oxidative damage being reciprocally positively correlated within the brain and negatively correlated with TAC. Thus, particularly in the brain, embryos with large TAC have smaller oxidative damage to lipids. In addition, TOS in the liver was associated with more severe lipid peroxidation.

Importantly, the correlations between markers of oxidative status in the brain and liver were generally weak, implying that oxidative damage in a particular organ does not allow inference on oxidative status in other organs. This weak relationship may also suggest a sort of ‘hierarchy’ of protection and/or differential sensitivity of the organs to oxidative stress. The brain contains the highest concentration of double bonds, especially C_{20} and C_{22} polyunsaturated fatty acids, which exposes the brain to the risk of oxidative damage (Surai, 2002). Embryos may prioritize antioxidant protection of the brain, thereby uncoupling oxidative damage to the brain from oxidative damage to other organs.

Question 3

Notably, the correlation between antioxidants and markers of oxidative status was consistent with the expectations for TAC, but not for TOS, LPO and PC. This result implies that oxidative damage cannot be inferred by the concentration of antioxidants. Hence, large amounts of dietary antioxidants that mothers allocate to the eggs do not necessarily result in lower oxidative damage, possibly because oxidation of lipids and proteins also largely depends on antioxidant defense afforded by other physiological pathways, mediated by enzymatic activity (Costantini and Verhulst, 2009).

Question 4

By measuring several antioxidants in the yolk and organs, we could test whether the statistical effect of the concentration of one antioxidant on morphological traits or oxidative status depended on the concentration of other antioxidants. Assuming that the relationships between embryo morphology or oxidative status markers and antioxidants considered individually at least partly reflect causation (see above), the present findings of no statistically significant interaction effects between antioxidants suggest that the effects of individual antioxidants on embryo traits do not depend on the concentration of other antioxidants, i.e. there are no measurable synergistic effects between antioxidants on embryo traits. This result is further corroborated by the results of the egg vitamin E supplementation experiment (see below), implying small integration of the components of the antioxidant system, at least under the experimental conditions of the present study.

Questions 5–8

As the increase of egg vitamin E level did not affect embryonic traits, we collectively discuss the results relevant to Q5–Q8. To the best of our knowledge, this is the first study in which the effects of a direct manipulation of egg concentration of one major antioxidant on the destination of other antioxidants and their relationships with morphological traits and oxidative status have been investigated in any species in the wild. The main outcome of the experiments is that there is no evidence for major functional interactions among maternal egg antioxidants of dietary origin. A major strength of the present experiment is that manipulation of the concentration of vitamin E in the egg occurred within the physiological range and directly into the eggs, rather than via the mother.

Several experimental studies have suggested that interactions exist among different antioxidants as well as between these and other components of the antioxidant system (Surai, 2002). These interactions occur, for example, in the form of reciprocal modulation of absorption or retention of antioxidants in specific tissues; modulation of the effects of other dietary or endogenous antioxidants; and/or in recycling of oxidized to non-oxidized forms or protection from auto-oxidation (Catoni et al., 2008; Surai, 2000). As a result of these interactions, the effects of individual antioxidants on physiological or morphological endpoints should depend on the combined effects of individual antioxidants. These experiments have typically been performed on domestic and/or artificially selected strains (e.g. mice, poultry; Jacob, 1995; Surai, 2000) and under captive conditions, and have very seldom concerned the effects of pre-natal exposure to (egg) antioxidants on pre-natal phenotype. In addition, experimental design, in terms of dosage of dietary antioxidants, has seldom been framed in terms of natural variation, possibly partly owing to the lack of natural reference conditions for domestic strains. The lack of measurable interaction effects between antioxidants on embryo phenotype (morphology or oxidative status) or effects of an experimental increase of vitamin E on distribution and effects of other antioxidants (i.e. no evidence for synergist effects) challenges the common wisdom that major functional interactions occur between exogenous egg antioxidants in determining embryo phenotype, at least in this species in the wild, and within physiological limits of variation of antioxidant concentrations.

Previous studies of birds led to partly inconsistent results on the interactions among components of the antioxidant system. A correlative study demonstrated that the correlations among some antioxidants (uric acid, carotenoids and vitamin E) and TAC varied across species. Overall, TAC strongly covaried with uric acid levels, both across species and within 23 of 30 studied species, while carotenoid concentrations positively covaried both among and within species. In contrast to our findings, vitamin E concentration did not strongly correlate with other antioxidants or with TAC (Cohen et al., 2008). Studies of the Leach’s storm petrel (*Oceanodroma leucorhoa*), a long-lived seabird, and of the Savannah sparrow (*Passerculus sandwichensis*), a short-lived migratory passerine, showed a significant correlation between vitamin E and total antioxidant capacity in the former but not in the latter species (Cohen et al., 2009a,b). All these findings, in combination with those from the present study, confirm the complexity of the relationships among antioxidants and phenotypic traits in birds.

Because vitamin E has a well-established role in antioxidant defense (Surai, 2002), we expected that vitamin E supplementation interfered with the relationships among embryo phenotypic traits and the concentration of other antioxidants or markers of oxidative status (see Introduction). One potential cause for the lack of such

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interaction effects is the low dose of vitamin E that we administered. We emphasize that we deliberately used a dose that did not result in post-manipulation concentrations exceeding the natural range of variation because we aimed at investigating the effects of variation in egg composition in a natural ecological and evolutionary setting. An additional possibility is that exogenous maternal egg antioxidants are contained in the eggs at or above their maximal effective dose, and any increase in their concentration is therefore ineffective. This interpretation is contradicted by the general tenet that dietary antioxidants are limiting in maternal diet (Møller et al., 2000). While this may obviously not apply to the particular population we studied, this interpretation is further contradicted by the positive effect that vitamin E egg supplementation had on post-natal growth in the same population (Parolini et al., 2015). In addition, the positive relationships between embryo morphological traits and antioxidant concentrations that we observed may be causal. If that is the case, the concentrations of individual antioxidants cannot be considered to be (at least) the maximal effective ones and interactions between the effects of individual compounds should be expected. Moreover, antioxidant concentrations have been shown to decline with laying order (Rubolini et al., 2011). This pattern can be interpreted as evidence that dietary limitation and/or maternal physiological constraints result in suboptimal composition of at least the last laid eggs. Hence, the availability of antioxidants to the average embryo does not seem to correspond to the maximal effective concentrations. Finally, an additional possibility is that statistical power of the tests was too low to detect significant effects. We deem the size of the sample as large, also given the experimental nature of the study. Thus, if 'real' effects existed that went undetected, these must be of small intensity and therefore they do not alter the main message of the study, that no marked combined effects among antioxidants on morphological or oxidative status endpoints occur.

In conclusion, for the first time in any experimental study in the wild, we explored the patterns of covariation between different components of the embryonic antioxidant defense system in the yolk and specific organs. Residual egg yolk shortly before hatching and embryo organs consistently differed in their concentration of the different exogenous antioxidants that we considered, and the antioxidant concentrations positively covaried among the yolk and the liver or brain. Eggs with larger antioxidant concentrations hosted larger embryos and had larger antioxidant capacity but not relatively low values at markers of oxidative damage, suggesting that other components of the antioxidant system intervene, with overwhelming effects, in protecting the egg from oxidation.

Vitamin E is among the main exogenous antioxidants, with well-documented effects on functional interactions between the branches of the antioxidant system and on oxidative status. The lack of consequences of increased vitamin E concentration on the other antioxidants and oxidative status markers therefore obviously do not completely dismiss this role of vitamin E. Rather, they suggest that under a natural selection regime in the wild and in a non-artificially selected population, physiological variation in the concentration of one major antioxidant has minor, if any, effects on other components of the antioxidant system and on their consequences for embryo growth and oxidative status.

Acknowledgements
We thank the Parco Regionale del Delta del Po (permit no. 657, 4 February 2014), which allowed both the manipulation and the collection of yellow-legged gull eggs. We also thank the referee for constructive criticism.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: N.S., D.R., M.P.; Methodology: C.D.P., M.P., M.C., F.K., G.C., A.M., I.D.D.; Resources: N.S.; I.D.D. and A.M.; Investigation: C.D.P., M.C., M.P., G.C., D.R. and N.S.; Statistical analyses: N.S., C.D.P., M.P. Writing–Original Draft: N.S.; Writing–Review and Editing: N.S., M.P. and C.D.P.; Supervision: N.S.

Funding
This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.146498.supplemental

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Figure S1

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Table S1. Amount (µg) of vitamin E (α : γ – tocopherol ratio *per* egg) injected into the yolk of yellow-legged gull eggs depending on egg mass at the time of deposition and laying order (first, second or third egg is a-, b-, or c-egg, respectively). The doses were designed to increase the post-manipulation vitamin E concentration of 1 standard deviation compared to that previously recorded in the same population for each class of egg mass and position in the laying sequence.

Laying order	*Egg mass (g)*	*Vitamin E (µg) (α : γ - tocopherol)*
a-egg	84-91	670 (623:47)
	92-95	748 (696:52)
	96-108	697 (648:49)
b-egg	80-88	509 (473:36)
	89-92	699 (650:49)
	93-99	688 (640:48)
c-egg	75-82	305 (283:22)
	82-87	616 (573:43)
	88-98	643 (596:45)

Table S2. Summary of the results of linear mixed models of morphological or oxidative status traits on two-way interactions between antioxidant concentrations. In the models we also included the effect of vitamin E treatment, sex, laying order and egg mass. Clutch was included as a random effect. Only the models where the effects of the two-way interaction between antioxidants were significant are reported. Overall, the effects of the interaction were tested in 135 models for all the possible pairs of antioxidants.

	F	d.f.	*P*	Coefficients (SE)
Skull Length				
Treatment	1.48	1,32	0.23	
Sex	1.68	1,32	0.20	
Laying Order	1.67	2,32	0.20	
Egg Mass	14.78	1,32	<0.001	
Carotenoids Yolk	8.85	1,32	0.006	-2.890 (0.971)
Tocopherols Yolk	0.61	1,32	0.44	-0.132 (0.169)
Carotenoids Yolk*Tocopherols Yolk	7.56	1,32	0.0097	0.023 (0.008)
Brain Mass				
Treatment	0.60	1,32	0.44	
Sex	2.38	1,32	0.13	
Laying Order	0.06	2,32	0.94	
Egg Mass	0.21	1,32	0.65	
Carotenoids Yolk	0.83	1,32	0.37	-0.004 (0.005)
Coenzyme Q10 Yolk	7.50	1,32	0.010	-0.059 (0.022)
Carotenoids Yolk*Coenzyme Q10	5.94	1,32	0.021	0.002 (0.001)
TOS Brain				
Treatment	0.32	1,30	0.58	
Sex	1.66	1,30	0.21	
Laying Order	0.19	2,30	0.83	
Egg Mass	2.70	1,30	0.11	
Retinol Brain	5.11	1,30	0.031	-259.99 (115.02)
Tocotrienols Brain	3.50	1,30	0.071	-10.081 (5.391)
Retinol Brain*Tocotrienols Brain	9.08	1,30	0.005	61.075 (20.265)

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Table S3. Summary of the results of linear mixed models of antioxidant concentrations or oxidative status markers on two-way interactions between vitamin E treatment and post-manipulation vitamin E concentration. In the models we also included the effect of sex, laying order and egg mass. Clutch was included as a random effect. Only the models where the effects of the two-way interaction between vitamin treatment and post-manipulation vitamin E concentration were significant are reported. Overall, the effects of the interaction were tested in 111 models for all the possible pairs of antioxidants. C: control; E: vitamin E treated.

	F	d.f.	*P*	Coefficients (SE)
α-tocotrienol Yolk				
Treatment	6.00	1,33	0.020	
Sex	0.80	1,33	0.38	
Laying Order	0.22	2,33	0.80	
Egg Mass	0.01	1,33	0.92	
Tocopherols Yolk	0.14	1,33	0.71	
Treatment*Tocopherols Yolk	5.99	1,33	0.020	C: 0.019 (0.010) E: -0.014 (0.009)
Tocotrienols Yolk				
Treatment	6.00	1,33	0.020	
Sex	0.85	1,33	0.36	
Laying Order	0.19	2,33	0.82	
Egg Mass	0.00	1,33	0.99	
Tocopherols Yolk	0.20	1,33	0.66	
Treatment*Tocopherols Yolk	5.99	1,33	0.020	C: 0.020 (0.010) E: -0.014 (0.009)

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Table S4. Summary of the results of linear mixed models of morphological or oxidative status traits on two-way interactions between treatment and antioxidant concentrations. In the models we also included the effect of sex, laying order and egg mass. Clutch was included as a random effect. Only the models where the effects of the two-way interaction between treatment and antioxidants were significant are reported. Overall, the effects of the interaction were tested in 122 models for all the possible pairs of antioxidants. C: control; E: vitamin E treated.

	F	d.f.	*P*	Coefficients (SE)
Liver Mass				
Treatment	2.46	1,30	0.13	
Sex	3.13	1,30	0.09	
Laying Order	0.66	2,30	0.52	
Egg Mass	10.31	1,30	0.003	
γ-tocotrienol Yolk	0.61	1,30	0.44	
Treatment*γ-tocotrienol Yolk	5.21	1,30	0.030	C: 2.145 (0.976) E: 1.105 (1.105)
Liver Mass				
Treatment	4.29	1,32	0.047	
Sex	3.91	1,32	0.057	
Laying Order	1.58	2,32	0.22	
Egg Mass	7.43	1,32	0.010	
Coenzyme Q10	0.65	1,32	0.43	
Treatment*Coenzyme Q10	6.22	1,32	0.018	C: 0.03235 (0.016) E:-0.01594 (0.012)
TOS Liver				
Treatment	1.77	1,31	0.19	
Sex	0.01	1,31	0.90	
Laying Order	7.71	2,31	0.002	
Egg Mass	10.35	1,31	0.003	
Retinol Liver	6.11	1,31	0.019	
Treatment*Retinol Liver	5.82	1,31	0.022	C: 176.47 (299.87) E:1044.80 (311.48)
PC Liver				
Treatment	1.57	1,28	0.22	
Sex	0.41	1,28	0.53	
Laying Order	0.25	2,28	0.78	
Egg Mass	0.19	1,28	0.67	
α-tocotrienol Liver	0.52	1,28	0.48	
Treatment*α-tocotrienol Liver	4.66	1,28	0.040	C: 0.123 (0.068) E: -0.060 (0.054)
PC Liver				
Treatment	2.25	1,28	0.14	
Sex	0.67	1,28	0.42	
Laying Order	0.32	2,28	0.73	
Egg Mass	0.201	1,28	0.66	
Tocotrienols Liver	0.39	1,28	0.54	
Treatment*Tocotrienols Liver	4.55	1,28	0.042	C: 0.118 (0.068) E: -0.064 (0.053)

	F	d.f.	*P*	Coefficients (SE)
Brain Mass				
Treatment	4.21	1,33	0.048	
Sex	0.50	1,33	0.48	
Laying Order	0.04	2,33	0.96	
Egg Mass	0.59	1,33	0.45	
α-tocotrienol Yolk	0.03	1,33	0.86	
Treatment*α-tocotrienol Yolk	5.04	1,33	0.032	C: 0.027 (0.018) E: -0.032 (0.018)
Brain Mass				
Treatment	4.39	1,33	0.044	
Sex	0.53	1,33	0.47	
Laying Order	0.05	2,33	0.95	
Egg Mass	0.64	1,33	0.43	
Tocotrienols Yolk	0.03	1,33	0.85	
Treatment*Tocotrienols Yolk	5.24	1,33	0.029	C: 0.027 (0.018) E: -0.032 (0.018)
TOS Brain				
Treatment	4.03	1,32	0.053	
Sex	6.27	1,32	0.018	
Laying Order	0.29	2,32	0.75	
Egg Mass	1.53	1,32	0.23	
Retinol Brain	0.35	1,32	0.56	
Treatment*Retinol Brain	5.23	1,32	0.029	C: -225.72 (136.95) E: 126.64 (85.260)
TOS Brain				
Treatment	6.05	1,31	0.020	
Sex	5.05	1,31	0.03	
Laying Order	0.15	2,31	0.86	
Egg Mass	5.23	1,31	0.029	
Tocotrienols Brain	0.30	1,31	0.59	
Treatment*Tocotrienols Brain	8.74	1,31	0.006	C: -5.574 (4.013) E: 8.089 (2.248)
PC Brain				
Treatment	4.80	1,27	0.037	
Sex	1.01	1,27	0.32	
Laying Order	0.45	2,27	0.64	
Egg Mass	0.11	1,27	0.74	
α-tocopherol Brain	0.58	1,27	0.45	
Treatment*α-tocopherol Brain	6.87	1,27	0.014	C: -0.008 (0.006) E: 0.014 (0.006)
PC Brain				
Treatment	1.31	1,25	0.26	
Sex	1.20	1,25	0.28	
Laying Order	1.01	2,25	0.38	
Egg Mass	0.91	1,25	0.35	
α-tocotrienol Brain	0.11	1,25	0.74	
Treatment*α-tocotrienol Brain	4.85	1,25	0.037	C: -0.014 (0.011) E: 0.019 (0.009)

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	F	d.f.	*P*	Coefficients (SE)
PC Brain				
Treatment	4.73	1,27	0.039	
Sex	0.97	1,27	0.33	
Laying Order	0.56	2,27	0.58	
Egg Mass	0.11	1,27	0.75	
Tocopherols Brain	0.56	1,27	0.46	
Treatment*Tocopherols Brain	6.79	1,27	0.015	C: -0.007 (0.006) E: 0.013 (0.005)

Fig. S1 a. Pairwise relationships between embryo morphological traits. The orientation of the ellipses indicates the sign of the observed correlation coefficient. Correlation coefficients were classified as: statistically significant ($P < 0.05$; black filling); larger than $|r| = 0.15$ (grey filling) though statistically non-significant; $-0.15 \leq r \leq 0.15$ (no filling, 'null' relationship). Single-colour black or grey filling indicates that the correlation was consistent with the expectation. Double colour filling indicates that the relationship was opposite to the expectation. Barred cells indicate the relationships that were not tested.

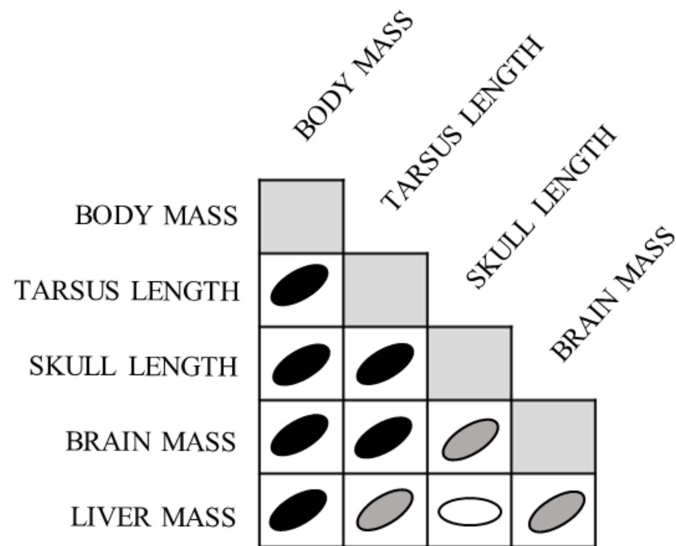


Fig. S1 b. Pairwise relationships between embryo morphological traits and antioxidant concentrations. The orientation of the ellipses indicates the sign of the observed correlation coefficient. Correlation coefficients were classified as: statistically significant ($P < 0.05$; black filling); larger than $|r| = 0.15$ (grey filling) though statistically non-significant; $-0.15 \le r \le 0.15$ (no filling, 'null' relationship). Single-colour black or grey filling indicates that the correlation was consistent with the expectation. Double colour filling indicates that the relationship was opposite to the expectation. Barred cells indicate the relationships that were not tested.

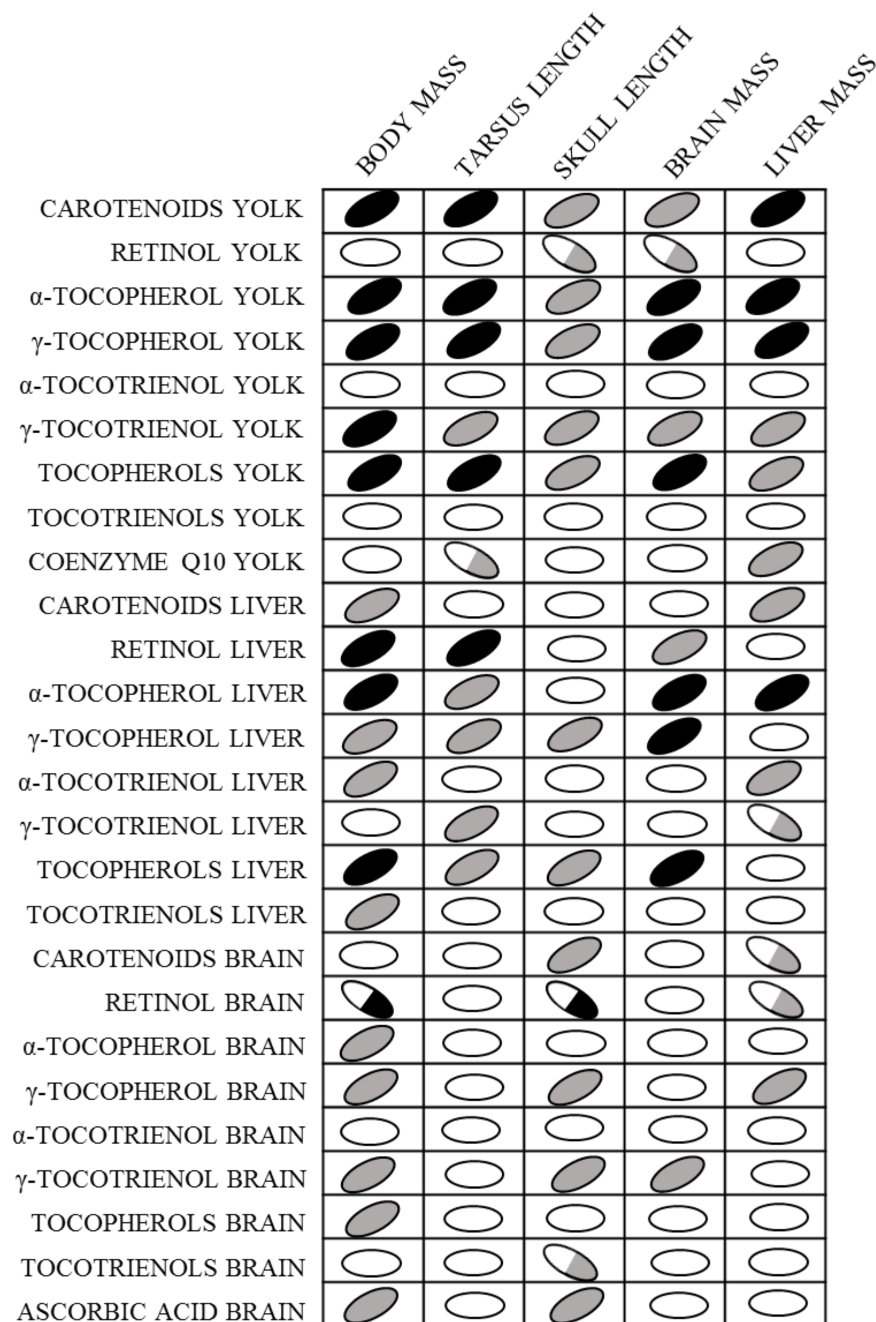


Fig. S1 c. Pairwise relationships between embryo morphological traits and markers of oxidative status. The orientation of the ellipses indicates the sign of the observed correlation coefficient. Correlation coefficients were classified as: statistically significant ($P < 0.05$; black filling); larger than $|r| = 0.15$ (grey filling) though statistically non-significant; $-0.15 \leq r \leq 0.15$ (no filling, 'null' relationship). Single-colour black or grey filling indicates that the correlation was consistent with the expectation. Double colour filling indicates that the relationship was opposite to the expectation. Barred cells indicate the relationships that were not tested.

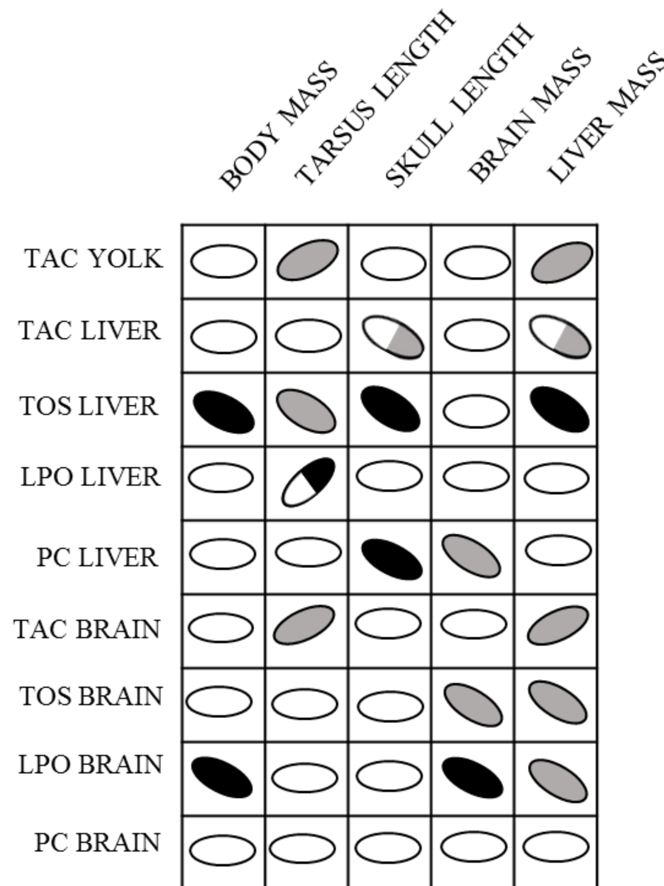


Fig. S1 d. Pairwise relationships between antioxidant conentrations. The orientation of the ellipses indicates the sign of the observed correlation coefficient. Correlation coefficients were classified as: statistically significant ($P < 0.05$; black filling); larger than $|r| = 0.15$ (grey filling) though statistically non-significant; $-0.15 \leq r \leq 0.15$ (no filling, 'null' relationship). Single-colour black or grey filling indicates that the correlation was consistent with the expectation. Double colour filling indicates that the relationship was opposite to the expectation. Barred cells indicate the relationships that were not tested.

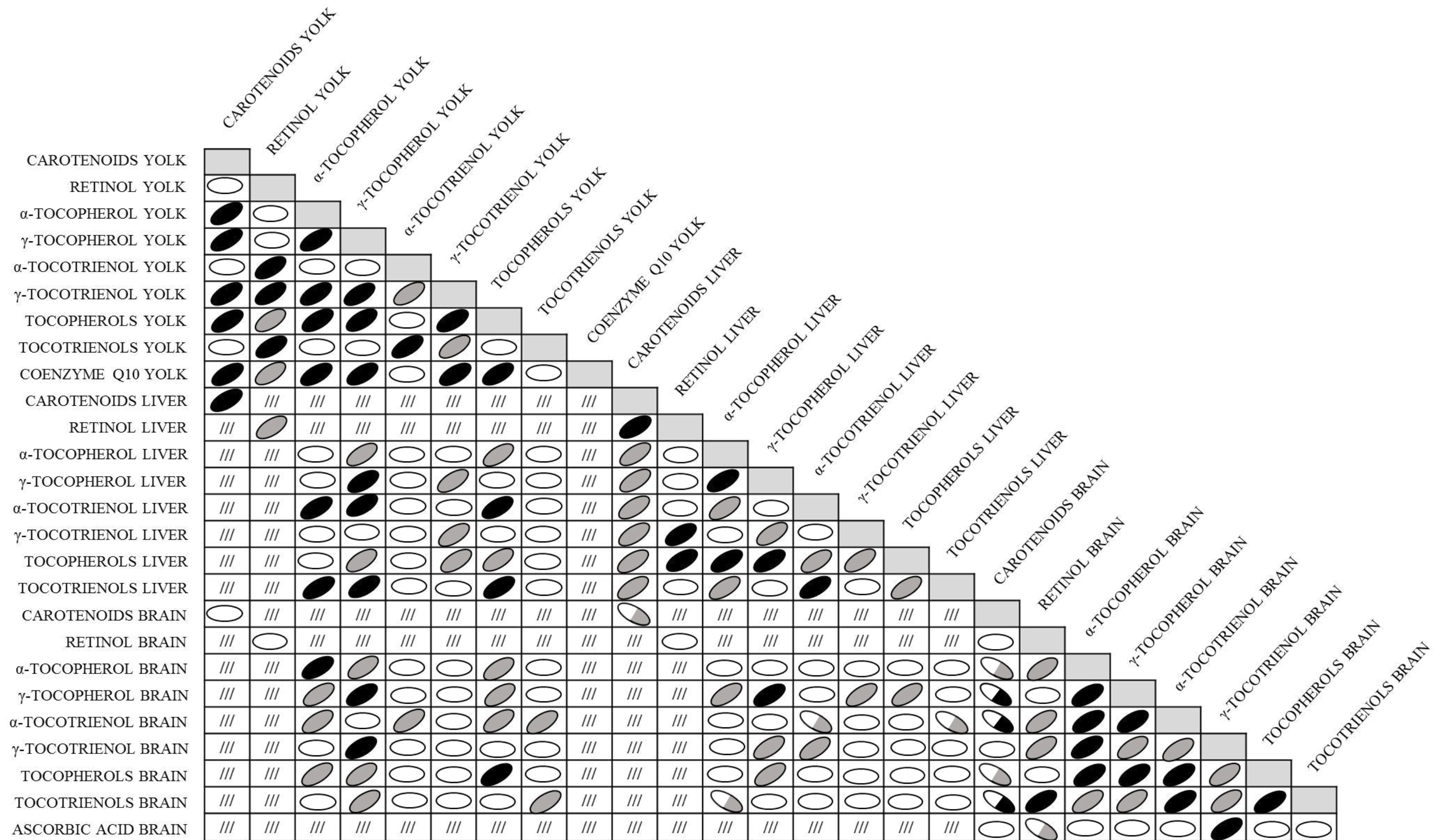


Fig. S1 e. Pairwise relationships between markers of oxidative status and antioxidant concentrations. The orientation of the ellipses indicates the sign of the observed correlation coefficient. Correlation coefficients were classified as: statistically significant ($P < 0.05$; black filling); larger than $|r| = 0.15$ (grey filling) though statistically non-significant; $-0.15 \leq r \leq 0.15$ (no filling, 'null' relationship). Single-colour black or grey filling indicates that the correlation was consistent with the expectation. Double colour filling indicates that the relationship was opposite to the expectation. Barred cells indicate the relationships that were not tested.

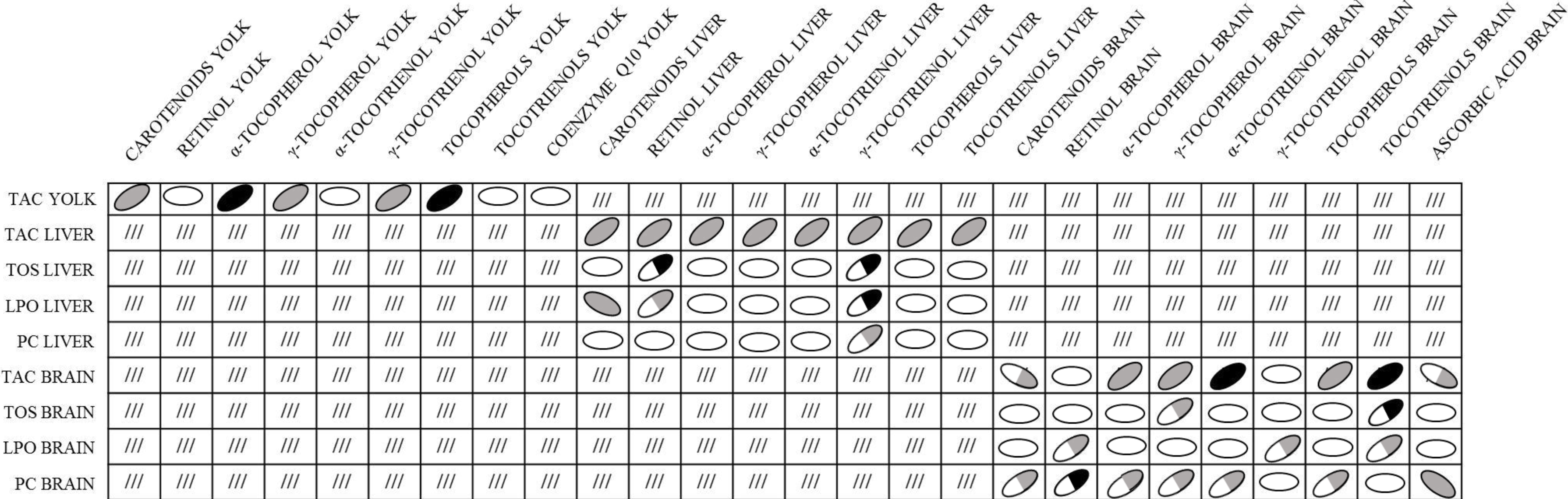
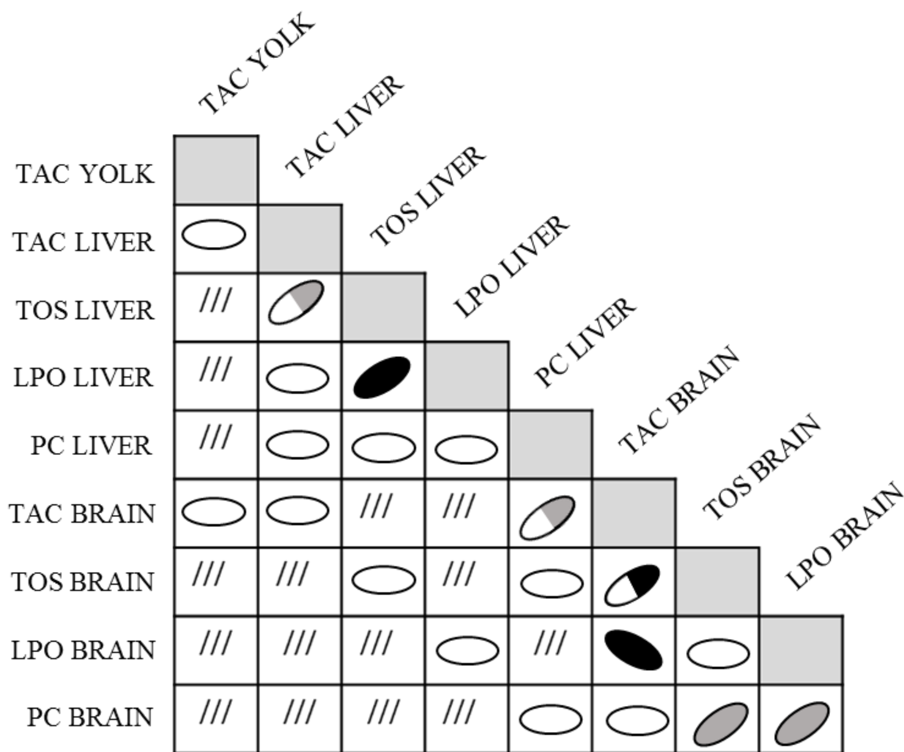


Fig. S1 f. Pairwise relationships between markers of oxidative status. The orientation of the ellipses indicates the sign of the observed correlation coefficient. Correlation coefficients were classified as: statistically significant ($P < 0.05$; black filling); larger than $|r| = 0.15$ (grey filling) though statistically non-significant; $-0.15 \leq r \leq 0.15$ (no filling, 'null' relationship). Single-colour black or grey filling indicates that the correlation was consistent with the expectation. Double colour filling indicates that the relationship was opposite to the expectation. Barred cells indicate the relationships that were not tested.



PART II

HORMONE-MEDIATED MATERNAL EFFECTS

Chapter 5

Contrasting effects of increased yolk testosterone content on development and oxidative status in gull embryos

Journal of Experimental Biology ***220*** *(2017): 625-633*

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RESEARCH ARTICLE

Contrasting effects of increased yolk testosterone content on development and oxidative status in gull embryos

Marco Parolini*, Andrea Romano*, Cristina Daniela Possenti, Manuela Caprioli, Diego Rubolini and Nicola Saino

ABSTRACT

Hormone-mediated maternal effects generate variation in offspring phenotype. In birds, maternal egg testosterone (T) exerts differential effects on offspring traits after hatching, suggesting that mothers experience a trade-off between contrasting T effects. However, there is very little information on T pre-natal effects. In the yellow-legged gull (*Larus michahellis*), we increased yolk T concentration within physiological limits and measured the effects on development and oxidative status of late-stage embryos. T-treated embryos had a larger body size but a smaller brain than controls. Males had a larger brain than females, controlling for overall size. T treatment differentially affected brain mass and total amount of pro-oxidants in the brain depending on laying order. T-treatment effects were not sex dependent. For the first time in the wild, we show contrasting T pre-natal effects on body mass and brain size. Hence, T may enforce trade-offs between different embryonic traits, but also within the same trait during different developmental periods.

KEY WORDS: Brain mass, Embryo, Growth, Oxidative status, Sexual dimorphism, *Larus michahellis*, Testosterone

INTRODUCTION

Epigenetic maternal effects mediated by egg size and biochemical composition have consequences for the phenotypic composition of the next generations, and can therefore have complex effects on the evolutionary dynamics of populations (Mousseau and Fox, 1998; Muriel et al., 2015). Ecological and evolutionary studies have framed the interpretation of maternal effects via the egg mostly in terms of their functional value to maximize parental fitness via adaptive transgenerational phenotypic plasticity (Mousseau and Fox, 1998; Müller et al., 2007). However, maternal effects evolve and are expressed under a number of potentially constraining conditions, suggesting that egg quality may be sub-optimal to Darwinian fitness of parents and/or individual offspring. In fact, maternal allocation of substances to the eggs may entail costs to the mother, and thus enforce trade-offs between offspring quality and maternal condition (Mousseau and Fox, 1998). In addition, mediators of maternal effects may have 'pleiotropic' and potentially contrasting effects on offspring fitness traits, thereby imposing trade-offs on maternal allocation strategies (Navara and Mendonça, 2008).

Maternal effects can occur via variation in egg mass and macro-constituents (e.g. albumen content) that have major effects on post-natal performance, as shown by correlational evidence and also egg-manipulation experiments (Bonisoli-Alquiati et al., 2007, 2008; Christians, 2002). Studies of the consequences of variation in quantitatively minor egg constituents have focused on antioxidants and steroid hormones (Groothuis and Schwabl, 2008; Groothuis et al., 2005; Navara and Mendonça, 2008; Romano et al., 2008; Saino et al., 2003). Androgens, in particular, have a special appeal in the evolutionary ecological study of maternal effects for at least three reasons. First, they are transferred to the eggs in amounts that partly depend on extrinsic conditions such as predation risk (Coslovsky et al., 2012), population density (van Dijk et al., 2013) and mate sexual attractiveness (Krištofík et al., 2014; but see Saino et al., 2006). Therefore, they have the potential to mechanistically and functionally link maternal experience of environmental conditions to offspring phenotype (Marshall and Uller, 2007). The observation that androgen deposition in the eggs varies with position in the laying sequence, and that these patterns of variation change across species, corroborates the idea that egg androgen concentrations are strategically modulated by females (Groothuis et al., 2005; von Engelhardt and Groothuis, 2011). Second, androgens are of pivotal importance to regulation of embryo differentiation and development of physiological and behavioural traits (Arnold, 2002; Groothuis and Schwabl, 2008; Pfannkuche et al., 2011). Third, androgens are drivers of sexual phenotypic differentiation, and can therefore participate in strategies of maternal sex allocation (e.g. Adkins-Regan et al., 2013; Navara and Mendonça, 2008; Riedstra et al., 2013; Ruuskanen and Laaksonen, 2010; Schweitzer et al., 2013).

The cleidoic egg of birds affords an ideal study system for maternal effects because it is isolated from the maternal physiological milieu. *In ovo* manipulation experiments on birds have provided extensive evidence for the pervasiveness and also for the differential and sex-dependent effects of androgens, and testosterone (T) in particular, on diverse offspring traits. Most studies have suggested that androgens are anabolic for muscle and skeletal growth (Eising et al., 2001; Lipar and Ketterson, 2000; Navara et al., 2006), and boost post-natal body mass gain (Navara et al., 2005; Pilz et al., 2004; Schwabl, 1996). However, other studies have failed to show such a positive effect or have even shown a negative effect on post-natal growth (Henry and Burke, 1999; Podlas et al., 2013; Possenti et al., 2016; Rubolini et al., 2006). In addition, *in ovo* T effects on growth have been shown to be sex dependent (Müller et al., 2009; Ruuskanen, 2015; Saino et al., 2006; Sockman et al., 2008). Elevated egg T levels are mostly suppressive to acquired immune processes (Groothuis et al., 2005; Navara and Mendonça, 2008; Navara et al., 2005; but see Rubolini et al., 2006; Tobler et al., 2010), with evidence for sex- and age-dependent effects (Tobler et al., 2010). Few studies have investigated the effect of T on oxidative status, with partly different outcomes. Some studies postulated that high amounts of maternally transferred androgens, including testosterone, may represent a cost for offspring in terms of increased susceptibility

Department of Biosciences, University of Milan, via Celoria 26, Milan I-20133, Italy.

*Authors for correspondence (marco.parolini@unimi.it; andrea.romano@unimi.it)

M.P., 0000-0003-0226-1709

Received 24 June 2016; Accepted 25 November 2016

Journal of Experimental Biology (2017) 220, 625-633 doi:10.1242/jeb.145235

List of abbreviations

LMM	linear mixed model
ROS	reactive oxygen species
T	testosterone
TAC	total antioxidant capacity
TOS	total pro-oxidant molecules

to oxidative stress as a consequence of accelerated growth (e.g. Groothuis et al., 2006; Martin and Schwabl, 2008). Enhanced offspring development rate mediated by egg androgens is associated with increased cell metabolism and a concomitant overproduction of reactive oxygen species (ROS; see Martin and Schwabl, 2008), which are produced during normal metabolic processes by the mitochondria and can cause severe toxic effects because they can oxidize cellular macromolecules (e.g. Finkel and Holbrook, 2000). Additionally, testosterone may directly induce oxidative stress in some tissues (Alonso-Alvarez et al., 2007 and references therein). For instance, yolk T decreased post-natal lipid peroxidation and increase total plasma antioxidant capacity (TAC) in one study (Noguera et al., 2011) but had no or negative sex-specific effects on post-natal TAC in other studies (Tobler and Sandell, 2009; Tobler et al., 2013). In addition, high egg T levels during pre-natal stages depress DNA repair after damage from acute stress (Treidel et al., 2013). Egg T enhances pre-natal (Boncoraglio et al., 2006) and post-natal begging behaviour (Eising et al., 2001; Rice et al., 2013; but see Boncoraglio et al., 2006; Saino et al., 2006; Smiseth et al., 2011) also in a sex-dependent way (Ruuskanen and Laaksonen, 2013; Ruuskanen et al., 2009), and affects chick territorial, neophobic, dispersal and other behaviours (e.g. Bertin et al., 2015; Müller et al., 2009; Tobler and Sandell, 2007). Finally, studies of egg T effects in adulthood have also provided evidence of contrasting effects both within and among fitness traits, such as fecundity (Müller et al., 2009; Rubolini et al., 2007), expression of male secondary sexual traits (Bonisoli-Alquati et al., 2011; Rubolini et al., 2006) and survival (Hegyi et al., 2011; Ruuskanen et al., 2012).

Thus, prominent features of experiments manipulating egg T on different fitness traits are that the effects are multi-tiered, can vary in sign, as well as according to the ontogenetic stage when the effects are measured, and they can be sex specific. In addition, there is evidence for mothers not being able to tune egg androgen concentration according to embryo sex (Aslam et al., 2013; Rubolini et al., 2011; but see Badyaev et al., 2008). The fact that egg androgens can have differential, and even opposite, effects on fitness traits, and that these effects can be sex dependent, may therefore enforce trade-offs in the amount of androgens that mothers allocate to their eggs.

Studies on prenatal effects of T are very rare (Hegyi and Schwabl, 2010; Muriel et al., 2013). This is unfortunate for several reasons. First, embryonic development and growth is a dynamic process where developmental conditions can preemptively imprint development and growth trajectories. In addition, the peri-natal period is often critical to individual offspring performance and survival because social relationships with competing siblings are established after birth. Furthermore, the effect of androgens may change over time even on the same trait (e.g. Hegyi and Schwabl, 2010). 'Longitudinal' trade-offs at high androgen levels may therefore occur, and, in order to interpret maternal allocation in androgen transfer to the eggs, we need to expand our view of the effects of androgens not only across traits but also over the entire ontogenetic process, starting from pre-natal development.

In the present study of the yellow-legged gull (*Larus michahellis* Naumann 1840), we aimed at contributing to fill this gap of knowledge of the effect of T on pre-natal development and physiology. Using a within-clutch experimental design, we administered a physiological dose of T and recorded the effects on embryo size and oxidative status while establishing a group of sham-injected control eggs. Shortly before hatching, the eggs were dissected to measure total embryo size and mass, residual yolk mass as an inverse measure of yolk absorption, as well as size of the liver and the brain. In addition, we measured TAC and the concentration of pro-oxidant molecules in the brain and liver.

Brain size was measured because the brain is a major target organ for the organizing effects of pre-natal androgens (Gahr et al., 1996; Garamszegi et al., 2007; Godsave et al., 2002) and also because inter-specific comparative evidence exists for coevolution between maternal effects mediated by androgens and brain size (Garamszegi et al., 2007). Liver size was measured because it is the main repository of antioxidants (Surai, 2002), and evidence exists that egg T can affect oxidative status after hatching (see above). Imbalance between antioxidant defenses and oxidative challenge to biological molecules is a major factor affecting bodily performance and fitness (Costantini, 2014; Halliwell and Gutteridge, 1999; Surai, 2002). We focused on TAC and the amount of pro-oxidant molecules (i.e. TOS according to terminology in Erel, 2005) in the liver, because of its role in antioxidant defense, and in the brain, because it is believed to be particularly sensitive to peroxidation of phospholipids (Surai, 2002; Surai et al., 1999).

Because of the heterogeneity in the observed effects of T, of the scanty number of studies on pre-hatching stages, and the absence of a general theoretical framework of the effects of T on the specific endpoints that we focused on, we refrained from making explicit predictions on the outcome of T manipulation on pre-natal morphology and physiology, and on its sex-dependent variation.

MATERIALS AND METHODS

Study organism

The yellow-legged gull is a large, mainly colonial, monogamous gull with altricial offspring and biparental care of progeny. Females lay one to three eggs (modal clutch size=3) that hatch asynchronously (hatching span 1–4 days) after 27–32 days of incubation (Cramp, 1998). Previous studies that are relevant to the present experiment have shown that eggs are a source of important maternal effects mediated by carotenoids, vitamins, corticosterone as well as T (e.g. Bonisoli-Alquati et al., 2007; Parolini et al., 2015; Romano et al., 2008; Rubolini et al., 2011; Saino et al., 2010). Specifically, a previous experiment, in which we only measured offspring phenotype after hatching and no oxidative stress endpoints were analysed, showed that *in ovo* T depressed body mass 4 days post-hatching (Rubolini et al., 2006). A subsequent experiment showed that *in ovo* T manipulation affected behavioural lateralization but had only weak effects on body mass at hatching (Possenti et al., 2016). In addition, *in ovo* manipulation of T boosts peri-natal signals of solicitation of care that offspring address to their parents at pipping-egg stage, but not post-natal begging behaviour (Boncoraglio et al., 2006). T levels decline with laying order. However, steroid hormone concentrations do not differ between eggs carrying a male or a female (Rubolini et al., 2011).

Field procedures

The present study was performed on a colony (>400 breeding pairs) in the Comacchio lagoon (NE Italy) during March–May 2015. The colony was visited every second day to monitor the progress of

laying and mark the newly laid eggs. When a new egg was found, it was temporarily removed from the nest for experimental manipulation while temporarily replacing it with a 'dummy' egg.

The experimental *in ovo* T manipulation was performed as described in Possenti et al. (2016).

We aimed at increasing the concentration of T by 1 standard deviation (s.d.) of the concentration recorded in the yolk of yellow-legged gull eggs from the same colony (Rubolini et al., 2011), by injecting an appropriate volume of a T solution directly into the yolk. Because the concentration of T in the yolk varies according to egg size and position in the laying sequence, we scaled the dose to be injected accordingly. Thus, we grouped first (a-), second (b-) or third (c-) laid eggs into three classes (tertiles) of size according to egg mass and calculated the s.d. of T concentration in the yolk for each tertile within each position in the laying sequence. We estimated the yolk mass for each class size and position in laying sequence according to the following equation: yolk mass=0.227 (0.039 s.e.) egg mass+ 1.815 (3.461 s.e.) ($F_{1,88}$=34.38, P<0.001). The amount of T due to be injected was computed as the product of the s.d. (in ng g^{-1}) of T concentration for each tertile and position in the laying sequence and the estimated yolk mass. The doses injected were as follows [laying order: class of size according to egg mass (g): amount of T injected (ng per egg)]: a-eggs: 84–91 g: 57 ng, 92–95 g: 59 ng, 96–108 g: 42 ng; b-eggs: 80–88 g: 74 ng, 89–92 g: 73 ng, 93–99 g: 81 ng; and c-eggs: 75–82 g: 95 ng, 82–87 g: 84 ng, 88–98 g: 76 ng.

T was injected in the yolk according to the validated procedure reported by Romano et al. (2008). Before being injected, the egg was weighed (to the nearest g) and placed with the longitudinal axis vertical. After disinfecting the eggshell, a hole was drilled using a sterile pin close to the acute pole. *In ovo* injection was performed by means of a 1-ml sterile syringe mounting a 0.6×30 mm needle while the egg was held firmly with its longitudinal axis vertical. Immediately after extracting the needle from the egg, the hole was sealed with a drop of epoxidic glue and a small piece of eggshell was superimposed on the hole. T solutions were prepared in sterile vials dissolving the hormone in corn oil to the final dilution required. Each vial contained the concentration of T to be injected in egg yolk depending on egg mass and position in the laying sequence. We adopted a within-clutch design, whereby both control and T-treated eggs were established within each clutch. We sequentially assigned the following treatment schemes to the clutches, according to the order in which the first egg was found (nest, a-, b-, c-egg): nest 1, T injection (T), control injection (C), T; nest 2, C-T-C; nest 3, T-C-C; nest 4, C-T-T and so forth with the following nests. T-treated eggs were injected with 30 µl of the appropriate T solution, while control eggs were injected with the same volume of corn oil only.

After T level manipulation, each egg was brought back to its nest of origin and regularly monitored until any sign of imminent hatching appeared. When eggshell fractures were observed, eggs were collected and frozen at −20°C until dissection.

The study was carried out under permission of the Parco Regionale del Delta del Po (#252015, 20 February 2015), which allowed both the manipulation and the collection of eggs when any sign of imminent hatching appeared. Eggs were experimentally manipulated by injecting a physiological dose of T at the time of laying. Even though the Guideline on The Use and Euthanasia Procedures of Chicken/Avian Embryos draft by Animal Care and Use Committee discourages hypothermia for euthanasia of avian embryos, we had to euthanize embryos through this procedure, placing the eggs into a −20°C freezer within 2 h of collection due to facility constraints. The Guidelines for the Euthanasia of Animals by American Veterinary Medical Association, physical methods of euthanasia, agree that this procedure may be necessary in some field situations if other methods are impractical or impossible to implement. This is the case because we performed a field experiment and we did not have a field laboratory with equipment to euthanize embryos by other methods [i.e. carbon dioxide (CO_2), anesthetic agents or decapitation], to dissect organs and to store them appropriately until biochemical analyses.

Laboratory procedures

In the laboratory, the eggs were left at room temperature for ca. 15 min and weighed (to the nearest g). Then, we removed the eggshell and the residual yolk sac was detached from the embryo and weighed (to the nearest g). Before dissection, the embryo was weighed (to the nearest g) and tarsus and head size (occipital-beak length) were measured by a caliper (to the nearest mm). The liver and brain were isolated from the embryo, weighed (to the nearest mg) and frozen at −80°C until biochemical analyses. All the measurements were performed by a single operator to ensure consistency. Molecular sexing of the embryo was performed according to Saino et al. (2008).

TAC and the concentration of total pro-oxidant molecules (TOS according to Erel, 2005) were measured in liver and brain homogenates from each embryo. Organs were homogenized in an appropriate volume of phosphate buffer (100 mmol l^{-1}, pH 7.4, 1 mmol l^{-1} EDTA and 100 mmol l^{-1} KCl) by an automatic homogenizer. Homogenates were centrifuged at 16,200 ***g*** for 10 min and an aliquot of the obtained supernatant was processed for measuring TAC and TOS. Briefly, TAC and TOS were measured according to colorimetric methods developed on plasma by Erel (2004 and 2005 respectively), and adapted to tissue homogenate samples. The TAC assay was calibrated by using Trolox and the results were expressed as µmol l^{-1} Trolox equiv. g^{-1} wet weight, while TOS was calibrated using hydrogen peroxide (H_2O_2) and the results were expressed as nmol l^{-1} H_2O_2 equiv. g^{-1} wet weight. Mean TAC intra-assay percentage coefficient of variation (CV%) was 5.01±4.24% and 5.01±4.24% (n=30 replicates), while the mean inter-assay CV (n=3 assay plates) was 6.97±5.42% and 6.9±2.5% for brain and liver homogenates, respectively. Mean TOS intra-assay CV% was 6.42±4.95% and 5.01±4.24% (n=30 replicates), while the mean inter-assay CV (n=3 assay plates) was 7.9±5.5% and 8.4±6.3% for brain and liver homogenates, respectively.

Statistical analyses

We relied on Gaussian linear mixed models (LMM) to analyze the independent and combined (two-way interaction) effects of treatment (T injection versus control), sex and laying order (fixed effect factors) on embryo traits. Where relevant, we included in the models egg mass at laying or embryo mass as covariates. Nest identity was always included as a random effect to account for non-independence of embryos from the same clutch. Egg mass, embryo and residual yolk mass, and mass of the liver and the brain were $\log_{10}$-transformed to account for allometry. In the analyses of liver and brain mass, embryo mass (covariate) was expressed as embryo mass minus liver or brain mass, respectively. The models in which all interactions were non-significant were simplified by removing all the interaction terms in a single step. We generally refrained from testing three-way interactions to avoid model over-parameterization. However, because of the evidence of an effect of T on brain size and sex differences in brain size, in the analysis of this variable, we tentatively investigated the three-way interaction between sex, treatment and embryo mass (see Results).

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For morphological analyses, the sample consisted of 30 clutches with three eggs each. Information on sex or morphology was not available for one a- and one b-egg. Thus, we considered 29 a-eggs (controls, T-injected: 15, 14), 29 b-eggs (14, 15) and 30 c-eggs (17, 13). Thirty-three eggs carried a female and 55 eggs carried a male embryo.

For analyses of markers of oxidative status, the sample consisted of 30 clutches with three eggs each. Information on sex was not available for one b-egg and information on individual markers of oxidative status was not available for some embryos, yielding the following sample sizes: 30 a-eggs (controls, T-injected: 16, 14), 28–29 b-eggs (14, 14–15) and 29–30 c-eggs (16–17, 13).

RESULTS

Testosterone and embryo morphology

In LMMs with brood as a random effect, T treatment did not affect the length of incubation (from laying to appearance of eggshell fractures) of embryos from T-treated eggs compared with controls ($F_{1,60}$=0.057, P=0.813), after controlling for embryo sex and laying order. However, T treatment was found to significantly enhance embryo mass and tarsus length while controlling for original egg mass (Table 1). These analyses did not disclose any significant effect of sex or laying order, nor any two-way interaction effects among sex, laying order and treatment. Conversely, T treatment caused a reduction in residual yolk mass, again independently of laying order or sex effects (Table 1).

Brain mass showed a complex pattern of variation according to the independent and combined effects of T treatment, sex and laying order. Brain mass increased with total embryo mass, as expected, but the slope of this relationship was more than twice as steep among controls compared with T-treated embryos (Table 1, Fig. 1), resulting in a significant treatment by embryo mass interaction effect (Table 1). The effect of T treatment depended on laying order: brain mass of embryos from T-treated b-eggs was significantly higher compared with that from control b-eggs, while brain mass from control b-eggs was significantly smaller than that from a- and c-control eggs. However, brain mass did not vary according to laying order among T-treated eggs. In addition, brain size was significantly smaller in females (Table 1). Hence, males had significantly larger brains compared with females also after controlling for the effect of general body size. All these significant effects on brain mass were confirmed (i.e. they were still statistically significant) when we controlled for a measured of head size (occipital-beak length) rather than for embryo body mass (details not shown). Differently from brain mass, liver mass was unaffected by T treatment, sex or laying order, whereas it increased with embryo mass, as expected (Table 1).

Inclusion of brain size in the model reported in Table 1 of the three-way interaction between sex, treatment and embryo mass showed that the relationship between brain size and embryo mass did not differentially vary in either sex depending on egg treatment (three-way interaction effect: $F_{1,45}$=1.73, P=0.195). Hence, there was no statistically significant evidence that T had a differential effect on brain mass of male or female embryos.

Testosterone and markers of oxidative status

Testosterone treatment did not affect TAC either in the liver or in the brain. In addition, TAC did not vary between the sexes or between positions in the laying sequence.

Testosterone treatment caused a reduction in TOS in the liver after controlling for the statistically non-significant effects of sex and

Table 1. Linear mixed models of morphological embryo traits in relation to the main and two-way interaction effects of egg testosterone treatment, sex, laying order and egg/embryo mass in the yellow-legged gull

	F	d.f.	P	Estimated marginal means/Coefficients (s.e.)		
Embryo mass						
Treatment	6.83	1, 53	0.011	Controls: 1.617 (0.007)	T-treated: 1.637 (0.008)	
Sex	0.49	1, 53	0.486	Males: 1.630 (0.006)	Females: 1.624 (0.008)	
Laying order	1.03	2, 53	0.365	a-eggs: 1.621 (0.008)	b-eggs: 1.633 (0.008)	c-eggs: 1.626 (0.009)
Egg mass	21.19	1, 53	<0.001	0.785 (0.171)		
Tarsus length						
Treatment	9.64	1, 53	0.003	Controls: 2.329 (0.005)	T-treated: 2.348 (0.005)	
Sex	0.52	1, 53	0.474	Males: 2.341 (0.005)	Females: 2.336 (0.006))	
Laying order	0.87	2, 53	0.425	a-eggs: 2.333 (0.006)	b-eggs: 2.341 (0.006)	c-eggs: 2.342 (0.007)
Egg mass	9.49	1, 53	0.003	0.402 (0.130)		
Residual yolk mass						
Treatment	6.84	1, 53	0.012	Controls: 1.222 (0.013)	T-treated: 1.178 (0.013)	
Sex	0.23	1, 53	0.635	Males: 1.196 (0.012)	Females: 1.204 (0.015)	
Laying order	0.74	2, 53	0.481	a-eggs: 1.214 (0.016)	b-eggs: 1.190 (0.015)	c-eggs: 1.196 (0.016)
Brain mass						
Treatment	5.60	1, 47	0.022			
Sex	22.88	1, 47	<0.001	Males: 0.171 (0.007)	Females: 0.129 (0.008)	
Laying order	2.77	2, 47	0.073	a-eggs: 0.159 (0.009)	b-eggs: 0.138 (0.009)	c-eggs: 0.153 (0.008)
Embryo mass	21.69	1, 47	<0.001	See Fig. 2		
Treatment×Sex	0.26	1, 47	0.612			
Treatment×Laying order	7.75	2, 47	0.001			
Laying order×Sex	1.29	2, 47	0.284			
Treatment×Embryo mass*	5.37	1, 47	0.025	Controls: 0.676 (0.134)[a]	T-treated: 0.264 (0.135)[b]	
Liver mass						
Treatment	0.85	1, 53	0.400	Controls: –0.149 (0.017)	T-treated: –0.134 (0.017)	
Sex	0.00	1, 53	0.486	Males: –0.142 (0.019)	Females: –0.141 (0.019)	
Laying order	0.53	2, 53	0.365	a-eggs: –0.129 (0.019)	b-eggs: –0.148 (0.019)	c-eggs: –0.148 (0.019)
Embryo mass	3.87	1, 53	0.055	0.471 (0.240)		

*See also Fig. 1.
[a] t=5.06, P<0.001.
[b] t=1.95, P=0.057.

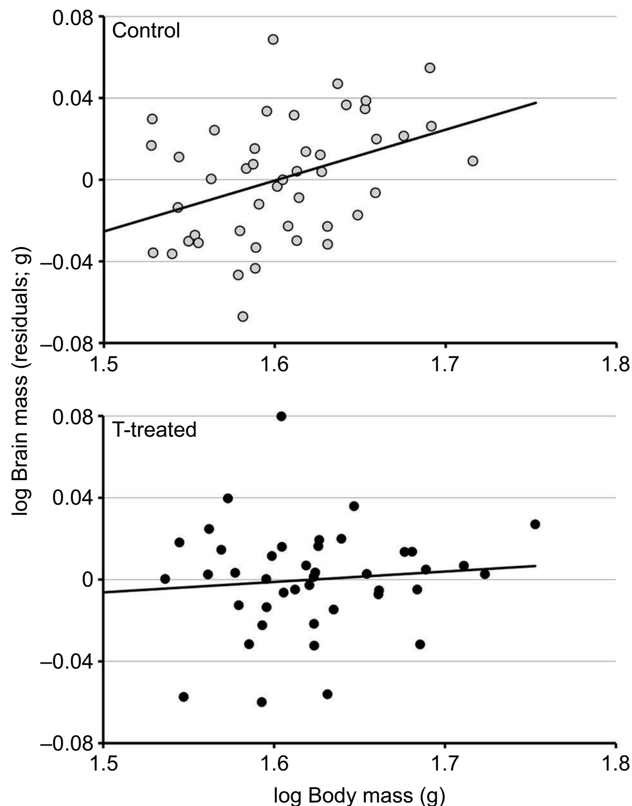

Fig. 1. Relationship between brain mass and embryo body mass in control or testosterone (T)-treated yellow-legged gull eggs. Brain mass is expressed as residuals from a linear mixed model on log brain mass data including treatment, sex, laying order and their interactions as fixed effects. Nest was also included in the model to calculate the residuals. The relationship was significantly positive for controls and marginally non-significant for T-treated embryos. The slope was significantly larger for the controls (see Table 1). Linear regression lines are fitted to better show the trend.

laying order (Table 2). In the brain, T treatment had a highly significant complex differential effect on TOS depending on laying order. In essence, while TOS of embryo from control b-eggs was smaller than that from control a-eggs, the difference between TOS of a- and b-eggs was reversed in the T-treated group, as TOS was significantly larger in b-eggs than in a-eggs.

DISCUSSION

A large body of studies has unveiled the multi-faceted role of maternal effects mediated by transfer of maternal androgens to the egg in causing short- and long-term variation in offspring phenotype after hatching (Groothuis and Schwabl, 2008; Groothuis et al., 2005). Yet, understanding how egg substances affect pre-natal development is of pivotal importance because maternal effects during embryonic life can imprint development of physiological and behavioural traits, and complex longitudinal trade-offs may operate between contrasting androgen effects during pre- and post-natal life (Navara and Mendonça, 2008).

We studied the effect of a physiological increase in yolk T concentration in the yellow-legged gull on embryonic growth and oxidative status. The main result of our experiment was that T has contrasting effects on overall embryo and brain mass. Embryos from T-treated eggs had a larger body size but, for a given body size, had a smaller brain than controls. In addition, T treatment caused a reduction in residual yolk mass, suggesting that an ultimate mechanism by which T enhances body size is acceleration of yolk absorption. T is believed to be anabolic to muscle and bone tissues (Navara and Mendonça, 2008). The present results imply that such anabolic effects are expressed already during the late embryonic stages. Brain tissues, in contrast, are known to be one of the main targets of the organizing effects of pre-natal T. Receptors for T exist in the brain from very early embryo development (Godsave et al., 2002), and major avian body systems, including immune, metabolic, muscular and skeletal systems, are likely to respond to androgens early in embryonic development when the neural and physiological axes are organized (Gahr et al., 1996; see Groothuis and Schwabl, 2008; Navara and Mendonça, 2008). The contrasting effects on body size and brain mass may therefore suggest that T mediates a developmental trade-off between allocation to general somatic growth and energetically demanding brain growth at the embryonic stage (the 'expensive tissue hypothesis'; Aiello and Wheeler, 1995; see also Isler and van Schaik, 2006). This interpretation is supported by comparative studies in which negative associations between brain size and size of other costly organs have been demonstrated across species (Aiello and Wheeler, 1995; Isler and van Schaik, 2006; Navarrete et al., 2011; Tsuboi et al., 2014). Intriguingly, the present results are also consistent with the conclusion of a comparative study of the coevolution between maternal effects mediated by egg composition and brain size, which suggested that high T levels may be suppressive to brain size (Garamszegi et al., 2007). Developmental trade-offs between brain and somatic growth might therefore be a conserved trait throughout the bird lineage.

In a previous experiment on the same colony, we found a negative effect of T on body mass 4 days after hatching (Rubolini et al., 2006). In addition, in a study of the effect of egg T on peri-natal lateralization, we reported a negative effect on body mass of 1-day-old chicks from a-eggs only, but no effect on body mass of hatchlings from b- or c-eggs (Possenti et al., 2016). Hence, the positive effect of T on pre-natal size seems to vanish around hatching and then turns to negative in early post-natal growth. T effects may therefore enforce not only trade-offs between different traits (body size and brain size) at the embryonic stage, but also longitudinal trade-offs in body size at different ontogenetic stages.

These findings are consistent with those of a previous study of spotless starling (*Sturnus vulgaris*) showing a stronger effect of T during embryo development compared with the nestling period (Muriel et al., 2013; see also Schwabl et al., 2007). The mechanism behind such age-dependent variation may consist of differences in the secretion of metabolizing enzymes between embryos and hatchlings (Bruggeman et al., 2002) and/or an effect of embryonic T on the number and distribution of androgen receptors after hatching (Navara and Mendonça, 2008; Resko and Roselli, 1997). Unfortunately, no study has investigated the differences in the metabolizing pathways of androgens in embryos and hatchlings, although it has been suggested that extensive metabolization occurs during early incubation (Fivizzani et al., 1986; von Engelhardt et al., 2009).

Notably, brain mass was found to differ between the sexes. Present evidence prompted us to analyze brain mass data from the control group from a previous experiment on the same population (M.P., unpublished data). Consistently with the present experiment, we found that brain mass was significantly larger in male than in female embryos (mean±s.e.: males: 1.59±0.03 g n=10; females: 1.49±0.03 g, n=20; $F_{1,25}$=9.06, P=0.006). Sexual dimorphism in size and structure of specific brain nuclei has been reported in several taxa (Gahr, 1994; Jacobs, 1996). Examples of sexual dimorphism in brain size include species where the sexes differ in

 Journal of Experimental Biology (2017) 220, 625-633 doi:10.1242/jeb.145235

Table 2. Linear mixed models of markers of embryo oxidative status in relation to the main and two-way interaction effects of egg testosterone treatment, sex, laying order and egg/embryo mass in the yellow-legged gull

	F	d.f.	*P*	Estimated marginal means/Coefficients (s.e.)		
TAC in the brain						
Treatment	0.83	1, 55	0.367	Controls: 17.75 (1.34)	T-treated: 18.90 (1.34)	
Sex	0.56	1, 55	0.456	Males: 18.92 (1.32)	Females: 19.73 (1.41)	
Laying order	0.92	2, 55	0.406	a-eggs: 18.54 (1.40)	b-eggs: 19.96 (1.41)	c-eggs: 19.48 (1.39)
TOS in the brain						
Treatment	1.35	1, 50	0.251	Controls: 0.211 (0.030)	T-treated: 0.235 (0.030)	
Sex	3.06	1, 50	0.087	Males: 0.244 (0.029)	Females: 0.203 (0.032)	
Laying order	0.50	2, 50	0.610	a-eggs: 0.226 (0.031)	b-eggs: 0.211 (0.032)	c-eggs: 0.234 (0.031)
Treatment×Sex	0.27	1, 50	0.300	See Fig. 3		
Treatment×Laying order	7.21	2, 50	0.002			
Laying order×Sex	0.95	2, 50	0.394			
TAC in the liver						
Treatment	0.25	1, 54	0.618	Controls: 30.00 (1.86)	T-treated: 29.02 (1.88)	
Sex	0.79	1, 54	0.379	Males: 26.53 (1.78)	Females: 30.49 (2.08)	
Laying order	0.49	2, 54	0.614	a-eggs: 28.63 (2.05)	b-eggs: 30.82 (2.12)	c-eggs: 29.08 (2.04)
TOS in the liver						
Treatment	4.51	1, 54	0.038	Controls: 1.234 (0.073)	T-treated: 1.055 (0.073)	
Sex	0.65	1, 54	0.424	Males: 1.108 (0.069)	Females: 1.183 (0.081)	
Laying order	0.49	2, 54	0.614	a-eggs: 1.109 (0.082)	b-eggs: 1.202 (0.084)	c-eggs: 1.125 (0.082)

TAC, total antioxidant capacity; TOS, total pro-oxidant molecules.

the structural architecture of the brain (e.g. Garamszegi et al., 2005; see Kotrschal et al., 2012), but reports of sex differences on overall brain size are scarce and refer to adult individuals (see Kotrschal et al., 2012). These results are therefore the first, to the best of our knowledge, where a sex difference in brain size at the embryonic stage is documented in any vertebrate species in the wild.

Sex-dependent selection for specific cognitive tasks is believed to cause divergent evolution in brain size in either sex (e.g. Garamszegi et al., 2005; Jones and Healy, 2006; Sherry, 2006) based on the assumption that brain space is positively associated with cognitive ability (Lefebvre et al., 1997; Striedter, 2005). For example, selection experiments have shown that large-brained males have faster learning ability in mate-finding tasks (Kotrschal et al., 2014). In the yellow-legged gull there is no evidence so far that late-stage embryos or newly hatched chicks are exposed to divergent selection for cognitive tasks between the sexes. Post-natal behaviour, including lateralization, anti-predator behaviour and begging, which are major fitness traits during early post-natal stages, show no sex-dependent variation (Possenti et al., 2016). However, sex differences in lateralization have been demonstrated in the ‘tonic immobility’ response to acute stress (Romano et al., 2015). The function of the sexual dimorphism in brain size that we observed thus remains to be elucidated. At the mechanistic level, differently from oestrogens, which participate in the orchestration of sexual differentiation of several organs, yolk androgens seem not to interfere with sexual differentiation of the brain (Groothuis and Schwabl, 2008). Our results are consistent with the notion of no effect of T on sexual differentiation of the brain, because we found no differential effect of T treatment on brain size in either sex, after controlling for variation in embryo size, and imply that processes not mediated by egg T concentration cause early onset of brain sexual dimorphism. For example, sex-dependent variation in the expression of androgen receptors, which may occur independently of androgen regulation, could cause the observed sexual dimorphism in brain size (Gahr, 2001).

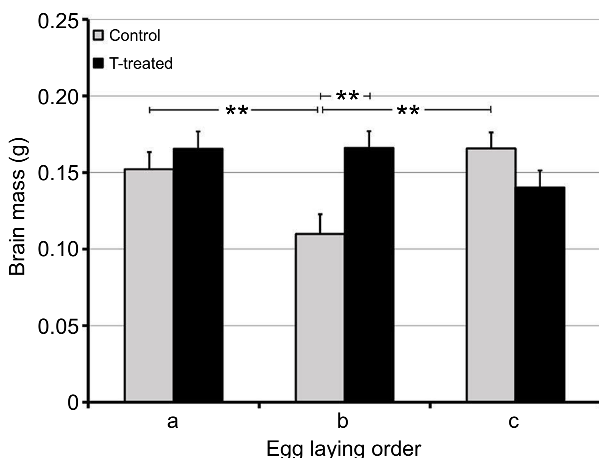

Fig. 2. Yellow-legged gull brain mass (estimated marginal means+s.e.) from a model with the same design as in Table 1 in relation to treatment and laying order. Significant pairwise differences (LSD test) between embryos of the same treatment or laying order are indicated by asterisks (***P*<0.01).

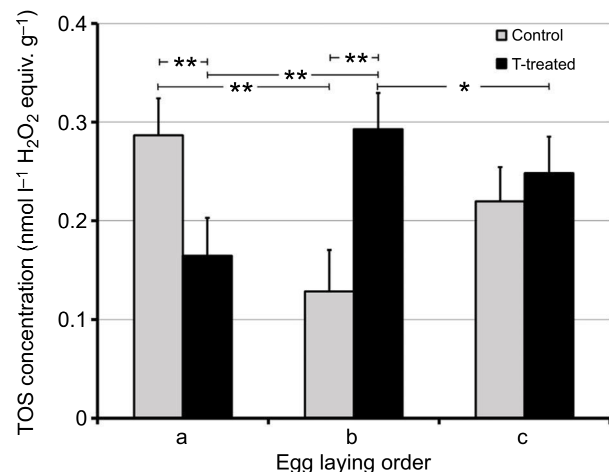

Fig. 3. Total pro-oxidant molecules (TOS; expressed as nmol l^{-1} H_2O_2 equivalents g^{-1} wet weight) in the yellow-legged gull brain in relation to treatment and laying order. Significant pairwise differences (LSD test) between embryos of the same treatment or laying order are indicated by asterisks (**P*<0.05; ***P*<0.01).

T treatment affected the pattern of variation of brain size according to laying order. Brain size of embryos of control b-eggs was smaller than that of embryos from control a- or c-eggs, whereas no such variation according to laying order was observed among embryos from T-treated eggs. The proximate causes and any possible function, from a parental perspective, of variation in offspring pre-natal brain size according to laying order are not clear. b-Eggs from the same colony are intermediate in size and biochemical composition between a- and c-eggs (Rubolini et al., 2011), suggesting that maternal effects via steroid hormones and antioxidants do not mechanistically cause b-egg embryos to grow smaller brains at the specific developmental stage when we collected them. Yet, the present results show that egg T has the potential to interfere with the developmental processes that cause differential pace of growth of brain tissues, relative to embryo size, according to laying order.

Interestingly, the differential pattern of variation of brain size according to laying order in control as compared with T-treated embryos was paralleled by variation in TOS in the brain. Control embryos in b-eggs had lower TOS compared with TOS in a- or c-eggs. Conversely, TOS of T-embryos from b-eggs did not significantly differ or was significantly larger compared with TOS from c- or a-eggs from the T-treated group, respectively. The studies of the effect of T on markers of oxidative status in young birds are few, have focused on post-natal stages and have provided mixed evidence (Noguera et al., 2011; Tobler and Sandell, 2009; Tobler et al., 2013). One interpretation of the present results is that T, via an unknown mechanism, specifically enhances growth of the brain of b-egg embryos and this causes increased production of oxidative molecules, leading to relatively large TOS estimates. This interpretation is supported by the observation that T is anabolic and that increased growth rate is generally considered to increase the production of molecules of high oxidative potential, although the evidence for an effect of T on metabolic rates is conflicting (Eising et al., 2003; Tobler et al., 2007; Wikelski et al., 1999) and is available for post-natal but not for embryonic life stages. Differently, the significant decrease in TOS levels measured in the brain of embryos from a-eggs could be related to the higher amount of antioxidants (including vitamins and carotenoids) that mothers allocate to the first-laid eggs compared with second- and third-laid eggs (Rubolini et al., 2011), which efficiently counteracted the free radical production imposed by early development.

We found no evidence that T affected the total antioxidant activity in the brain or in the liver. In the same species that we studied here, Noguera et al. (2011) showed that egg T enhanced plasma TAC during chick growth (age 8 days). Because we did not document any effect of egg T on liver or brain TAC in the present study, we conclude that T effects on TAC depend on ontogenetic stage and/or that they vary between tissues. The negative effect of T on liver TOS that we observed here is partly consistent with Noguera et al. (2011), who did not show an effect of T on TOS but showed that T prevented the increase in lipid peroxidation during chick post-natal growth that was observed among control chicks. In fact, these results combined suggest that reduced oxidative damage to lipids could be due to reduced production of oxidative compounds.

However, our results on the effect of T on TAC and TOS indicate that T has no general direct effect on the oxidative status in different organs via an effect on body growth. Hence, an alternative interpretation is that T has independent effects on growth and TOS in selected organs, with no effect of growth on antioxidant capacity or TOS.

In conclusion, this study shows that physiological increase in T levels has major, contrasting effects on pre-natal development, boosting body growth but depressing brain growth, which was differentially affected depending on laying order. In the same population, T has been shown to depress post-natal body size. Such contrasting effects of egg T on different traits and on the same trait during different ontogenetic stages may enforce a trade-off on maternal decisions regarding T transfer to the eggs. Testosterone also had complex effects on the production of pro-oxidant molecules both in the liver and in the brain, which may be independent of any effect on growth. Finally, we documented for the first time sexual dimorphism in pre-natal brain size. Further studies on other species are needed to assess the generality of pre-natal T effects and of sex-related variation in brain size.

Acknowledgements

We thank the Parco Regionale del Delta del Po (#252015, 20 February 2015), which allowed both the manipulation and the collection of yellow-legged gull eggs. We also thank the referee for constructive criticism.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: N.S., D.R., M.P.; Methodology: C.D.P., M.P. and M.C.; Resources: N.S.; Investigation: C.D.P., M.C., A.R., M.P. and N.S.; Statistical analyses: N.S.; Writing–Original Draft: M.P. and N.S.; Writing–Review and Editing: N.S., M.P and C.D.P.; Supervision: N.S.

Funding

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

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Chapter 6

Yolk testosterone affects growth and promotes individual-level consistency in behavioral lateralization of yellow-legged gull chicks

Hormones and Behavior ***80*** *(2016): 58-67*

Hormones and Behavior 80 (2016) 58–67

Contents lists available at ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh

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Cristina Daniela Possenti [a], Andrea Romano [a,*], Manuela Caprioli [a], Diego Rubolini [a], Caterina Spiezio [b], Nicola Saino [a], Marco Parolini [a,*]

[a] Department of Biosciences, University of Milan, via Celoria 26, Milan, Italy
[b] Research and Conservation Department, Parco Natura Viva, Verona, Italy

ARTICLE INFO

Article history:
Received 21 July 2015
Revised 11 January 2016
Accepted 29 January 2016
Available online 1 February 2016

Keywords:
Larus michahellis
Lateralization
Maternal effect
Population-level lateralization
Testosterone

ABSTRACT

Behavioral lateralization is common in animals and may be expressed at the individual- and at the population-level. The ontogenetic processes that control lateralization, however, are largely unknown. Well-established sex-dependence in androgen physiology and sex-dependent variation in lateralization have led to the hypothesis that testosterone (T) has organizational effects on lateralization. The effects of T exposure in early life on lateralization can be efficiently investigated by manipulating T levels in the cleidoic eggs of birds, because the embryo is isolated from maternal and sibling physiological interference, but this approach has been adopted very rarely. In the yellow-legged gull (*Larus michahellis*) we increased yolk T concentration within the physiological limits and tested the effects on the direction of lateralization in two functionally fundamental behaviors (begging for parental care and escape to cover) of molecularly sexed hatchlings. We also speculated that T may intervene in regulating consistency, rather than direction of lateralization, and therefore tested if T affected the 'repeatability' of lateral preference in consecutive behavioral trials. T treatment had no effect on the direction of lateralization, but enhanced the consistency of lateral preference in escape responses. Sex did not predict lateralization. Neither behavior was lateralized at the population-level. We therefore showed for the first time in any species an effect of egg T on consistency in lateralization. The implications of the effect of T for the evolution of trade-offs in maternal allocation of egg hormones, and the evolutionary interpretations of findings from our studies on lateralization among unmanipulated birds are discussed.

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Introduction

Behavioral lateralization, whereby behavioral functions are consistently biased towards either the right or the left side generally as a result of asymmetric control by either brain hemisphere, is common among vertebrates (Adret and Rogers, 1989; Franklin and Lima, 2001; Reddon and Hurd, 2008; Rogers, 2002, 2008; Rutledge and Hunt, 2004; Ströckens et al., 2013; Vallortigara, 2000; Vallortigara and Rogers, 2005; see Pfannkuche et al., 2009), and has also been documented in invertebrates (Frasnelli, 2013). Behavioral lateralization in animal populations may be expressed at different levels (Rogers et al., 2013): its occurrence at the individual-level, meaning that individuals perform a behavior preferentially on either side, may or may not translate into population-level lateralization (i.e. alignment of the direction of the lateralization in the majority of individuals) depending on the relative frequency of individuals showing a specific lateral preference. In humans, for example, handedness is apparent at the individual-level and also at the population-level, because the frequency of right-handed people largely exceeds that of left-handers (Schaafsma et al., 2009). Conversely, in fiddler crabs where antisymmetric distribution in the size of the chelae is associated with strongly lateralized socio-sexual behavior at the individual-level, no or low lateralization occurs at the population-level because right- and left-'handed' individuals occur at approximately the same frequency (Jennions and Backwell, 1996; Pratt et al., 2003). Thus, where no lateralization occurs at the population-level, individual-level lateralization may still exist. It has also been shown that lateralization may vary in 'strength', with lateralized individuals differing in the intensity of the preference for a specific side. Whether we see population-level lateralization or not will thus ultimately depend on genetic and epigenetic control of individual-level lateralization in combination with the frequency of individuals that inherit or develop any specific lateral preference. An additional dimension of individual-level variation in lateralization, which has seldom been investigated, is the consistency of individuals in their lateralization, i.e. the extent to which any particular individual systematically prefers either side or shows no specific lateral preference. Any particular lateralized individual may show highly or, conversely, poorly consistent lateral preference (i.e. the variance in lateral preference may vary) or

* Corresponding authors at: Department of Biosciences, University of Milan, via Celoria 26, 20133 Milan, Italy.
E-mail addresses: andrea.romano@unimi.it (A. Romano), marco.parolini@unimi.it (M. Parolini).

http://dx.doi.org/10.1016/j.yhbeh.2016.01.007
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may even show no lateral preference in a highly consistent way. We speculate that this component of individual-level lateralization may be partly influenced by the same developmental and physiological mechanisms that influence direction and strength, and may also be important in an evolutionary perspective, because of its consequences in e.g. social as well as predator–prey interactions.

Mechanistic studies of individual-level lateralization and, at a different level, functional interpretations of lateralization and of the selection processes that lead to the evolution of lateralized populations have flourished over the decades (Csermely and Regolin, 2013; Ocklenburg and Güntürkün, 2012; Rogers et al., 2013; Rogers and Vallortigara, 2015). However, much of this matter is still vividly debated.

The common, though not uncontroversial, observation of sex-dependent lateralization in diverse functions has directed attention to the potential role that 'sex hormones', and testosterone ('T' hereafter) in particular, may have in the development of lateralized behavior (Rajendra and Rogers, 1993; Schwarz and Rogers, 1992; Vallortigara et al., 1999; see Pfannkuche et al., 2009 and references therein). Stemming from the organizational-activational theory (Phoenix et al., 1959; see also Arnold, 2009; Wallen, 2009) of sexual differentiation of the brain, extensive experimental work has demonstrated that androgens have pervasive organizational but also activational effects on brain structures and functions (see Adkins-Regan, 2007; Groothuis et al., 2005; Partecke and Schwabl, 2008; Rogers, 2008).

Because many of the anatomical and physiological differences between the sexes are in fact rooted in differences in steroid hormone profiles since early life (Arnold and Breedlove, 1985; Balthazart and Ball, 1995; Ketterson and Nolan, 1999; Schlinger, 1997; Wingfield et al., 1994; see Adkins-Regan, 2005, 2007), it has been hypothesized that androgens affect lateralization, causing sex-dependent but also individual-level variation in lateralization. At least three hypotheses have been presented for the mechanisms that may mediate the effects of early exposure to T, mainly in the context of sex-dependent variation in lateralization in humans and other mammals (see Pfannkuche et al., 2009 for a review). Here, we will not go into the details of the putative mechanisms and specific predictions that these hypotheses generate because they may not be readily generalized across taxa, owing to major differences in brain structure and in the control pathways of steroid hormones on sex-dependent development and expression of behavior.

In birds pre-natal exposure to different T levels affects diverse morphological, physiological and behavioral traits that are expressed in early post-hatching stages but also in adulthood, suggesting the existence of organizational besides activational effects (Bonisoli-Alquati et al., 2007; Groothuis et al., 2005; Strasser and Schwabl, 2004; see Williams and Groothuis, 2015). These effects, however, can be independent of the process of sexual differentiation (Groothuis and Schwabl, 2008; Schlinger, 1998; Williams and Groothuis, 2015). Hence, exposure to prenatal T in birds may have different consequences as compared to other vertebrates, possibly because of differences in the processes of sexual differentiation (Carere and Balthazart, 2007). In addition, sexual differentiation in birds is considered to be induced mainly by the action of estrogens in females, rather than by the masculinizing effects of T in males (Carere and Balthazart, 2007). Hence, variation in pre-natal T levels in birds may be expected to have consequences on post-natal phenotype, which are independent of sex or, alternatively, that depend on sex partly because of aromatization of T to estrogens and the feminizing effects of estrogens on females.

Experimental studies of epigenetic effects of pre-natal T in eutherian mammals are hampered by the intimate physiological connection between the offspring and their mother, as well as, in multiparous species, among siblings, which prevents controlled manipulation of the pre-natal milieu, besides of course ethical constraints to experimentation on humans. Pathological conditions such as CAH (Congenital Adrenal Hyperplasia), which entails altered prenatal exposure to androgens (Mathews et al., 2004, 2009; see also Hines, 2006), may not be fully representative of the consequences of variation in the pre-natal hormone milieu in the general, healthy population. Conversely, birds represent excellent models to investigate the influence of pre-natal environment as determined by maternal allocation of materials to the eggs (Gil, 2008; Mousseau and Fox, 1998; Riedstra et al., 2013) because their eggs are cleidoic, meaning that no exchange of materials occurs between the internal egg milieu and the outer environment, except for oxygen, carbon dioxide and water vapor (Groothuis et al., 2005). Being isolated from the maternal body, bird eggs afford the opportunity of testing how experimentally altered concentrations of egg substances influence pre-natal offspring development, without incurring any interference by maternal or sibling physiology.

Several studies of birds have been used for insightful experimental approaches to investigate the role of epigenetic effects mediated by pre-natal exposure to T on a wide variety of traits, ranging from pre- and early post-natal development, growth and immunity, social or other forms of behavior, and survival (Rubolini et al., 2006; Rutkowska and Cichoń, 2006; Siefferman et al., 2013; Tobler et al., 2010; von Engelhardt et al., 2006; see also Muriel et al., 2013 and references therein; see Schaafsma and Groothuis, 2012 for an experiment on fish). Some experiments have even extended to the investigation of organizational T effects, like those on socio-sexual behavior or susceptibility to activational effects of T that are expressed in adulthood (e.g. Bonisoli-Alquati et al., 2011a, 2011b; Cooke et al., 1998; Groothuis et al., 2005; Rhen and Crews, 2002; Rubolini et al., 2007; Strasser and Schwabl, 2004). Given these advantages of cleidoic bird eggs as experimental models, it is surprising that the *in ovo* manipulation approach to the mechanistic study of behavioral lateralization has been previously adopted, to the best of our knowledge, only once (Riedstra et al., 2013). In that study, no evidence for an effect of egg experimental T manipulation on the direction or strength of lateralization in three behavioral tasks could be observed in chicken (*Gallus gallus domesticus*) chicks.

In the present study we manipulated the concentration of T in unincubated eggs of the yellow-legged gull (*Larus michahellis*) and tested for the effect on lateral preference exhibited by newly hatched chicks, relative to chicks from sham-injected, control eggs, in two tasks: solicitation of food provisioning by pecking at the bill of dummy parental heads (hereafter 'begging') (Romano et al., 2015) and escape to a safe, dark place in a test apparatus, reflecting response to fear (hereafter 'escape response'). We tested if T treatment influenced the direction but also the consistency of individual-level lateralization (see above for a definition). An effect of T on consistency in lateralization was expected based on the evidence that in poultry chicks T increases persistence of attention in searching for a particular type of food or in a particular place (Andrew, 1972, 1975; Andrew and Jones, 1992; Andrew and Rogers, 1972; Klein and Andrew, 1986), suggesting that T treatment may also induce greater consistency in lateral behavior by boosting attention to the stimulus. Indeed, the consequences of exposure to T on lateralization may be seen not only in terms of direction but also in terms of 'strength' of lateralization, but this component of lateralization has been relatively neglected in favor of a prominent focus on the direction of lateralization.

Moreover, we tested if lateralization existed at the population-level. Finally, we analyzed variation in the direction and consistency of lateralization according to sex and position of the original egg in the laying sequence. The effect of laying order was tested because in the same population that we studied here (Rubolini et al., 2011), as well as in many other bird species (e.g. Badyaev et al., 2006; Groothuis et al., 2006), variation in T concentration according to egg laying order has been observed, suggesting strategic maternal effects mediated by T according to laying order and/or that chicks differ in susceptibility to T effects depending on laying order.

In designing the experiment we paid special attention to administer a dose of T into the egg yolk that resulted in post-manipulation yolk T levels within the natural range of variation of the yolk T concentration

recorded in the same study colony (Rubolini et al., 2011). Indeed, the extent of manipulation of hormone concentrations relative to natural variation is a crucial procedural aspect in experiments on epigenetic effects mediated by egg composition (Groothuis and von Engelhardt, 2005; Riedstra et al., 2013). This is the case because the amenability of the results of such experiments to evolutionary interpretation may considerably differ if the hormone is dosed within the natural range of variation or if, conversely, supra-physiological manipulations are performed.

A previous study on the same population using a physiological dose of T larger than the dose we used decreased post-hatching body mass, whereas no effect of prenatal T exposure on post-hatch begging behavior was observed (Boncoraglio et al., 2006). In order to test if the smaller yolk T dose that we administered in the present experiment was also effective, we also recorded body mass and size soon after hatching to test for an effect of T on somatic growth.

The two tasks we considered are of major functional importance to yellow-legged gull chicks. Begging by pecking at the red patch on the otherwise yellow bill of parents is a key stimulus that serves to solicit food provisioning by regurgitation by the attending parent (Cramp, 1998; Romano et al., 2015). Pecking may be reiterated several times over a short time until the parent regurgitates a food bolus which is passed directly into the chick mouth (Cramp, 1998; Romano et al., 2015). We studied lateralization in begging by symmetrically and simultaneously presenting two realistic, identical dummy parental heads on either side of the chick and recording the side of the chick on which each pecking episode occurred over repeated trials lasting 1 min each (Romano et al., 2015). Chicks are vagile and may leave the nest soon after hatching to hide in the herbaceous vegetation in order to escape from aerial (i.e. raptors) and terrestrial (i.e. rats) predators, harassment from parents of neighboring nesting territories, and perhaps also for shading from direct sunlight. We used a Y-shaped maze with two terminal dark symmetric chambers and recorded the side that the chick chooses to hide, escaping from the starting, unshaded position.

Methods

Study species

The yellow-legged gull is a large (700–1000 g) semi-colonial charadriform bird with a socially monogamous mating system and biparental care of the offspring (Cramp, 1998). Females lay clutches of 1–3 eggs (modal clutch size is 3 eggs) at 1–3 days intervals. Incubation lasts 27–32 days. Sibling eggs hatch asynchronously over 1–4 days. Chicks depend on their parents for food and have low vagility, but may wander around in the parental territory soon after hatching (Cramp, 1998; our personal observations). Parents regurgitate food boluses for their offspring under stimulation of the chicks that peck at the sub-terminal red patch on the lower mandible of parents' bill (see Cramp, 1998). This 'begging' display is an innate behavior which is readily performed by chicks even in response to artificial red stimuli. Adults respond to provisioning solicitation by regurgitating food (Cramp, 1998). In food provisioning events, chicks may perform several pecks at parental bill in a relatively short time (e.g. up to tens of events per minute).

Chicks tend to hide in the herbaceous vegetation in order to escape predation, being harassed by adults of the neighboring territories, and to get shading from direct sunlight.

General field procedure and testosterone injection in the eggs

The study was performed in a large colony (ca. 400 breeding pairs) in the Comacchio lagoon (NE Italy). The colony was visited daily or every second day, starting on 23 March 2015. All nests that were found were marked individually and so were the eggs that were found to have been laid. Thus, we knew whether an egg was the first, second or third (a-, b-, or c-egg, respectively) that had been laid. The day when a new egg was found, it was temporarily removed from the nest and taken to a nearby tent for manipulation. The removed egg was temporarily replaced with a 'dummy' egg to avoid nest desertion and the interference with incubation behavior of parents. We adopted a within-clutch experimental design, whereby sham-injection (control) and T-injection were performed in different eggs of the same clutch, to minimize the consequences of environmental and other parental effects. The following treatment schemes (nest, a-, b-, c-egg) were assigned sequentially to the clutches according to the order in which the first egg was found: nest 1, T injection (T), control (C), T; nest 2, C-T-C; nest 3, T-C-C; nest 4, C-T-T and so forth with the following nests. Injection was performed into the yolk (see Romano et al., 2008 for a validation of the procedure of egg manipulation). Before being injected, the eggs were weighted (to the nearest g) and placed with the acute pole pointing upwards for ca. 15 min. The eggshell close to the acute pole was disinfected and a hole was drilled using a sterile pin 30 μL of T solution in corn oil were injected in the yolk by means of 1-mL sterile syringe mounting 0.6 × 30 mm needle while the egg was held firmly with its longitudinal axis vertical. Control eggs were injected with the same amount of corn oil. After injection, the hole was sealed with a drop of epoxidic glue and plugged with a small piece of eggshell. Importantly, the amount of T injected into individual eggs was scaled according to egg mass and laying order. This was required because the proportional increase in T level caused by injection of any specific amount of T depends on mass of the egg (and thus of the yolk; see below) in which the injected T will dissolve. In addition, T concentration is known to vary according to position in the laying sequence (Rubolini et al., 2011). We aimed at increasing the concentration of T by ca. 1 SD of the concentration measured in the yolk; therefore the amount due to be injected varied depending on the egg (and thus yolk) size. Based on previous data (Rubolini et al., 2011), we estimated the yolk mass based on total egg mass according to the following equation: yolk mass = 0.227 (0.039 SE) egg mass + 1.815 (3.461 SE); $F_{1,88} = 34.38$, $P < 0.001$). Then, we grouped a-, b- or c-eggs into three classes (tertiles) of size according to egg mass and the standard deviation of T concentration in the yolk for each tertile was calculated based on data in Rubolini et al. (2011). The amounts of T that were required to increase the concentration of the hormone by 1 SD and were injected in the three classes of egg mass for any specific position in the laying sequence were as follows (laying order: class of egg mass (g): amount of T injected (ng/egg): a-eggs: 84–91: 57; 92–95: 59; 96–108: 42; b-eggs: 80–88: 74; 89–92: 73; 93–99: 81; and c-eggs, 75–82: 95; 82–87: 84; 88–98: 76. Thus, the dose of T due to be injected was finely tailored on the particular class of size and laying order of the experimental eggs, according to the variance in T concentrations that had been previously estimated for individual egg classes in the same population (Rubolini et al., 2011).

The solutions containing the amount of T per 30 μL corn oil appropriate for any class of egg mass and laying order were prepared in advanced and stored in sterile vials. Because we injected an amount of T equal to that needed to increase the final concentration of yolk T by 1 SD, the final concentration of T could be assumed to be within the natural range of variation in the vast majority of eggs. Hatching success was 0.667 (n = 153 eggs) for control and 0.75 (n = 164) for T injected eggs, implying that T eggs had non-significantly ($\chi^2_1 = 2.69$, $P = 0.102$) larger hatching success compared to controls.

When the eggs reached the 'pipping' stage, they were injected with a drop of food dye of different non-toxic colors through the cracks or small hole to temporarily mark the chick and assign it to its original egg after hatching. On the first day when the chick was found to have hatched and to have dry down, meaning that it had hatched several hours before, we made it recognizable by banding with a colored elastic rubber ring on either tarsus. We then started the behavioral tests (see below), and measured body mass and tarsus length, which was considered as an indicator of skeletal size. In addition, we took a small drop of blood for molecular sexing (Saino et al., 2008).

Begging test

The description of the begging test protocol is derived from Romano et al. (2015), as the protocol we used here was strictly similar. The chick was placed in a begging test apparatus, which consisted of a wooden box with a 15 × 33 cm base and three 15 cm high walls. The chick was placed in the testing position, at the open end of the apparatus, with the head pointing to the opposite end. Two realistic, identical, natural-sized yellow-legged gull heads with yellow bill and the subterminal red patch on the lower mandible were attached symmetrically on the lateral walls, pointing towards the chick, and with their sagittal plane vertical. The heads were attached to the wall at the level of the base of the neck so that the anterior end of the head could move freely, parallel to the walls. The chick could reach the bill of both dummy heads by moderately stretching the neck from its testing position. The heads were operated simultaneously and symmetrically, by means of a single lever to which both heads were connected, by making them oscillate on their sagittal plane within ca. 25° from the horizontal plane. Each complete oscillation took ca. 2 s. The chick was initially held firmly by the experimenter using both hands and completely covering it. The chick's sagittal plane was held parallel to the planes of the two stimulus heads. Care was taken that the base of the apparatus was horizontal, and the bill was aligned on the chick's sagittal plane and pointed slightly downwards. The experimenter was always placed behind the chick and was out of its sight, and the sun (whether visible or not) was approximately behind the chick and the experimenter. The hands were slowly removed from the chick by taking care of moving them symmetrically on either side of the chick. The position of the stimulus heads relative to the chick was closely similar to the position that real parents' head may have during interactions with the chicks (Cramp, 1998). For all pecking events that occurred within 1 min of the start of the trial, we recorded the side on which the head that was pecked at was located relative to the chick.

For each chick we performed two or three begging trials. The first two trials were normally performed on the first day, with ca. 1 h elapsing between consecutive trials, whereas the third was conducted on the next day (see the Results section for information on number of trials per chick). However, some chicks failed to respond to the dummy parental stimulus during some trials. For the analysis of lateralization in begging, for each chick we therefore had one-three data points, one for each trial, which consisted in the number of pecks that were directed rightwards relative to the total number of pecks performed at either dummy parental head. The leftward or, respectively, rightward direction of the first peck in a trial could affect the chances that later pecks occurred on either side: specifically first pecks in a given direction likely increased the chances that second pecks also occurred in the same direction. However, chicks that did more than one peck (and therefore had the opportunity of changing pecking side) and changed the direction of pecking during the begging trial, did so on average after four pecks (mean number of pecks before changing $\pm$SE: 4.01 ± 0.39) while the number of pecks in their trial was approximately 12 on average (11.79 ± 0.84). While carry-over effects of first peck direction could have mildly inflated the evidence for individual-level lateralization, there was therefore ample scope for chicks to change the direction before the trial ended. On the other hand, because no positive reinforcement from pecking, in terms of food provisioning, occurred, chicks were likely facilitated in changing the direction of pecking along individual begging trials, making the evidence for individual-level lateralization conservative. We had no clue to weigh the relative importance of either effect on the observed begging behavior. Because the sequence of begging events that we observed genuinely reflected decisions by chicks that were allowed to decide over alternative directions of begging in a natural scenario where hatchlings are attended by two parents at the nest and no food rewards from begging occurs, we retained the information from all pecking events in the analyses. Moreover, we emphasize that our main goal in the present study was to test for an effect of T treatment on lateralization. Because behavior of T- and control chicks was tested under the same experimental conditions and their responses were recorded with the same protocol, any confounding effect should have operated in the same way on either experimental group and should therefore have produced no bias in the results of the main test.

Escape test

After completion of a begging trial, the chick was subjected to the escape test, which normally consisted of two trials, each of which included three repetitions of the test procedure. In all cases when both the begging and the escape tests were performed in a particular session, the former preceded the latter.

In all repetitions of the escape response trial, the chick was placed in a Y-shaped test apparatus consisting of a wooden base (38 × 30 cm) and a start corridor with transparent plastic lateral walls and roof (section: 8 × 8 cm; length 16 cm, i.e. approximately two times the body length of a resting chick), ending with a 90° bifurcation. The width of the corridor was such that the chick could not easily turn back but was also free to move with no friction on the walls. Both bifurcations of the start corridor led to a dark chamber where the chick could enter. At the start of the test, the chick was placed at the open, unshaded end of the corridor. The chick normally started moving along the corridor towards the bifurcated end within seconds/tens of seconds after it had been placed at the entrance. After it started moving, it usually reached the bifurcation within a few seconds, and entered either dark chamber. Choice was assumed to have occurred when the chick had entered either dark chamber by least one-third of its body length. In all cases, when the chicks had entered the chamber they calmed and squatted down and did never turn back to enter the other chamber or walk back along the start corridor. The chick was then removed from the apparatus and other two repetitions of the first escape test were performed, with the same procedure as above. The second trial was performed on the same day (ca. 1 h after the first trial) while the third trial, if any, was performed on the next day. For each trial, we thus performed three repetitions. For each repetition we recorded the side of the chamber that the chick entered relative to the direction along which the chick moved along the entrance corridor. When the chick failed to move from the start position within 2 min after the test had started, the repetition was considered to have given a null result.

In the analysis of the effect of treatment on lateralization we considered the number of repetitions in a trial when the chick chose the right chamber relative to the total number of repetitions in that trial that did not give a null result. Thus, for each chick we had one-three data points, one for each trial, which consisted in the number of instances when the right chamber was chosen relative to the total number of valid choices in that trial.

The begging and escape tests were normally scheduled as follows: we first performed the first 1 min begging trial and then the first escape trial (including three repetitions of the test). Approximately 1 h later, we performed the second begging and escape trials. Finally, depending on time and weather constraints, the next day we carried out a third trial of begging and/or escape tests.

The study was carried out under license of the Parco Regionale del Delta del Po (#252015, 20 February 2015). The chicks were always handled for the shortest time needed to perform the behavioral tests and blood sampling. The manipulations did not appear to cause any harm to the chicks and we are confident that they did not permanently alter their general state and viability.

Statistical analyses

We mainly relied on generalized (binomial) linear mixed models (LMM) where the response variable was the number of rightward begging or escape responses relative to the total number of responses in any particular trial. Chick identity was included as a random effect

to account for non-independence of the proportion of rightward responses and also to test for consistency of individual-level lateral responses. In the LMM we included the effect of T treatment of the original egg, sex, and position of the egg in the laying sequence as fixed effect factors. The two-way interaction terms were initially included in the model, and the model was then simplified by removing all the non-significant interaction terms in a single step, in order to reduce the number of parameters in the model and the risk of incurring type I statistical errors. All the main effects were always retained in the final models. Individual-level consistency in lateralization was tested by comparing log-likelihood values estimated for the model including the random effect of chick identity or, respectively, the random effect of intercept alone. Log-likelihood values were obtained by Laplace approximation, which can be applied to compare models assuming a binomial error distribution (Bolker et al., 2009; Zuur et al., 2009). To test if lateralization changed between trials, we also tested if the inclusion of 'trial' as a random factor enhanced the fit of the models, by likelihood ratio tests. In order to test if T treatment caused a change in consistency in lateralization, we tested the contribution of chick identity to the fit of the models run on control or T-chicks separately. In addition, to estimate the amount of variation in lateral response that was attributable to chick identity in the control or the T-treated groups we computed: 1) conditional R^2 (see Nakagawa and Schielzeth, 2013) for a model of direction of pecking events or escape responses where we included the main effects of sex and laying order, as well as the random effect of chick identity, and 2) marginal R^2 for a model with the same fixed effects only (Nakagawa and Schielzeth, 2013). To the best of our knowledge, no method has been devised to assess the uncertainty (e.g. confidence intervals) around the estimate of R^2, and no analysis of the usefulness of such uncertainty estimates has been presented for marginal or conditional R^2 from generalized linear mixed models (Nakagawa and Schielzeth, 2013). To assess the importance of chick identity in explaining the observed variation we simply relied on the difference between conditional and marginal R^2 values. Population-level lateralization was assessed by testing deviation of the intercept of the model including the main effects of sex, egg treatment and laying order from the logit-transformed value of the response variable of 0, which was expected under the null hypothesis that half of the responses were rightwards. To represent the consistency of lateralization of individual chicks, we estimated the binomial variance of the proportion of escape responses that were rightwards.

Thirty-nine out of the 66 broods involved in the study were represented in the sample by more than one chick. Inclusion of the random effect of brood identity, besides that of chick identity, in the models never improved model fit. This may suggest no parentage effects on lateralization, possibly reflecting no genetic variation. This evidence would be consistent with a previous study of begging and reversal to prone posture in the same colony (Romano et al., 2015). However, because of the relatively small sample of broods, and the within-brood experimental design that we adopted, we refrain from deriving conclusions on parentage effects on lateralization, which will therefore not be discussed further.

Variation in chick body mass and tarsus length was analyzed in Gaussian LMM where we included the random effect of brood and the fixed effects of treatment, sex, laying order and their two-way interactions. In the models we also included the effect of estimated age at measurement (which was either 0 or 1, implying that the chick was measured either on the day of hatching or on the next day) as well as of mass of the original egg, which is a strong predictor of body size traits at hatching and at later stages before fledging. These models were simplified as described above for lateralization variables. Finally, begging rate (expressed as the number of pecking events in 1 min trials) was analyzed in Poisson LMM, with the same terms as for the analyses of morphological variables.

All LMM were run using the MIXED or GLIMMIX routines in SAS 9.2 software. For binomial models, the events/trials syntax was adopted. R^2 values of the binomial LMM were estimated using the *lme4* routine in R 2.15.2 using the procedure devised by Nakagawa and Schielzeth (2013). Statistical parameters are presented with their associated standard error, unless otherwise specified.

Results

Hatchling phenotype and begging rate in relation to in ovo testosterone treatment

The effect of *in ovo* T treatment on body size and intensity of begging response (i.e. the number of pecks per minute) was tested in 108 chicks that were sexed molecularly. A Gaussian LMM with brood identity as a random effect revealed a significant T treatment by laying order effect on body mass (Table 1). Post-hoc LSD tests revealed a marginally significant ($t_{99} = 2.02$, $P = 0.046$) difference between chicks from control or T-injected a-eggs, with the latter being smaller (Fig. 1), whereas the differences between control and T-chicks from b- or c-eggs were not statistically significant ($P > 0.12$ in both cases). Thus, T treatment differentially affected chick body mass depending on laying order and, in particular, depressed body mass of chicks from a-eggs, which have the smaller concentration of yolk T (Rubolini et al., 2011). Importantly, these results were unaffected by the size of the original egg, which varies according to the laying sequence (e.g. Saino et al., 2010), and significantly predicted body mass around hatching (Table 1).

The analysis of body size, as indexed by tarsus length, showed a significant sex by laying order effect (Table 1), whereby male chicks from a- and b-eggs, but not those from c-eggs were significantly larger than female chicks.

Both body mass and tarsus length were significantly, positively predicted by original egg mass (Table 1).

Overall, 257 begging trials lasting 1 min were performed by the 108 chicks (mean number of trials per chick: 2.4, range: 1–3). On average, the chicks (including also those that did not perform any begging), performed 4.82 pecks (range 0–30) per trial. The twenty-four chicks that did not perform any pecking were included in the analyses of begging intensity but had to be excluded from the subsequent analyses of lateralization. The simplified model included the significant effect of laying order, whereby b-chicks performed more begging pecks than c-chicks. In addition, begging rate significantly declined with chick age (Table 1). However, pecking rate was not predicted by interaction or main effects of egg treatment (Table 1).

Lateralization according to in ovo testosterone treatment

Eighty-four of the 108 chicks that were tested for begging performed at least one peck at the dummy parental heads during the one-three begging trials and were therefore used in the analysis of lateralization in begging. In a binomial LMM with chick identity as a random factor and the main and two-way interaction terms of T treatment, sex and laying order as fixed factors we found no significant effects of the two-way interaction terms ($P > 0.52$ in all cases). The simplified model obtained after excluding the non-significant effects of the two-way interactions showed no significant main effects (Table 2). In addition, the intercept did not significantly differ from 0. In fact, out of the 14.75 (1.44) pecks that chicks that did respond to stimulation performed on average, 7.32 (0.97) were rightwards, yielding a proportion of rightward pecks (49.6%), very close to that (50%) expected in case of no population-level lateralization. Hence, lateralization did not vary according to T treatment, sex, laying order, or their combined (interaction) effects, and no significant lateralization existed at the population-level.

A likelihood ratio test comparing the model in Table 2 with a model only including the main effects (i.e. excluding the random effect of chick identity) showed that lateralization occurred at the individual-level, as implied by the large, statistically significant difference in log-likelihood values associated to either model (Table 3). The R^2 of the

Table 1
Gaussian linear mixed models of body mass and tarsus length, and Poisson linear mixed model of begging rate in relation to T treatment of the original egg, sex, position of the original egg in the laying sequence and their interaction terms. Age at measurement and mass of the original egg or chick mass were included as covariates. Brood identity and chick identity (begging rate analysis only) are included as random effects in the models. The initial models were simplified by removing all the non-significant interactions in a single step. Samples sizes according to treatment, sex and laying order were: control chicks: 42; T-chicks: 66; males: 58; females: 50; a-chicks: 32; b-chicks: 41; c-chicks: 35 (see also the Methods section).

	Excluded terms			Retained terms			
	F	df	P	F	df	P	Coeff. (SE)
Body mass							
Treatment				0.04	1,99	0.839	
Sex				0.17	1,99	0.683	
Laying order				0.10	2,99	0.909	
Treatment × Sex	0.24	1,96	0.623				
Treatment × Laying order				3.13	2,99	0.048	
Sex × Laying order	0.59	2,96	0.554				
Egg mass				304.10	1,99	<0.001	0.729 (0.042)
Age				1.97	1,99	0.164	0.851 (0.607)
Tarsus length							
Treatment				0.88	1,99	0.352	
Sex				7.18	1,99	0.009	
Laying order				2.37	2,99	0.098	
Treatment × Sex	0.54	1,96	0.465				
Treatment × Laying order	2.34	2,96	0.101				
Sex × Laying order				6.03	2,96	0.003	
Egg mass				11.08	1,99	0.001	0.417 (0.125)
Age				15.70	1,99	<0.001	7.200 (1.817)
Begging rate							
Treatment				3.69	1,147	0.057	
Sex				1.52	1,147	0.220	
Laying order				3.45	2,147	0.035[a]	
Treatment × Sex	0.34	1,148	0.560				
Treatment × Laying order	0.12	2,148	0.884				
Sex × Laying order	0.84	2,148	0.433				
Egg mass				3.33	1,147	0.070	−0.047 (0.026)
Age				26.44	1,147	<0.001	−0.687 (1.340)

[a] Estimated marginal means (SE) for a-chicks: 0.766 (0.281); b-chicks: 1.284 (0.239); and c-chicks: 0.426 (0.273). The estimated marginal mean for b-chicks was larger than that of c-chicks ($t_{147} = 2.57$, $P = 0.011$).

model including the random effect of chick identity was considerably larger than that of the model with fixed effects only, demonstrating large individual-level consistency in the side on which begging events occurred (Table 3). Models on chicks of either treatment groups showed significant individual-level lateralization with R^2 values being large and differing by only ca. 4% (Table 3). Models including the random effect of trial nested within chick identity provided a better fit of the data for all chicks pooled ($\chi^2_1 = 88.88$, $P < 0.0001$) and for control chicks ($\chi^2_1 = 99.97$, $P < 0.0001$), but not for T-chicks ($\chi^2_1 = 1.75$, $P = 0.186$). These effects of trial imply that T-chicks were more consistent in their response among trials as compared to control chicks.

Analyses similar to those of lateralization in begging were run for escape response, on a total of 106 chicks. Individuals were subjected on average to 5.8 (range 1–7) repetitions of the escape test. We found no significant two-way interaction effects among treatment, sex and egg laying order in a model including chick identity as a random effect. The simplified model only including main effects showed that lateral preference in escape response did not depend on treatment, sex or egg laying order (Table 2). The estimated intercept did not significantly deviate from 0, implying no population-level lateralization also in escape response. Escape responses were rightwards in 56.2% (2.70 SE) of the repetitions.

A likelihood ratio test comparing the model in Table 2 with the model including the same fixed effects but excluding the effect of chick identity disclosed significant individual-level lateralization in the whole sample of chicks (Table 3). However, when the sample was split according to T treatment, chick identity was found to significantly contribute to the fit of the model on chicks from T but not from control eggs. The R^2 value of the model for T-chicks was markedly larger than for control chicks (Table 3). Fig. 2 shows that the proportion of chicks that were consistent in the preference for either side during the escape response tests was more than twice as large among T- compared to control chicks. Among T-chicks, likelihood ratio tests showed that individual identity significantly contributed to the fit of the models for both males ($\chi^2_1 = 4.82$, $P = 0.028$; $R^2 = 18.1\%$) and females ($\chi^2_1 = 15.28$, $P < 0.001$; $R^2 = 29.1\%$), with an apparently stronger effect for females. Thus, there was a hint that the effect of T on the consistency of lateralization was larger among females as compared to males. Models including the random effect of trial nested within chick identity did not provide a better fit of the data for all chicks pooled or for chicks of either treatment groups ($\chi^2_1 < 2.99$, $P > 0.08$ in all cases),

We found no significant correlation in individual-level lateral preference in either task ($r = 0.128$, $P = 0.241$, $n = 85$ chicks). The strength of the correlations between lateral preference in either task did not differ between control and T-chicks (control chicks: $r = 0.00$, $n = 36$; T-chicks: $r = 0.199$, $n = 49$; $z = 0.884$, $P = 0.377$).

Discussion

We studied the effect of pre-natal exposure to elevated T levels *in ovo* on behavioral lateralization of yellow-legged gull chicks in two tasks: soliciting parents to regurgitate food by the innate begging display, and escaping to a shaded, safe position. The direction of lateralization was not affected by *in ovo* T treatment in both sexes. No lateralization existed at the level of population in both tasks. Chicks from both T- and control eggs showed strong individual-level variation in lateral preference in begging behavior. Conversely, individual-level consistency in lateral preference in the escape response was observed among chicks from T-eggs but not among controls. Finally, we found no sex differences in the direction of lateralization.

Our study does not support the general idea that T affects the direction of lateralization. This hypothesis ultimately stems from the observation that in some species and traits, the two sexes differ in the direction of lateralization (Pfannkuche et al., 2009). In fact, we could also uncover no evidence that the direction of lateralization differed between male and female chicks independently of *in ovo* T treatment.

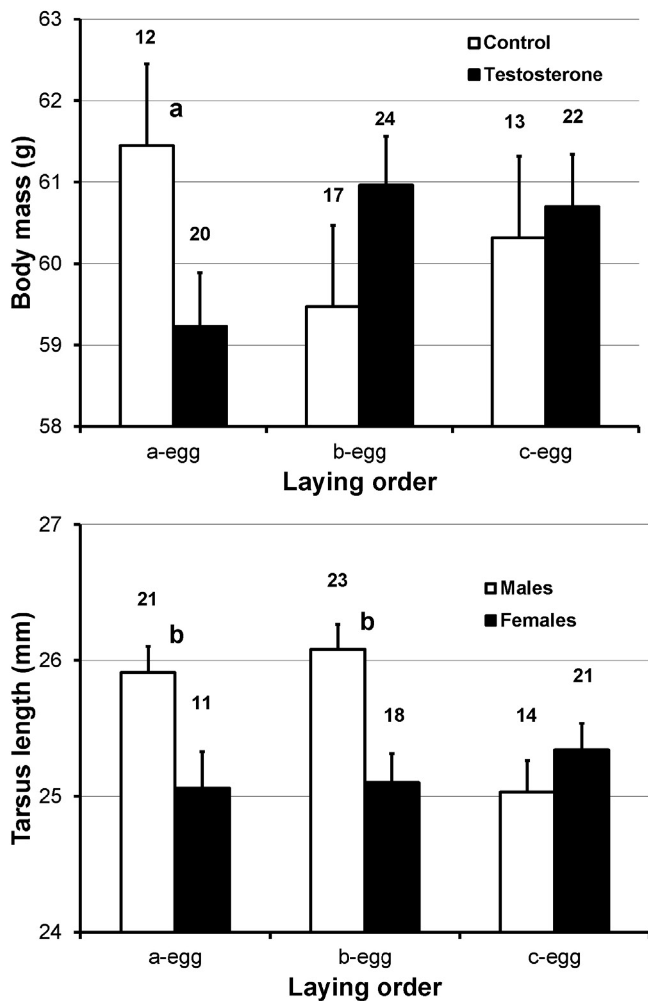

Fig. 1. Estimated marginal means (+SE) of body mass and tarsus length of chicks from testosterone injected or control eggs of different laying order. a: significance of the difference between treatments: P = 0.046; b: significance of the difference between sexes: P < 0.02. The number of chicks for each treatment is shown.

Because sample sizes were reasonably large and the effect of sex on lateralization in either task was far from statistical significance (see Table 2), we are confident that lack of sex-dependence was not attributable to low power of statistical tests. The only previous study where the effect of physiological T doses in the egg on lateralization was investigated (Riedstra et al., 2013) did also not provide evidence for an effect on direction of lateralization in three visually guided behaviors. Hence, in birds there is no evidence arising from the only two studies so far, for an effect of pre-natal exposure to elevated T levels on direction and strength (sensu e.g. Riedstra et al., 2013) of behavioral lateralization.

However, pre-natal T apparently produced an increase in the consistency that chicks showed in their lateral preference in escape behavior, meaning that, independently of whether an individual displayed a preference for either the left or the right side or showed no specific lateral preference, its pattern of lateral preference was reinforced by exposure to elevated pre-natal T levels. This is implied by the significant effect of chick identity in the models of lateral preference in the escape response in T but not in control chicks, and by the markedly larger proportion of the variance in lateral preference that was accounted for by chick identity in the former as compared to the latter experimental group. In addition, there was a hint that T affected the pattern of consistency in lateral preference in begging behavior. Indeed, lateral preference of T-chicks did not vary among trials whereas the opposite was the case for control chicks, as indicated by the fact that the random effect of trial was significant for control but not for T-chicks (see Table 3). This study is therefore the first where the effect of T on consistency (see above) in lateral preference has been investigated and shows that pre-natal T may act on lateralization by stabilizing individual preference. The generality of the pattern that we observed requires confirmation by further experimental investigation in other models.

At the present stage, the interpretation of the mechanisms that mediate the observed effects of T on consistency in lateral preference is bound to be speculative. Egg hormones, and T in particular, have been shown to have both organizational and activational effects on behavior and morphological traits (Groothuis et al., 2005). Distinguishing between organizational and activational effects in early post-natal behavioral tests, however, is difficult. Because yolk is absorbed gradually by the developing embryo and substantial amounts of residual yolk remain in the yolk sac around hatching in our model species (M. P., unpublished results; see also von Engelhardt et al., 2009), yolk T manipulation may result in exposure to elevated exogenous T levels enduring shortly after hatching, when behavioral tests were performed. Moreover, the effects of maternal hormones after hatching may in fact represent a form of 'transient activational effects' that occur soon after hatching, as suggested by the fact that some of these effects have been shown to vanish at later post-hatching stages (see Carere and Balthazart, 2007). On the other hand, steroid hormones have been shown to affect the development of visual lateralization induced by light. The visual system of birds is asymmetrical (Güntürkün, 2002; Rogers and Sink, 1988), and the asymmetry is located in the projections that cross the midline of the brain, from one side of the thalamus to the contralateral wulst: there are more projections from the left thalamus to the right forebrain than from the right thalamus to the left forebrain (Rogers and Deng, 1999). Light stimulation just prior to hatching influences the asymmetry

Table 2
Binomial linear mixed models of the frequency of rightward begging pecks or escape responses in relation to testosterone treatment of the original egg, sex and laying order of the original egg. Two-way interaction terms were removed in a single simplification step as their effect was non-significant. In all models, chick identity was included as a random effect factor. The inverse-linked least square means (SE) estimated from the models are reported. Samples sizes according to treatment, sex and laying order for begging analyses were: control chicks: 35; T-chicks: 49; males: 51; females: 33; a-chicks: 24; b-chicks: 36; c-chicks: 26; for escape response sample sizes were: control chicks: 42; T-chicks: 64; males: 57; females: 49; a-chicks: 32; b-chicks: 40; c-chicks: 34. (see also the Methods section).

	t/F[a]	df	P	Parameter estimates (SE)
Begging				
Intercept	0.68	79	0.501	0.332 (0.491)
Treatment	0.60	1,60	0.440	Control: 0.415 (0.086) Testosterone: 0.503 (0.073)
Sex	0.00	1,60	0.958	Males: 0.462 (0.071) Females: 0.456 (0.091)
Laying order	0.84	2,60	0.437	a: 0.358 (0.099) b: 0.480 (0.086) c: 0.542 (0.102)
Escape response				
Intercept	0.24	101	0.814	0.058 (0.244)
Treatment	0.95	1,103	0.332	Control: 0.608 (0.047) Testosterone: 0.549 (0.039)
Sex	1.06	1,103	0.307	Males: 0.547 (0.041) Females: 0.610 (0.045)
Laying order	1.15	2,103	0.320	a: 0.618 (0.054) b: 0.604 (0.047) c: 0.512 (0.054)

[a] t-Value is given for the test on the intercept.

Table 3
Log-likelihood (LL) values from models of the frequency of rightward begging pecks or escape responses including or, respectively, excluding the random effect of chick identity on all chicks pooled or on chicks belonging to either the control or the T-treated group. The R^2 values of the models computed according to Nakagawa and Schielzeth (2013) are also presented. Δ values are the difference between the LL or the R^2 values (%) for the models including or, respectively, excluding the random effect of chick identity. Asterisks are the ΔLL values which are associated with a significant (P always <0.0001) difference in the fit of the models according to the likelihood ratio test based on χ^2_1.

	Begging		Escape response	
	LL	R^2	LL	R^2
All chicks				
Model with sex, treatment laying order; chick ID	642.72	48.02	555.45	18.35
Model with sex, treatment laying order; intercept	953.79	1.19	576.05	0.73
Δ	311.07**	46.83	20.6**	17.62
Control chicks				
Model with sex, laying order; chick ID	342.80	50.46	209.20	10.31
Model with sex, laying order; intercept	531.71	1.82	210.70	3.63
Δ	188.91**	48.64	1.50	6.68
Testosterone chicks				
Model with sex, laying order; chick ID	298.62	45.56	339.72	24.24
Model with sex, laying order; intercept	419.37	0.84	359.87	0.43
Δ	120.75**	44.72	20.15**	23.81

of the visual projections from the thalamus to the wulst of the chick forebrain. A relatively short period of monocular light deprivation in the early post-hatching period of pigeons was shown to alter tectal morphology and behavioral asymmetry in adulthood (Manns and Güntürkün, 1999). Thus, asymmetrical light stimulation during critical ontogenetic periods has the potential to trigger visual asymmetry in diverse model organisms. T and also other steroid hormones interfere with the effect of light on the development of visual lateralization, by affecting the structural asymmetry in the thalamofugal projections (Rogers, 2008). Sex hormones may act by preventing loss of neurons from the right side of the thalamus, or by promoting growth of neurons on both sides of the thalamus (Schwarz and Rogers, 1992). Thus, the development of these visual projections depends on the interaction of light stimulation and pre-hatching exposure to T. Hence, T affects the development of neuroanatomical structures relevant to lateral preferences. It could be speculated that T treatment increases consistency in lateral preferences, as observed in the present study, via a general positive effect on persistence of behavioral patterns (Andrew, 1972, 1975; Andrew and Jones, 1992; Andrew and Rogers, 1972; Klein and Andrew, 1986).

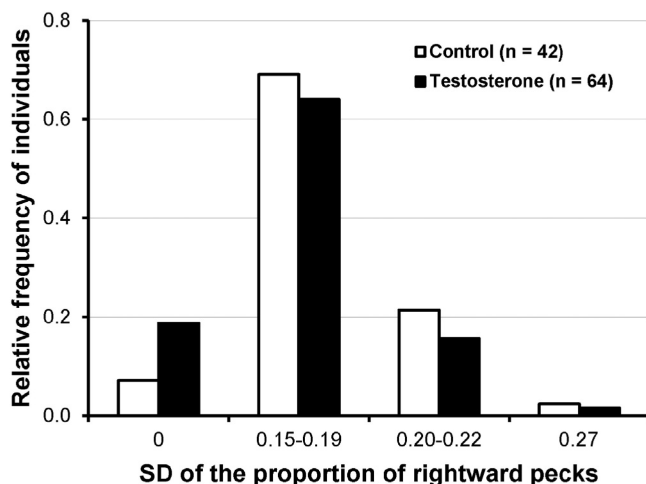

Fig. 2. Frequency distribution of individual-level binomial estimated standard deviations (SD) in the overall proportion of rightward escape responses for control chicks and T-chicks. Given the number of repetitions of the tests and the observed frequency of rightward responses the variances took 8 discrete values. The limits of the 4 classes in which these values were classified are thus explicitly given for better accuracy. 0 indicates that all escape responses were performed on the same side. Increasing variance values indicated that individuals were less consistent in lateral preference. The proportion of individuals which chose the same side on all repetitions was more than twice among T- as compared to control chicks.

Post-hatching T-treated males had reversed asymmetry in visual discrimination learning compared to controls, while negatively affecting learning performance of females in pecking discrimination tasks, implying that T can induce or reverse asymmetry of function in males (Greenspon and Stein, 1983; Zappia and Rogers, 1987). Post-hatching T administration has also been shown to reduce the strength of lateralization in visual discrimination learning (Zappia and Rogers, 1987) and to increase population-level lateralization in a rotational task in fish (Schaafsma and Groothuis, 2011). However, the androgen DHT (dihydrotestosterone) promotes the strength in lateralization in copulatory behavior (Bullock and Rogers, 1992). Our results therefore imply that, contrary to post-natal exposure to elevated T levels, pre-natal exposure has no effect on the direction of behavioral lateralization at tasks that show no sex-dependent variation in the natural population.

The dose of T that we administered was adjusted for egg mass and laying order and corresponded to that needed to elevate yolk post-manipulation concentration by 1 SD of the concentrations previously recorded in the same natural population (Rubolini et al., 2011). This design was chosen in order to ensure that we did not caused supra-physiological post-manipulation concentrations (see also Groothuis and von Engelhardt, 2005; Riedstra et al., 2013). While the dose of T was chosen to be relative low, it proved to be effective on somatic growth. Specifically, T appeared to cause a reduction in body mass of chicks from a-eggs, after controlling for the potentially confounding effect of original egg mass and sex. This finding is consistent with a previous experiment where a double dose compared to the one we used here caused a reduction in body mass of 4 days old chicks, although in that study the effect was also observed for chicks from b- and c-eggs (Rubolini et al., 2006), whereas in the present study was observed for a-chicks only, as implied by the significant treatment by laying order interaction effect. The fact that mass was measured at a later stage and that the dose we used here was lower, in combination with the increase in T concentration with laying order, may provide an explanation for the lack of effect of T treatment on b- and c-chicks, potentially due to larger responses to T by embryos from eggs with smaller absolute concentrations of the hormone. In addition, similarly to another previous study, no effect of T on begging rate was observed (Boncoraglio et al., 2006).

Our study was carried out in a wild population not subjected to artificial selection. This is important because artificially selected strains held in captivity may not entirely represent their wild, unselected counterparts in several respects. First, artificial selection may cause inadvertent evolutionary change via direct selection on the trait of interest or selection on a correlated trait, resulting in change in mean phenotypic values but also in genetic variance in the trait. Second, any early maternal effect mediated by egg composition, including hormone concentrations, may be affected both because of the genetic makeup of the artificially selected strain and because of captivity conditions, in terms of e.g. stress and

social effects, by acting on maternal phenotype may cause the biochemical composition of the eggs to deviate from that of populations in the wild. The information we provide here is therefore novel and complementary to that from other studies on artificially selected, captive strains that used different protocols in terms of developmental stage at T treatment and criteria to dose T, compared to those that we used here.

Because here and in a previous study (Romano et al., 2015) we focused on a natural population and on functionally important tasks, which concern parent-offspring communication and access to food, as well as escape from predators and social harassment, we deem our findings on individual- and population-level lateralization in the unmanipulated chicks amenable to interpretation from an evolutionary perspective. The patterns of lateralization were found to vary according to the specific task under scrutiny. Begging behavior was lateralized at the individual- but not at the population-level in both studies. Conversely, the present study showed that escape response was not lateralized at either the individual- and at the population-level. Another functionally important behavior (i.e. reversing from supine to prone position; see Romano et al., 2015) was found to be lateralized both at the individual- and at the population-level. Moreover, no correlation existed between the direction of lateralization in begging and the reversal to prone position (Romano et al., 2015) or, respectively, escape response (present study). In general, these findings show that lateralization at different traits may differ within individuals, as also found in other studies (Izawa et al., 2005; Schiffner and Srinivasan, 2013; Vince, 1964). Escape response was the only trait for which we could detect neither individual-level nor population-level lateralization. No population-level lateralization in escape response may be maintained by negative frequency-dependent selection whereby individuals lateralized in the same direction as most of the individuals in the population would suffer increased risk of predation owing to specialization of predators on the most frequent escape phenotype. Individual-level lateralization may also be negatively selected because it makes behavior of the escaping prey more predictable by a predator reiterating its capture attempts during a single attack or upon repeated encounters of the same individual prey. Individual-level lateralization in begging, that was confirmed in the present study, may instead increase the efficiency in food provisioning, which consists in direct transfer of food boluses from the parents to the chick, and prevent food from being lost to the competing sibling or falling on the ground, as long as the chick's side on which begging is performed is the same where the passage of food also occurs. This could be particularly advantageous in young chicks, whose motor skills are still poor. Lateralization in reversal to prone position (Romano et al., 2015) may well be adaptive because individual directional preference in reversal attempts likely make successful reversal faster and thus reduces the time the chick takes to resume a more cryptic, less vulnerable posture. No negative frequency-dependent selection, which may instead operate on escape behavior (see above) on lateralization in this task and consistent postural asymmetry among embryos, also exposing to lateralization effects of differential exposure of either embryonic eye to light (Güntürkün and Manns, 2010; Kuo, 1932; Manns and Güntürkün, 1999; Rogers and Bolden, 1991; Rogers and Deng, 1999) may cause directional lateralization in early post-hatching period (Rogers and Workman, 1993), translating into moderate population-level lateralization.

The fitness consequences of the observed patterns of individual-level lateralization are generally unknown in intensity but also in sign. Depending on selection being positive or, conversely, negative, larger or smaller transfer of maternal T to the eggs may be promoted by selection on lateralization. However, high T levels have been claimed to have positive effects on competitive ability but also have negative consequences for somatic growth, as we showed here and in a previous study (Rubolini et al., 2006). Hence, trade-offs may exist among the contrasting effects of T on lateralization and other fitness traits. The concentration of T in bird eggs varies non-randomly according to laying order, as it increases in last laid eggs in some species, including the yellow-legged gull, while showing the opposite pattern in other species. Adaptive interpretations of the observed patterns of maternal T variation in the eggs may thus also require the consequences of T on lateralization to be taken into account.

In conclusion, we found no experimental evidence for an effect of exposure to physiological increase in T levels in the egg on the direction of lateralization in two important behaviors of yellow-legged gull chicks. Moreover, no sex-dependent nor population-level lateralization were observed, irrespective of T treatment of the eggs. However, escape response was found to be more consistent in chicks from T treated eggs. If consistency in lateral preferences has fitness consequences, the present results imply that adaptive transfer of maternal T to the eggs may have to be tuned according to its effects on lateralization, besides the other effects, physiological and behavioral effects, which have been documented in the yellow-legged gull and in other bird species.

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Chapter 7

Effect of yolk corticosterone on begging in the yellow-legged gull

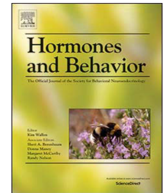
Hormones and Behavior ***97*** *(2018): 91-97*

Hormones and Behavior 97 (2018) 121–127

Contents lists available at ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh



Effect of yolk corticosterone on begging in the yellow-legged gull

Cristina Daniela Possenti*, Marco Parolini, Andrea Romano, Manuela Caprioli, Diego Rubolini, Nicola Saino

Department of Environmental Science and Policy, University of Milan, via Celoria 26, 20133 Milan, Italy

ARTICLE INFO

Keywords:
Begging behavior
Corticosterone
Larus michahellis
Lateralization
Maternal effects

ABSTRACT

Behavioral lateralization is widespread across vertebrates. The development of lateralization is affected by both genetic and environmental factors. In birds, maternal substances in the egg can affect offspring lateralization via activational and/or organizational effects. Corticosterone affects the development of brain asymmetry, suggesting that variation in yolk corticosterone concentration may also influence post-natal behavioral lateralization, a hypothesis that has never been tested so far. In the yellow-legged gull (*Larus michahellis*), we increased yolk corticosterone concentration within physiological limits and analyzed the direction of lateralization of hatchlings in reverting from supine to prone position ('RTP' response) and in pecking at dummy parental bills to solicit food provisioning ('begging' response). We found that corticosterone treatment negatively affected the frequency of begging and it may cause a slight leftward lateralization. However, the direction of lateralization of the RTP response was not affected by corticosterone administration. Thus, our study shows a maternal effect mediated by corticosterone on a behavioral trait involved in parent-offspring communication during food provisioning events. The findings on lateralization are not conclusive due to the weak effect size but provide information for further ecological and evolutionary studies, investigating mechanisms underlying the development of lateralization.

1. Introduction

Behavioral lateralization occurs whenever left-right brain hemispheres asymmetrically control behavioral functions, resulting in their consistent bias toward either side of the body (Güntürkün et al., 2000; Halpern et al., 2005; Vallortigara, 2006). Behavioral lateralization has been long thought to be unique to humans but there is a growing body of evidence showing that it is widespread among vertebrates (Adret and Rogers, 1989; Bisazza et al., 1998; Halpern et al., 2005; Reddon and Hurd, 2008; Rogers, 2002, 2006, 2008, 2012; Ströckens et al., 2013; Vallortigara, 2000) as well as among invertebrates (Byrne et al., 2004; Frasnelli, 2013; Hobert et al., 2002; Pascual et al., 2004). For instance, several species of ants, parrots and wallabies exhibit preferential use of limbs of either side for specific behavioral tasks, such as foraging or manipulating objects (Giljov et al., 2012; Harris, 1989; Heuts et al., 2003). Behavioral lateralization in animal populations may exist at two different levels. Population-level lateralization occurs when the majority of the individuals shows an alignment in the direction of the asymmetry. On the other hand, when the pronounced left-right biases in lateral preference vary among individuals, lateralization at the individual-level exists. The two forms of lateralization may occur together, but if no lateralization at the population-level occurs, individual-level lateralization may still exist.

Lateralization has attracted attention on its functional interpretation and on the selection process that lead to the evolution of lateralized populations as well as on the genetic and physiological mechanisms underlying its development (Bisazza et al., 1998; Halpern et al., 2005; Vallortigara, 2006). Although it is well-known that lateralization has a genetic component, there is a flourishing body of evidence showing that environmental influences on the development of lateralization can occur (Annett, 1978; Bishop, 2001; Laland et al., 1995; Provins, 1997; Rife, 1940; Schaafsma et al., 2009).

Environmental effects, including pre-natal intra-uterine or egg conditions, may influence individual-level lateralization. In birds, post-natal lateralization may partly develop as a consequence of monocular light stimulation of the visual system in the embryo (Rogers, 1982). During the late stages of incubation, the embryo maintains an asymmetrical position that entails a larger light exposure of its right eye through the eggshell (Koshiba et al., 2003; Manns and Güntürkün, 1999; Rogers, 1990, 2002, 2006). Pre-natal postural asymmetry may also affect post-hatching motor lateralization because of the differential use of either embryo foot to maintain position or to emerge from the eggshell (Casey, 2005; Casey and Martino, 2000; Rogers and Workman, 1993). Other pre-natal environmental effects on lateralization may

* Corresponding author at: Dept. Environmental Science and Policy, via Celoria 26, 20133 Milan, Italy.
E-mail address: cristina.possenti@unimi.it (C.D. Possenti).

https://doi.org/10.1016/j.yhbeh.2017.10.014
Received 3 January 2017; Received in revised form 25 October 2017; Accepted 27 October 2017
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result from transfer of maternal substances to the eggs that can have activational and/or organizational effects. Steroid hormones are transferred by the mothers to their eggs in amounts that can vary among mothers as well as among eggs from the same clutch, depending for example on embryo sex and the position in the laying sequence (Badyaev et al., 2006; Groothuis et al., 2006; Royle et al., 2001; Rubolini et al., 2011; Schwabl, 1999). Corticosterone, for example, is believed to have a pervasive effect on visual system asymmetry, suggesting that it may influence post-hatching lateralization. Corticosterone is the main adrenocorticoid hormone in birds and is secreted by the adrenal glands under stimulation of the hypothalamo-pituitary-adrenocortical (HPA) axis (Costantini, 2014; Henriksen et al., 2011; Wingfield and Romero, 2001). Maternal corticosterone is transferred into egg yolk and albumen (Kalliecharan and Hall, 1974; Rubolini et al., 2005, 2011; Saino et al., 2005) in amounts that can vary depending on mother's quality and environmental conditions at the time of laying (Hayward and Wingfield, 2004; Love et al., 2008; Saino et al., 2005). Modulation of maternal transmission and endogenous production of this stress hormone can thus shape post-natal phenotype, including brain structure, visual asymmetry and behavioral traits (Freire et al., 2006; Rogers, 2005; Rogers and Deng, 2005; Sui et al., 1997). Administration of exogenous corticosterone into the eggs during incubation has been applied in few studies in order to investigate the effects on hatchling phenotype. Elevated corticosterone levels have been shown to affect behavioral traits, such as the intensity of chick food solicitation displays (i.e. begging behavior), fearfulness, competitiveness or exploratory behaviors, although the results have been often found to be inconsistent across species (Davis et al., 2008; Love and Williams, 2008; Janczak et al., 2006; Rubolini et al., 2005; see review Henriksen et al., 2011). Furthermore, an *in ovo* increase of corticosterone concentration has been shown to prevent the development of visual asymmetry in response to light (Rogers and Deng, 2005), leading to an impairment of the ability of chicks to perform more than one task simultaneously: corticosterone-treated chicks have poorer performance at detecting an overhead predator while feeding than control ones (Dharmaretnam and Rogers, 2005; Freire et al., 2006; Rogers et al., 2004). Despite the potential role of corticosterone in the development of visual asymmetry, however, the effect of corticosterone treatment on the direction of lateralization has not been tested directly.

In the present study, we manipulated corticosterone concentration in the egg yolk of the yellow-legged gull (*Larus michahellis*) by *in ovo* injection in order to test the effect of a physiological increase in egg corticosterone concentration on behavioral lateralization of newly hatched chicks. We focused on two behavioral tasks that have been previously used as proxies of lateralization: 'begging behavior', which is the solicitation of food provisioning by pecking at parental bill (Cramp, 1998); and Reversal-To-Prone response (RTP) after tonic immobility (Romano et al., 2015), which is a temporary supine catatonic-like stage of reduced responsiveness induced by physical restraint, including temporary suppression of the righting response (Jones et al., 1988). An effect of corticosterone treatment on individual lateral preference was expected since in some bird species corticosterone reduces the degree of visual asymmetry during the late stages of embryonic development. In the analyses, we accounted for the potential effects of sex and laying order because these factors have been previously shown to affect lateralization in the same species (Romano et al., 2015).

2. Methods

2.1. Study species

The yellow-legged gull is a large, mainly colonial, monogamous charadriiform bird with biparental care of the progeny (Cramp, 1998). Females lay 1–3 eggs (modal clutch size is 3 eggs) at 1–3 days intervals. Eggs from the same clutch hatch asynchronously (hatching spread: 1–4 days) after 27–31 days of incubation. The chicks are altricial and nidifugous, and can leave and wander around the nest soon after hatching (Cramp, 1998; our personal observations), typically to escape from predators or to get shading from sunlight. Hatchlings are fed by both parents that regurgitate food boluses under stimulation of the chicks pecking at the sub-terminal red patch on the lower mandible of parents' bill (see Cramp, 1998). Such 'begging' display is a highly stereotyped, innate behavior, which may consist of several pecking events in a relatively short time (up to tens of events per minute until food is eventually provided by the attending parent).

2.2. General field procedures and in ovo corticosterone manipulation

The study was performed in a large colony (> 400 pairs) in the Comacchio lagoon (44° 20′ N-12° 11′ E, NE Italy). The colony was visited every second day in order to monitor the progress of laying and mark the newly laid eggs. When a new egg was found, it was removed for experimental manipulation while being temporarily replaced with a 'dummy' egg to avoid interference with incubation behavior.

We adopted a within-clutch design, whereby both control and corticosterone-treated groups of eggs were established within each clutch to minimize the consequences of environmental and parental effects, because chicks from the same brood experience more similar micro-ecological conditions and interactions with adults compared to chicks from different broods (see also Parolini et al., 2015; Possenti et al., 2016 for a similar experimental design). In the same population, corticosterone concentration does not vary among eggs from different clutches (likelihood ratio test between linear mixed models including the effect of laying order and including or, respectively, excluding the random effect of clutch $\chi^2 = 0.810$, df = 1, $P = 0.370$; variance between clutches = 1.26; residual = 3.77 based on data from Rubolini et al., 2011). However, other egg components vary among clutches (e.g. testosterone; $\chi^2 = 4.67$, df = 1, $P = 0.03$; variance between clutches = 2.80; residual = 4.99). We sequentially assigned the following treatment schemes to the clutches, according to the order in which the first egg was found (nest, laying order): nest 1, egg 1, corticosterone injection (C); egg 2, control injection (O); egg 3, C; nest 2, O-C-O; nest 3, C-O-O; nest 4, O-C-C and so forth with the following nests. We aimed at increasing the concentration of yolk corticosterone by 1 standard deviation of the concentration measured in the yolk of eggs from the same colony (Rubolini et al., 2011), so that the final concentration of corticosterone was within the natural range of variation. We tuned the dose to be injected *in ovo* according to the egg size and the position in the laying sequence. Therefore, based on Rubolini et al. (2011), we grouped first, second or third laid eggs each into three classes (tertiles) of size according to egg mass and we calculated the standard deviation of corticosterone concentration in the yolk for each tertile, within each position in the laying sequence. Then, we estimated yolk mass based on total egg mass according to the following equation: yolk mass = 0.227 (0.039 SE) egg mass + 1.815 (3.461 SE) ($F_{1,88} = 34.38$, $P < 0.001$) (Parolini et al., 2015). The amount of corticosterone due to be injected was calculated as the product of the relevant standard deviation value and yolk mass as estimated suing the above equation. The amount of corticosterone injected in the three classes of egg mass for the three positions in the laying sequence was as follows (laying order: class of egg mass (g): amount of corticosterone injected (ng *per* egg): eggs 1: 84–91 g: 54 ng; 92–95 g: 63 ng; 96–108 g: 57 ng; eggs 2: 80–88 g: 39 ng; 89–92 g: 33 ng; 93–99 g: 75 ng; and eggs 3: 75–82 g: 41 ng; 83–87 g: 46 ng; 88–98 g: 38 ng. The corticosterone solutions were prepared in advance and stored in sterile vials, which contained the desired concentration of corticosterone solubilized in corn oil due to be injected in egg yolk depending on egg mass and laying order. Treated eggs were injected with 30 μL of the appropriate solution of corticosterone, while control eggs were injected with 30 μL of corn oil only. Corticosterone was injected in the yolk with the same procedure reported in Romano et al. (2008) and also used in other studies (Parolini et al., 2015; Possenti et al., 2016). Before being injected, the egg was

weighed (to the nearest g) and placed with the longitudinal axis vertical. After disinfecting the eggshell, a hole was drilled using a sterile pin close to the acute pole. *In ovo* injection was performed by means of 1 mL sterile syringe mounting a 0.6 × 30 mm needle while the egg was held firmly with its longitudinal axis vertical. Immediately after extracting the needle from the egg, the hole was sealed with a drop of epoxidic glue and a small piece of eggshell superimposed to the hole.

When the eggshell had been fractured by the chick at the start of the hatching event (i.e. the 'pipping' stage), the egg was injected with a drop of blue or green food dye through the small fracture to associate the hatchling to its original egg. The chick was then made individually recognizable by banding it with a colored elastic rubber ring on either tarsus. Then, we started behavioral tests (see below) after that a blood sample had been collected for molecular sexing (Saino et al., 2008).

2.3. Begging test

The begging test was performed as reported in Romano et al. (2015). The chick was placed in a wooden box apparatus consisting of 15 × 33 cm base and three 15 cm high walls. The chick was placed at the open end of the apparatus with the head pointing to the opposite side. Two identical, realistic, natural-sized yellow-legged gull heads with the typical sub-terminal red patch on the lower mandible were attached symmetrically on the lateral walls, pointing toward the chick, and with their sagittal plane vertical so that the heads could move parallel to the walls. The heads were moved simultaneously and symmetrically, using a single lever to which both heads were connected, by making them oscillate on their sagittal plane within 25° from the horizontal plane. The chick could reach the bill of both dummy heads by moderately stretching the neck from its test position. The chick was initially held firmly with its sagittal plane parallel to the plane of dummy heads by the experimenter while completely covering it with the hands. Care was taken that the base of the apparatus was horizontal, the experimenter was always placed behind the chick and thus was out of sight, and the sun (whether visible or not) was approximately behind the chick and the experimenter. The position of the stimulus heads relative to the chick was closely similar to the position that real parents' head has during interactions with the chicks (Cramp, 1998). Each begging trial lasted 1 min. During the trial, we recorded the side of the head that was pecked at. We carried out the begging test at the earliest possible age, as soon as the chick was found to have hatched and the down was dry.

The begging tests were carried out as described in Possenti et al. (2016). For each chick, we performed two or three begging trials. The first two trials were performed on the first day when the chick was found to have hatched, with ca. 1 h elapsing between consecutive trials, whereas the third trial was conducted on the next day if the chick failed to respond in any of the trials on the first day (see Results for information on the mean of number trials per chick). However, some chicks failed to perform any pecking response in all trials. In the analyses of lateralization, we only considered the chicks that responded at least in one trial.

2.4. Reversal-to-Prone response (RTP)

After completion of the begging test, we recorded RTP response after tonic immobility as reported in Romano et al. (2015). Indeed, when the chick was placed in supine position, it initially displayed an immobility response (tonic immobility) but then reverted to the prone position. The chick was initially placed supine in a test apparatus consisting of a shallow bowl (diameter: 15 cm, maximum depth 3 cm) glued to a 18 × 24 base with 14 cm high walls on three sides. The experimenter stood on the open side of the apparatus and held the chick firmly with both hands symmetrically placed on top of the chick and completely covering its body until the chick stopped struggling to escape. Care was taken that the base of the apparatus was horizontal, the sagittal plane of the chick was vertical, and the bill was aligned on the sagittal plane and pointed vertically. The experimenter was always placed behind the chick and thus out of its sight, and the sun (whether visible or not) was approximately behind both the chick and experimenter. From its position, the chick could only see the walls of the apparatus and the sky. For each trial, we recorded the side on which the chick rolled to reverse to the prone position (i.e. RTP response) and the duration (hereafter 'latency') in the tonic immobility test (i.e. seconds elapsing before rolling over).

For each chick, we recorded two or three RTP responses after tonic immobility trials that lasted a maximum of 3 min. The trials were performed on the first day when the chick was found to have hatched, with ca. 1 h elapsing between consecutive trials, whereas the third trial was conducted on the next day if the chick failed to revert to the prone position in any trials on the first day. For all chicks included in the analyses of lateralization, we recorded two RTP responses.

3. Statistical analyses

Individual proportion of rightward pecks was expressed as the mean of the within-trial proportion of rightward pecks, which was in turn expressed as the number of pecks that were rightwards relative to the total number of pecks performed in the trial.

Individual proportion of rightward pecks was analyzed in a generalized linear mixed model (GLMM) assuming a binomial error distribution. In the model, we included the random effect of brood identity and the fixed effects of egg treatment, sex, laying order (factors) as well as of their two-way interaction. Lateralization in either treatment group was also analyzed in binomial GLMM with brood identity as a random effect, and sex and laying order as fixed effects. Age was excluded from the models because it did not significantly predict lateralization ($P > 0.36$ for both treatment groups). These models served to test if the individual proportion of rightward pecks significantly deviated from − 0.5, i.e. the estimated intercept deviated from logit = 0.5. In all these models, the individual data were weighted for the square-rooted absolute number of peck responses provided by chicks, to account for larger accuracy of estimates of individual proportion of pecks on either side for chicks that performed more responses.

The individual absolute number of pecks on either side was treated as a count and was therefore analyzed in a GLMM model assuming a negative binomial error distribution while including brood identity as a random effect and egg treatment, sex, laying order (factors) as well as of their two-way interaction as fixed effects. To test for a differential effect of treatment on the number of pecks on either side, we included the interaction effect between treatment and side. To account for the dependency of the number of pecks on either side by the same chick we therefore also included in the model chick identity as a random effect. In these models, we also included the number of 1-min begging trials performed for each chick, because this can obviously affect the absolute number of pecking responses, as well as mean age at the tests, because age can affect motor skills.

Lateralization in RTP response in either treatment group was analyzed in binomial GLMM with brood identity as a random effect, and sex and laying order as fixed effects. Again, the effect of age was excluded from the models because it was non-significant ($P > 0.620$ for both treatment groups). The individual proportion of rightward RTP responses was analyzed in binomial GLMM with the same design as for individual proportion of rightward pecks (see above). However, no weight term was added because two RTP responses were available for all chicks. The effect of mean age at tonic test was excluded from the model because of its non-significant effect ($P = 0.913$).

Latency in the tonic immobility test was expressed as the mean of the within-trial seconds taken before the chick rolled over. This variable was $\log_{10}$-transformed to improve normality. Latency was analyzed in a linear mixed model (LMM) assuming a normal error distribution including brood identity as a random effect and egg treatment, sex, laying

order as fixed factors as well as of their two-way interaction.

We estimated the effect size (Cohen's d) for the effect of treatment based on Eq. (22) in Nakagawa and Cuthill (2007) for the LMMs whereas we adopted the specification from Snijders and Bosker (1999) for logit-link GLMMs with binomial errors. No effect size could be estimated for the effect of treatment on the number of pecks because, to the best of our knowledge, no method to estimate effect size has been devised for GLMM assuming a negative binomial error distribution and two random effects. Following Cohen (1992), the cut-off values of 0.8, 0.5 and 0.2 were used to identify “large”, “medium” or, respectively, “small” effect sizes.

Repeatability analyses were performed using a likelihood ratio test applying Laplace approximation comparing the (‘null’) model only including a random intercept effect with a model that also included the random effect of chick identity (Bolker et al., 2009).

All the statistical analyses were performed using PROC GLIMMIX in SAS 9.3.

4. Results

4.1. Begging response

Out of 145 chicks tested, 23 did not performed any pecking. We thus analyzed begging response in a sample of 122 chicks (55 from control eggs and 67 from corticosterone eggs; 47 from first-laid eggs; 46 from second-laid eggs; 29 from third-laid eggs) from 73 broods, with 48 nests providing more than one chick. The chicks were subjected to two or three begging trials (mean ± SD: 2.46 ± 0.50).

A likelihood ratio test comparing a binomial model including the random effect of chick identity with a null model showed that the proportion of begging responses on either side within each trial was not repeatable between trials at the individual level (χ^2 = 1.94, df = 1, P = 0.160) after controlling for the effect of treatment, sex, laying order and their two-way interactions. Thus, the chicks were not consistent in the side of the response, i.e. they were not lateralized at the individual level.

A binomial GLMM of individual proportion of rightward pecks with brood identity as a random effect showed that chicks from corticosterone-treated eggs performed a significantly smaller proportion of rightward pecks to the stimulus parental heads compared to controls (Table 1; Fig. 1a), while accounting for the non-significant effects of sex, laying order and the two-way interactions between main effects (Table 1). The effect size (Cohen's d) for treatment effect was 0.20. Separate binomial GLMMs on chicks of either treatment group, with sex and laying order as fixed effects showed that corticosterone chicks had significant leftward lateralization (H_0: mean individual proportion of rightward pecks = 0.5; mean observed proportion: 0.34; t = 2.40, df = 55, P = 0.0197), whereas control chicks did not show significant lateralization although they exhibited a larger proportion of leftward than rightward pecks (mean observed proportion: 0.45; t = 0.42, df = 48, P = 0.678).

In a GLMM analysis assuming a negative binomial error distribution, egg treatment was found to significantly reduce the number of pecks to the stimulus parental heads (Table 2; Fig. 1b) while controlling for the non-significant effects of sex, laying order and age at the begging trials (Table 2). In this model, the interaction between side and treatment was non-significant, implying that the negative effect of corticosterone on the absolute number of begging events was similar on either side (Table 2).

These results combined suggest that corticosterone treatment might cause leftward lateralization by reducing the absolute number of pecks on either side and also weakly effect the rightward response.

Table 1
Binomial generalized linear mixed model (GLMM) of individual proportion of rightward pecks in relation to the main and two-way interaction effects of egg corticosterone treatment, sex and laying order. Brood identity was included as a random effect. N = 122 chicks.

	F	df	P
Treatment	4.72	1,40	0.036
Sex	0.51	1,40	0.479
Laying order	1.17	2,40	0.321
Treatment × sex	0.89	1,40	0.352
Treatment × laying order	0.83	2,40	0.444
Sex × laying order	0.77	2,40	0.468

4.2. Reversal-To-Prone response

The RTP response was analyzed in a sample of 139 chicks (63 from control eggs and 76 from corticosterone eggs; 51 from first-laid eggs; 53 from second-laid eggs; 35 from third-laid eggs) from 80 broods. Two RTP responses were available for each chick. A likelihood ratio test comparing a binomial model including the random effect of chick identity with a null model showed that the side of the RTP response was not repeatable at the individual level (χ^2 = 0.06, df = 1, P = 0.800) after controlling for the effect of treatment, sex, laying order and their two-way interactions. Separate binomial GLMMs on chicks of either treatment group, with sex and laying order as fixed effects showed that no significant lateralization occurred in both treatment groups (details not shown).

In a binomial GLMM with brood as a random effect, egg treatment did not affect the individual proportion of rightward RTP responses either per se or in combination with the effects of sex or laying order (Table S1; Fig. 2a). The effect size for treatment effect was 0.00.

Latency in RTP response was also not repeatable (χ^2 = 0.00, df = 1, P > 0.990) after controlling for treatment, sex, laying order and the two-way interactions. The effect of egg treatment on the (log_{10}-transformed) latency in the tonic immobility test was marginally non-significant (Table 3) in a LMM model controlling for the non-significant effects of sex, laying order and the two-way interactions between main effects (Table 3; Fig. 2b). The effect size for treatment effect was 0.16.

5. Discussion

We tested the effect of a physiological increase in corticosterone concentration in yellow-legged gull eggs on lateralization of newly hatched chicks at two behavioral tasks. Our results suggest that chicks from corticosterone-treated eggs might have a leftward preference when begging for food behavior whereas no lateral preference was observed among control chicks. However, the direction of lateralization of the RTP response after tonic immobility test was not affected by *in ovo* corticosterone treatment. There was no differential effect of corticosterone on lateralization depending on sex or laying order. The lack of the effect of laying order was expected because the hormone concentration into gull eggs did not vary with laying order (Rubolini et al., 2011) and the dose of corticosterone was adjusted according to the variance in concentration recorded for eggs of different laying order. Unexpectedly, however, we did not find significant lateralization in begging response according to laying order nor in RTP response according to sex, while controlling for treatment effects.

The majority of the studies on the ontogeny of lateralization in birds has focused on two model species, the precocial domestic chicken (*Gallus gallus*) and the altricial pigeon (*Columbia livia*), that have different brain structural asymmetry in the visual pathway (Halpern et al., 2005; Manns and Ströckens, 2014; Rogers, 2008). In both species, individual-level behavioral lateralization has been suggested to be affected by the asymmetrical light stimulation of the embryo due to postural asymmetry during pre-hatching developmental period (Rogers, 1982). As a consequence of asymmetrical stimulation by light, in chicken the thalamofugal projections from the left brain hemisphere develop to a greater extent than their counterparts from the right side

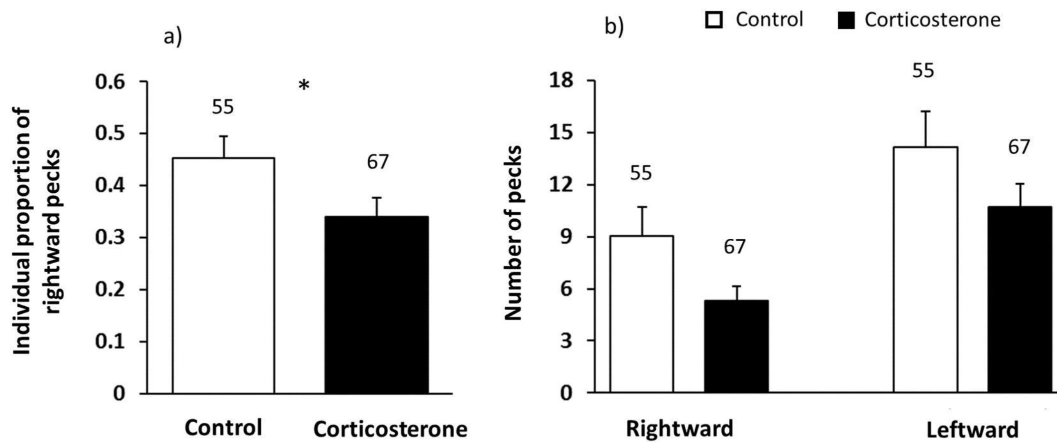

Fig. 1. Estimated marginal means (+SE) of: a) the individual proportion of rightward pecks, and b) the number of rightward or leftward pecks to the dummy stimulus heads by chicks from corticosterone or control eggs. Significant differences between chicks from corticosterone-treated and control eggs are indicated by asterisks (*: $P < 0.05$). The number of chicks for each group is shown.

Table 2
Negative binomial generalized linear mixed model (GLMM) of the individual absolute number of pecks on either side in relation to the main effects of egg corticosterone treatment, sex, laying order as well as their two-way interaction and age at the begging trials. We also included the interaction effect between treatment and side. Chick and brood identity were included as a random effect.

	F	df	*P*
Treatment	5.99	1,120	0.016
Sex	0.01	1,120	0.919
Laying order	0.82	2,120	0.444
Side	23.88	1,120	< 0.001
Treatment × sex	0.07	1,120	0.799
Treatment × laying order	0.13	2,120	0.875
Sex × laying order	0.47	2,120	0.624
Side × treatment	0.79	1,120	0.377
Age at begging test	7.59	1,120	0.007
Number of begging trials	58.83	1,120	< 0.001

Table 3
Linear mixed model (LMM) of latency (seconds taken before the chick rolled over, expressed as $\log_{10}$) in the tonic immobility test in relation to the main and two-way interaction effects of egg corticosterone treatment, sex and laying order. Brood identity was included as a random effect. N = 139 chicks.

	F	df	*P*
Treatment	3.22	1,50	0.079
Sex	1.66	1,50	0.204
Laying order	0.79	2,50	0.460
Treatment × sex	2.27	1,50	0.138
Treatment × laying order	1.50	2,50	0.232
Sex × laying order	0.42	2,50	0.662

(Rogers and Bolden, 1991), leading to a functional brain lateralization. This structural asymmetry of thalamofugal visual pathways is partly related to visual behavior. However, in pigeons light-stimulated asymmetry affects the projections of the tectofugal pathway leading to stronger projections from the right optic tectum to the left brain hemisphere (via contralateral nucleus rotundus) than from the left optic tectum to the right one (Güntürkün et al., 1998). Some studies have suggested that the asymmetrical light exposure puts the exposed right eye and its connected (left) hemisphere in charge of visual behavior (Andrew et al., 2004; Deng and Rogers, 1997; Rogers, 1996; Rogers and Deng, 2005). Furthermore, there is a large body of evidence showing that the left hemisphere develops a functional dominance and is specialized in foraging behavior with the particular ability to learn to peck at food and avoid pecking at distracting targets (Manns and Güntürkün, 1999; Rogers, 2006, 2008, 2012).

Early exposure to corticosterone and also other steroid hormones (e.g. testosterone and estrogens) plays a role in shaping post-natal phenotype, as these hormones can interfere with the effect of light on the development of visual system lateralization, affecting the structural asymmetry in the thalamofugal projections in chicken (Rogers, 2008; Rogers and Deng, 2005; Rogers and Rajendra, 1993; Schwarz and Rogers, 1992). Experimental studies, where corticosterone levels were manipulated, typically have focused on the effect of treatment on the development of the structural brain asymmetry that improves the ability to perform more than one task simultaneously (Freire et al., 2006; Rogers and Deng, 2005). Moreover, to the best of our knowledge,

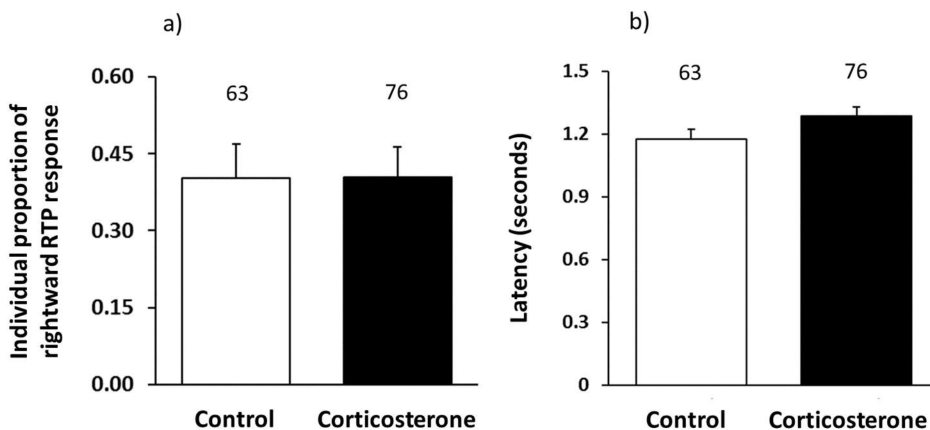

Fig. 2. Estimated marginal means (+SE) of: a) the individual proportion of rightward Reversal-To-Prone (RTP) response and b) latency in the tonic immobility test, which is expressed as $\log_{10}$ of seconds elapsing before rolling over, of chicks from corticosterone or control eggs. The number of chicks for each groups is shown.

no study to date has investigated the effect of early exposure of corticosterone on visual asymmetry in pigeon hatchlings. Therefore, the present is the first study that assesses the effect of corticosterone administration on the direction of lateralization of two behavioral tasks in a non-model species, whose resemblance to either the chicken-type or the pigeon-like visual anatomy is unknown.

A physiological increase in yolk corticosterone concentration seems to affect the direction of food-soliciting behavior (i.e. 'begging') of gull hatchlings. In the yellow-legged gull, begging is an innate visual behavior exhibited by the newly hatched chicks when they peck at the red patch on the parents' bill (Goethe, 1937). In the same species, begging intensity has been previously found to be negatively affected by an experimental increase in corticosterone levels, although lateralization was not investigated (Rubolini et al., 2005). Our present results showed that corticosterone treatment reduces the intensity of begging behavior with a subtle effect on righting responses, leading to leftward lateralization. We may speculate that corticosterone exposure during the pre-hatching period interferes with the effect of light on the development of the structural asymmetry of the visual system, by reducing neuron tectofugal or thalamofugal projections from the left hemisphere (Manns and Güntürkün, 1999; Manns and Ströckens, 2014; Rogers and Deng, 2005). The reduction of projections from the left side may thus lead to an impairment in the ability to perform pecks increasing the (non-significant) tendency for leftward lateralization. Two putative mechanisms may be involved in the reduction of brain asymmetry structure. First, if cerebral asymmetry results from a smaller attrition of neurons from the left hemisphere by light stimulation, corticosterone may impair light-stimulated protection causing a loss of neurons from both hemispheres (experiment in chicken from Rogers and Deng, 2005). Second, we can speculate that an increase in corticosterone levels, simulating an increased release of corticosterone caused by a stress-inducing stimulus and mediated by the HPA axis, affects functional brain organization, as suggested by Korte et al. (2005). Marked differences in brain structure and hormonal pathways among taxa (see review Ocklenburg et al., 2016), however, prevent from further speculating on the effects that corticosterone treatment produces on brain structure in our study species.

Despite the functional dominance of the left hemisphere in visual pathways in both model species, there is evidence that stronger bilateral input is guided to the left hemisphere in pigeons but to the right one in chickens (Diekamp et al., 2005; Güntürkün et al., 1998; Rogers and Deng, 1999). However, it has been suggested that the degree of bilateral input affects the dominant hemisphere and encoding strategy. Indeed, an example of a dissociation between hemispheric specializations and strategy is represented by right hemispheric superiority for spatial orientation tasks: chickens and pigeons exhibited more leftward pecks at grain spread over an area in front of them (Chiandetti, 2011; Diekamp et al., 2005). However, we are not in the position of identifying which brain hemisphere is dominant in the yellow-legged gull.

The functional implications, if any, of the observed effect of corticosterone treatment on lateralization in begging behavior remain to be elucidated. Because lateralization induced by corticosterone treatment was accompanied by a reduction in the absolute frequency of begging events, exposure to high corticosterone levels *in ovo* may impair efficient stimulation of parents to provision food to the chick particularly when a chick faces the attending parent on its own right side, thus reducing access to food in approximately half of the food provisioning events.

Pre-natal exposure to corticosterone did not affect lateralization of the RTP response after tonic immobility. Because sample size was reasonably large and the effect of treatment was far from statistical significance (see Results), we are confident that the lack of statistically significant effect of corticosterone administration was not attributable to low power of statistical tests. Tonic immobility consists in a reduction of responsiveness induced by physical restraint and its duration is considered to be positively related to fearfulness (Jones et al., 1988). Our findings disclosed that chicks hatched from treated eggs showed a marginally non-significant ($P = 0.079$; see Results) increase in the latency in the tonic immobility compared to controls. The present result is consistent with previous reports where an increase in plasma corticosterone levels prolonged tonic immobility duration in adult hens (Ahmed et al., 2014; Cockrem, 2007; Henriksen et al., 2013; Jones et al., 1988). Experimentally elevated corticosterone concentration may thus reliably mimic the physiological consequences of exposure to a natural stressful situation, such as exposure to the risk of predation, leading to reduced physical responsiveness.

Lack of differences in lateralization of begging response according to laying order and of RTP response according to sex is at odds with previous findings by Romano et al. (2015). The causes of such inconsistency are unclear. We might speculate that the expression of variation in lateralization according to sex and laying order depends on general ecological conditions whereby stress as mediated by availability of food and thus hunger level affect the expression of lateralization at different tasks. However, we have no clue at which ecological conditions varied between either study year (2014 vs 2016). Future studies should therefore also aim at assessing the generality of variation in lateralization according to sex and position along the laying sequence in different years and ecological contexts.

In conclusion, we found a negative effect of a physiological increase in pre-natal exposure to corticosterone on the intensity of begging behavior in yellow-legged gull hatchlings. This effect might affect the direction of lateralization in favor of the leftward side via a slight reduction of the number of begging pecks on right side. Therefore our findings highlight the importance of maternal effects mediated by the transfer of corticosterone to the eggs that may affect a behavioral trait involved in parent-offspring communication. However, the present study is not conclusive because the effects of corticosterone on lateralization, prompting for more experiments to shed light on the mechanisms underlying the development of lateralization in birds.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2017.10.014.

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Table S1. Binomial generalized linear mixed model (GLMM) of individual proportion of rightward Reversal-To-Prone (RTP) response in relation to the main and two-way interaction effects of egg corticosterone treatment, sex and laying order. Brood identity was included as a random effect. N = 139 chicks.

	F	d.f.	*P*
Treatment	0.00	1,50	0.979
Sex	0.02	1,50	0.876
Laying Order	0.13	2,50	0.880
Treatment × Sex	0.53	1,50	0.471
Treatment × Laying Order	0.36	2,50	0.702
Sex × Laying Order	0.14	2,50	0.874

Chapter 8

Predation risk affects egg mass but not egg steroid hormone concentrations in yellow-legged gulls

The Science of Nature (submitted)

Predation risk affects egg mass but not egg steroid hormone concentrations in yellow-legged gulls

Cristina Daniela Possenti[1], Alexandra Bea Bentz[2], Andrea Romano*[1], Marco Parolini*[1], Manuela Caprioli[1], Diego Rubolini[1], Kristen Navara[2] and Nicola Saino[1]

[1]. *Department of Environmental Science and Policy, University of Milan, via Celoria 26, 20133 Milano, Italy*

[2]. *Department of Agricultural and Environmental Sciences, University of Georgia, 203 Poultry Science Building, Athens (GA), USA*

Correspondence should be addressed to:

*Andrea Romano, andrea.romano@unimi.it;

*Marco Parolini, marco.parolini@unimi.it

Short title: Non-consumptive predator effects on gull eggs

20 **Abstract**

21 Predators have both direct, consumptive effects on their prey and non-consumptive effects on
22 physiology and behaviour, including reproductive decisions, with major cascading effects on
23 prey ecology and evolution.

24 Here, we experimentally tested such non-consumptive effects of exposure to increased
25 predation risk on the concentration of maternal steroid hormones, clutch size and egg mass in
26 the yellow-legged gull (*Larus michahellis*). We simulated increased predation risk by
27 displaying stuffed predators (adult fox, *Vulpes vulpes*, and adult buzzard, *Buteo buteo*) to
28 breeding adults before egg laying.

29 The concentration of corticosterone, which has been shown to increase under exposure to
30 maternal predation risk in other species, and of testosterone did not differ between eggs from
31 mothers exposed to the predators and control eggs. The concentration of the two hormones
32 negatively covaried. Clutch size did not vary according to experimental treatment, whereas
33 egg mass was markedly larger in clutches from nests exposed to predators than in clutches
34 from control nests.

35 By increasing egg mass, mothers may reduce the risk of cooling of the eggs when incubation
36 is impeded by predators, boost energy reserves, reduce post-natal detectability caused by food
37 solicitation, and/or enhance development at hatching, thus increasing the chances of offspring
38 survival.

39 In general, the present results are inconsistent with most of the few previous studies on similar
40 non-consumptive predator effects and suggest that such effects may vary idiosyncratically
41 among species according to ecological conditions and developmental mode.

42 **Key words**: Clutch size; Corticosterone; Egg size; Predation effects; Testosterone

43

44 **Significance**

45 In addition to direct consumption, predators can have indirect, non-consumptive effects on
46 physiology and behavior of their preys. Here, we experimentally investigated effects of
47 increased predation risk on the concentration of maternal steroid hormones, clutch size and
48 egg mass in the yellow-legged gull. The exposure of stuffed predators before egg laying did
49 not affect the levels of corticosterone and testosterone in the eggs, as well as the clutch size.
50 However, egg mass was larger in clutches from nests exposed to predators than from control
51 ones. An increase in egg mass can be adaptive to limit the risk of egg cooling when
52 incubation is prevented by predators, reduce chick detectability due to food solicitations,
53 and/or limit offspring starvation. Despite these findings are inconsistent with previous studies,
54 they suggest that non-consumptive effects vary among species according to ecological
55 conditions and developmental mode.

56

Introduction

Predation is a major force molding the evolution of morphological, physiological and behavioural traits of prey (Agrawal et al. 1999; Eggers et al. 2006; Fontaine and Martin 2006; Massaro et al. 2008; Peluc et al. 2008; Storm et al. 2010; Coslovsky et al. 2011; Giesing et al. 2011). Predators, however, also greatly impact the populations of their prey over ecological time frames (Pianka et al. 1970; Ricklefs 2000; Kokko and Lopez-Sepulcre 2007; Griebeler et al. 2010). The most obvious ecological effect of predators is killing of prey, which has direct consequences for prey population dynamics by affecting births and deaths. Recent research, however, has emphasized that the ecological effect of predators may extend far beyond those arising from the mere killing of prey (Creel et al. 2007; Creel and Christianson 2008; Travers et al. 2010). Indeed, predation risk can broadly impact on the physiology and behaviour of prey and this can also have indirect, cascading effects on prey demography. Such 'non-consumptive' predator effects can vary according to whether predation risk mostly concerns adults rather than offspring/eggs, and this is expected to be reflected into the flexible physiological and behavioural response specifically adopted by prey (Lima 2009; Travers et al. 2010).

Non-consumptive effects of predation on breeding birds have been shown to extend from macro- and micro-habitat choice including nesting habitat (Greig-Smith 1982; Dow and Fredga 1983; Hakkarainen et al. 2001; reviewed by Lima 2009), social (e.g. flocking and coloniality; Gotmark and Andersson 1984; Robinson 1985; Beauchamp 2003; Caro 2005; reviewed by Lima 2009), parental (e.g. nest and brood attendance; see review by Lima 2009) and filial (begging) behaviour (Redondo and De Reyna 1988; Briskie et al. 1999; reviewed by Lima 2009). In addition, variation in predation risk affects the expression of phenotypically plastic life-history traits like clutch and egg size (Doligez and Clobert 2003; Eggers et al.

81 2006; Fontaine and Martin 2006). When predators mostly impact on eggs/offspring, life-
82 history theory leads to expect that the experience of high predation risk results in a reduction
83 in clutch size during the current reproductive event. This is expected because of lowered
84 reproductive value of current offspring under intense predation risk and a trade-off between
85 current and future reproduction (Slagsvold 1984; Magnhagen 1991; Nager et al. 2000; Hauber
86 2003; Griebeler et al. 2010). In addition, reduction in clutch size under high predation risk can
87 function to decrease detectability of parents attending large broods, and of large broods
88 themselves, or can result from reduced foraging efficiency of laying mothers (Martin at al.
89 2000a,b; Ghalambor and Martin 2002; Fontaine and Martin 2006; Lima 2009). The
90 relationship between predation risk and clutch size has thus been most often shown to be
91 negative, as expected, although with notable exceptions (Doligez and Clobert 2003; Eggers et
92 al. 2006; Fontaine and Martin 2006; Massaro et al. 2008; Cassey et al. 2009).

93 The consequences of high predation risk on egg size have been tested experimentally only
94 rarely and on a taxonomically limited spectrum of altricial species, and have been found to
95 range from negative to null depending on the focal species (Safriel 1975; Slagsvold 1984;
96 Martin 1995; Cassey et al. 2009; Lima 2009; Coslovsky et al. 2011). Negative effects of
97 predation risk on egg size may again stem from the perception of a reduction in the
98 reproductive value of the current progeny but also from reduced parental foraging activity to
99 reduce detectability by predators (Lima 1987; Thomson et al. 1998; Martin at al. 2000a,b;
100 Ghalambor and Martin 2002; Zanette et al. 2006). However, depending on the biology of the
101 species, a positive effect of predation risk on egg size may be expected (e.g. Fontaine and
102 Martin 2006). For example, large eggs typically produce large offspring (e.g. Amundsen
103 1995; Smith and Bruun 1998; Styrsky et al. 1999; reviewed in Krist 2011) with faster
104 development that can fledge earlier (Krist 2011), thus reducing vulnerability to predation,
105 which is high particularly during the pre-fledging period. Moreover, large offspring resulting

106 from large eggs can better resist peri-natal starvation periods in cases when parental
107 attendance is temporarily impeded by the presence of predators than offspring originating
108 from small eggs (e.g. Magrath 1991, 1992; Rhymer 1988: reviewed in Krist 2011). Finally, it
109 can be speculated that large eggs may reduce the negative effects of egg cooling on embryo
110 viability when incubation is impeded by the proximity of the predator to the nest (Gillooly et
111 al. 2002).

112 In oviparous organisms, mothers transfer to the eggs major constituents (e.g. proteins and
113 lipids) but also quantitatively minor components like antioxidants, immune factors and
114 hormones that can profoundly impact on the development of the offspring, thereby having
115 both short- and long-term effects on morphological, physiological as well as behavioural traits
116 (Mousseau and Fox 1998; Bonduriansky and Day 2009). Non-consumptive effects of
117 predation can also be subtly expressed in terms of the biochemical composition of the eggs,
118 although this hypothesis has been tested experimentally only in few studies (Cockrem and
119 Silverin 2002; Saino et al. 2005; Coslovsky et al. 2011; Pitk et al. 2012). Predation risk can
120 impact on the concentration of egg maternal steroid hormones. Corticosterone is the main
121 hormonal mediator of the acute stress response via the hypothalamo-pituitary-adrenal axis in
122 birds (Wingfield and Romero 2001; Henriksen et al. 2011; Costantini 2014). Female birds
123 exposed to increased risk of predation before laying have been found to increase the amount
124 of corticosterone that they transfer to their eggs, both in the albumen and in the yolk, and a
125 similar effect also occurs in response to other forms of stress (e.g. Romero and Remage-
126 Healey 2000; Romero et al. 2000; Saino et al. 2005). Modulation of maternal corticosterone
127 in the egg can be generally interpreted in an evolutionary perspective as an adaptive tool at
128 the disposal of the mother to better equip the offspring to their post-natal environment (see
129 Groothuis et al. 2005; Engelhardt and Groothuis 2011). In a predator-prey interaction
130 scenario, increased egg corticosterone concentration can be adaptive because it enhances anti-

131 predatory behavior both in adult and immature individuals. Alternatively, environmental
132 effects on egg composition may be simply seen as the inevitable consequence of
133 environmental factors on maternal physiology (Mousseau and Fox 1998). Under this
134 perspective, increased corticosterone concentration in the eggs after maternal exposure to high
135 predation risk may simply mirror the consequences of maternal stress response in terms of
136 increased secretion of corticosterone.

137 Predation has also been shown to affect testosterone concentration in the eggs (Coslovsky et
138 al. 2011). Egg maternal testosterone is believed to have major activational and organizational
139 effects on the developing embryo as well as post-natally (Strasser and Schwabl 2004;
140 Groothuis et al. 2005; Bonisoli-Alquati et al. 2007; Gil 2008; see Williams and Groothuis,
141 2015). Maternal modulation of testosterone under high predation risk can be adaptive, for
142 example, if it boosts growth of traits (e.g. wing growth) that advance timing of fledging
143 (Schwalb 1996; Navara et al. 2006; Ketternson et al. 2001). Again, an alternative
144 interpretation is simply that egg composition reflects the non-adaptive consequences of the
145 effects of predation risk on maternal physiology.

146 In addition, corticosterone and testosterone concentrations in the eggs have been found to
147 negatively covary (Duckworth et al. 2001; Henriksen et al. 2011; but see Rubolini et al.
148 2011), suggesting that transfer of the two hormones to the egg depends on reciprocally
149 constraining effects or that mothers adaptively down-regulate the concentration of either
150 hormone in response to an increase in the concentration of the other.

151 Overall, however, the field experimental studies of the effects of predation risk on egg
152 biochemical composition and on egg size are few, mostly limited to altricial species, and have
153 provided inconsistent results. In this study we tested the effect of simulated exposure to two
154 common predators in the study area (adult fox, *Vulpes vulpes*, and adult buzzard, *Buteo buteo*)

155 during the period between the rapid yolk development (RYD) phase and laying on yolk
156 concentration of corticosterone and testosterone, on clutch size and on egg mass of the
157 yellow-legged gull (*Larus michahellis*). We expected that corticosterone concentration
158 increased as a consequence of exposure to predators because of increased passive transfer of
159 the hormone from the mother to the egg and/or because of active modulation of maternal
160 corticosterone in the egg, if corticosterone has adaptive effects peri- and post-natal anti-
161 predatory behaviour. We had no directional expectation on the direct effect of exposure to the
162 predators on testosterone concentration in the yolk because of the lack of theoretical and
163 empirical background on the effect of perceived risk of predation on maternal transfer of
164 androgens to the yolk.

165 According to life-history theory and previous experimental evidence on different species, we
166 expected predation risk to reduce clutch size (Slagsvold 1984; Magnhagen 1991; Nager et al.
167 2000; Hauber 2003; Griebeler et al. 2010). The predictions on the effect of exposure to
168 predators on egg mass were ancipital. According to one adaptive scenario of egg mass
169 modulation, an increase in egg mass under increased perceived risk of predation of the chicks
170 could be expected. This effect could be expected because large eggs can hasten post-natal
171 development and thus time of fledging, can buffer reduced parental food provisioning if large
172 predation risk impairs parental behaviour, or can enhance post-natal anti-predator behaviour.
173 In addition, larger egg reserves can result in reduced solicitation (i.e. begging) activity by the
174 hatchlings to the attending parents, which involves both acoustic and visual displays, thereby
175 reducing the risk of predation (Redondo and De Reyna 1988; Briskie et al. 1999). Finally,
176 large egg mass can reduce egg cooling when incubation is impeded by the presence of
177 predators close to the nest. On the other hand, an alternative adaptive scenario can lead to
178 expect a reduction in egg mass in clutches from nests exposed to the predators if large

179 predation risk causes a decline in reproductive value of the offspring and/or because exposure

180 to predators reduces egg laying performance via a negative effect on maternal physiology.

181

182 **Materials and Methods**

183 *Study organism*

184 The yellow-legged gull is a large charadriiform widely distributed across the Mediterranean
185 basin (see Cramp 1998 for information on the natural history of the species). Monogamous
186 pairs lay one clutch of 1-3 eggs per breeding season. Eggs are laid at 2-4 days intervals. A
187 replacement clutch may be laid if the first clutch is lost precociously. Egg size declines with
188 laying order, particularly from the second to the third egg. The duration of the rapid yolk
189 development (RYD) phase, when maternal substances including hormones are deposited in
190 the yolk, is unknown in this species. However, in a similar-sized species, the Audouinii's gull
191 (*Larus audouinii*), RYD occurs in approximately 7 days before laying (range: 6-8 days; Ruiz
192 et al. 2000). Because the timing of the RYD phase correlates with egg size and thus with body
193 size, in this study we assume that the RYD phase of the yellow-legged gull also starts
194 approximately 1 week before laying of the individual egg. The yolk of the eggs contains
195 corticosterone and testosterone of maternal origin in amounts that vary among mothers, as
196 observed in the same colony where the present study was conducted (Rubolini et al. 2011).
197 Incubation of the eggs gradually starts already upon laying of the first egg, causing
198 asynchronous hatching (hatching spread: 1-4 days). Before laying, adults in our study colony
199 are typically found in their nesting territories inside the breeding colonies during the middle
200 of the day (personal observation) because foraging activity typically occurs early or late
201 during daytime and presumably also during the night.

202 Adult yellow-legged gulls have no predators in our study area. However, chicks may be
203 preyed upon by rats (*Rattus norvegicus*), foxes (*Vulpes vulpes*), herons (*Ardea* sp.) and
204 falconiformes, as well as by feral dogs. Predators are actively mobbed by colonial gulls.

205

206 *Experimental procedures*

207 In spring 2016, we identified 5 separate sub-colonies in the northern part of the Comacchio
208 Lagoon (44° 20' N-12° 11' E; norther Italy). Three sub-colonies were assigned to a control
209 treatment whereby a piece of brown cloth was placed in the center of the sub-colony, hanged
210 on a support at ca. 1 m from the ground. The piece of cloth did not cause any apparent
211 reaction by the nesting gulls, as expected also because artificial objects are common on the
212 islets where the gull colonies in the Comacchio lagoon are settled. Two sub-colonies were
213 assigned to the predator treatment. In the center of each sub-colony, we placed either a stuffed
214 fox clearly visible from a distance on the ground or a stuffed buzzard hanged on a support ca.
215 1 m from the ground. Predators were presented every second day on average for two hours
216 late in the morning (range 9.00 am - 1.00 pm), alternating them between the two sub-colonies.
217 Presentation of the predators lasted longer than any attempted predation episode ever
218 observed during hundreds of hours of observation on the colonies in the same area since year
219 2005.

220 All the adults from the entire sub-colonies where the predators were placed actively reacted to
221 the predator stimuli by mobbing them and then typically by sitting in the water at a distance
222 while performing episodic mobbing flights to the predator. This was the case also for the fox
223 despite it is a typically crepuscular/nocturnal predator.

224 On days 11 to 15 after the start of the predator or control treatment, we collected all the first
225 eggs that had just been laid in the nests within a radius of 30 m from the site where the
226 predator or the control stimuli were located. The eggs were weighted to the nearest gram and
227 then stored at -20 °C for subsequent hormonal analyses. To minimize observer bias, blinded
228 methods were use when all behavioral data were recorded and/or analyzed.

229

230 *Yolk hormone analyses*

231 Yolk steroids were extracted from homogenized yolk samples with a double ether extraction
232 followed by liquid column chromatography according to methods described by Schwabl
233 (1993). Briefly, 50 mg of yolk was weighed and vortexed with 1000 µL of deionized water.
234 Next, 3 mL of petroleum:diethyl ether (30:70 vol/vol) was added, the mixture was vortexed
235 for 30 sec and was allowed to settle for 20 min. Samples were then snap frozen and the
236 supernatant was poured off and dried. The sample was reconstituted in 1 mL 10% ethyl
237 acetate in isooctane and steroids were separated using celite column chromatography.
238 Testosterone was eluted in 20% ethyl acetate in isooctane and corticosterone was eluted in
239 50% ethyl acetate in isooctane. Testosterone and corticosterone were quantified with
240 commercial enzyme immune-essay (EIA) kits (ENZO, NY, USA). Anti-testosterone had a
241 cross-reactivity of 100% with testosterone, 14.6% with 19-hydroxytestosterone, 7.20% with
242 androstendione, and <1% with all other steroids. Anti-corticosterone had a cross-reactivity of
243 100% with corticosterone, 21.3% with deoxycorticosterone, 21.0% with
244 desoxycorticosterone, and <1% with all other steroids. Average recoveries were 95.0% for
245 testosterone and 93.7% for corticosterone. Average intra-assay variation was 4.9% for
246 testosterone and 2.2% for corticosterone. Testosterone was analyzed over 4 assays and inter-
247 assay variation was 1.9%. Corticosterone was analyzed over 2 assays and inter-assay variation
248 was 4.4%. A single datum for corticosterone concentrations was excluded as it was a positive
249 outlier.

250

251 *Statistical analyses*

252 We mainly relied on generalized linear mixed models in which the effect of sub-colony and of
253 nest identity (models on egg mass only) were included as random effects, experimental

254 treatment (exposure to predator or control) and laying order (models on egg mass only) were
255 included as fixed effects, and laying date as a continuous covariate. In the analyses of
256 corticosterone and testosterone concentrations and of egg mass, we adopted a normal error
257 distribution while in the analysis of clutch size we adopted a Poisson error distribution. Two-
258 way interaction terms between treatment, laying order, laying date and egg mass (where
259 relevant) were always initially included in the models and then all excluded in a single step
260 because they did not significantly contribute to the models.

261 The test of the random effect of sub-colony in the models of hormone concentrations was
262 performed by likelihood ratio tests based on the normal LMM described above. The test of the
263 effect of sub-colony (random effect) on clutch size was performed by applying a Poisson
264 GLMM while adopting Laplace approximation of likelihood estimates. In the models of egg
265 mass, the effect of the random terms of sub-colony and nest identity was tested by likelihood
266 ratio test on the models including or, respectively, excluding the focal random effect.

267 To estimate effect sizes for the effect of treatment on the different variables we had to adopt a
268 mixed strategy. For clutch size, effect size (Cohen's d) was estimated based on equation 22 in
269 Nakagawa and Cuthill (2007) based on parameters provided by a normal LMM rather than a
270 Poisson GLMM because, to the best of our knowledge, no method to estimate effect sizes
271 from Poisson GLMM has been devised. The effect sizes for the effect of treatment on
272 hormone concentrations were estimate simply by equation 22 in Nakagawa and Cuthill
273 (2007). Finally, for egg mass, the effect size was estimated based on a normal LMM where
274 we included only the random effect of nest identity because sub-colony identity had no
275 significant effect and, to the best of our knowledge, no method to estimate effect sizes from
276 normal LMM with two random effects has been devised. The effect sizes were computed as
277 Cohen's d (Nakagawa and Cuthill 2007).

278 All statistical parameters are reported together with their associated standard error.

279 All analyses were performed using SAS 9.3 or SPSS 13.0 statistical packages.

280

281 **Results**

282 The sample for hormone analyses consisted of first-laid eggs from 25 clutches that were
283 exposed to predators and 24 control clutches.

284 Corticosterone concentration was not significantly affected by simulated exposure to
285 predators (Table 1; Fig. 1), with eggs from sub-colonies exposed to predators on average
286 showing smaller concentrations than control sub-colonies, contrary to the expectation. The
287 effect size (Cohen d) was -0.090. The significant effect of the random effect of sub-colony
288 (Table 1) indicates that some unidentified factor caused the eggs from different sub-colonies
289 to differ in corticosterone concentrations. Corticosterone concentration declined with laying
290 date and did not covary with egg mass (Table 1). When included in the model, the distance
291 between the individual nests and the place where the predator was presented did not predict
292 corticosterone levels (details not shown). The experimental treatment did not affect
293 testosterone concentration and there was no significant variation in testosterone concentration
294 among colonies (Table 1; Fig. 1). The effect size was 0.030. In addition, testosterone
295 concentration did not significantly vary with laying date and did not covary with egg mass
296 (Table 1). In a linear mixed model with sub-colony as a random effect, the interaction term
297 between treatment and corticosterone concentration did not predict the concentration of
298 testosterone in the yolk ($F_{1,44} = 0.21$, $P = 0.652$). A simplified model excluding the interaction
299 term showed that the concentration of testosterone significantly negatively covaried with that
300 of corticosterone ($F_{1,45} = 5.32$, $P = 0.026$; Fig. 2). Hence, there was a negative relationship
301 between the concentration of the two steroid hormones but the slope of the relationship did
302 not depend on experimental treatment.

303 Clutch size was not predicted by experimental treatment (Table 1) with very similar mean
304 clutch sizes in nests exposed to predators (2.92 (0.05), n = 36) or in control nests (2.85 (0.06),
305 n = 33). The effect size was 0.1178.

306 The sample for the analyses of egg mass consisted of 36 entire clutches that were exposed to
307 predators and 33 control clutches. Egg mass at laying was significantly larger in broods that
308 were exposed to predators (Table 1; Fig. 3). The effect size was 0.2486. The difference in
309 average mass (4.8 g) between the two groups of eggs estimated as least-squares means from
310 the model in Table 1 corresponded to 0.89 standard deviations of the mass of the eggs from
311 the control group computed from residuals of a linear mixed model controlling for nest
312 identity (random effect) and laying order and laying date (fixed effects). The difference in egg
313 mass between the control and the experimental groups corresponded to ca. 0.7 standard
314 deviations of egg mass in our population.

315 In addition, egg mass significantly declined with position in the laying sequence (Table 1;
316 Fig. 3). These effects were reciprocally independent, as shown by the non-significant effects
317 of the two-way interaction terms that were removed from the models. Thus, independently of
318 laying order and laying date, eggs in nests that were exposed to predators were larger than
319 control eggs. In addition, egg mass significantly varied among nests (Table 1).

320

321 **Discussion**

322 We experimentally tested the effects of simulated exposure to predators during the egg
323 formation period on the concentration of corticosterone and of testosterone in the eggs, and on
324 egg mass and clutch size in the yellow-legged gull. We did not record any significant effect of
325 experimental treatment on corticosterone concentration. This result contradicts our
326 expectation that was based on the relatively few studies exploring the non-consumptive effect
327 of predation on transmission of corticosterone to the eggs in birds (Cockrem and Silverin
328 2002; Saino et al. 2005). The lack of the effect of exposure to predators was unlikely to be
329 due to insufficient power of the statistical tests. This is the case because the size of the sample
330 was reasonably large and the difference between the control and the predator-exposed group
331 of eggs was opposite to that expected, as corticosterone concentration was larger, though
332 statistically non-significantly so, in control eggs as compared to the eggs from nests exposed
333 to predation. The interpretation that the experimental protocol was ineffective in stimulating a
334 response in the gulls is also unlikely because the gulls strongly reacted to the both stuffed
335 diurnal buzzard and nocturnal fox by actively mobbing them or by sitting in the water far
336 from the stuffed predators (our personal observations). This behaviour is very different from
337 that usually adopted by undisturbed adults, that typically stay in their nesting territory within
338 the colony during daytime. In addition, daily exposure to the predator last longer than any
339 episode of alarm induced by a natural predator ever observed in the same colonies (see also
340 Methods). Finally, the timing of presentation of the predators was likely appropriate to have
341 an effect on the biochemical composition of the egg because the experimental treatment
342 started approximately when the RYD phase can be estimated to start in our model species,
343 based on evidence from a closely related gull species of similar size (*Larus audouinii*; Ruiz et
344 al. 2000). Thus, our results suggest that exposure to predation risk has no detectable effect on
345 the concentration of corticosterone in the egg yolk. However, our results also suggest that

346 corticosterone concentration in the eggs depends on environmental effects, as suggested by
347 the observation that it varied significantly among sub-colonies and also declined along the
348 breeding season. The factors that caused such variation, however, remain unknown.

349 Testosterone was also apparently unaffected by experimental treatment, and testosterone
350 concentrations were very similar in either experimental group. In the absence of clear
351 predictions on the direct effect of exposure to predators on testosterone concentration in the
352 eggs, the main reason for investigating the effect was that corticosterone and testosterone
353 concentrations have been shown to be related in birds (Duckworth et al. 2001; Okuliarová et
354 al. 2010; Henriksen et al. 2011; see also Rubolini et al. 2011). The concentrations of either
355 hormone were negatively correlated and the slope of relationship was independent of
356 experimental treatment. Hence, the present study does not corroborate the idea that a positive
357 effect of exposure to predators on corticosterone concentration can be generalized across
358 species and also does not support the only previous record of a negative effect of predation
359 risk on egg testosterone concentration (Coslovsky et al. 2012). Notably, a recent meta-
360 analysis supported coloniality as the main factor predicting variation of egg testosterone
361 levels across species, with more colonial species having smaller concentrations of the
362 hormone (Bentz et al. 2016). It might be speculated that selection for reduced aggressiveness
363 in colonial species results in smaller response to external stressful conditions in terms of
364 allocation of steroid hormones to the eggs.

365 Previous studies of the effect of predation risk on clutch size disclosed either negative or null
366 effects (Doligez and Clobert, 2003; Eggers et al. 2006; Fontaine and Martin 2006; Massaro et
367 al. 2008; Cassey et al. 2009). The present study thus adds to the body of evidence that
368 predators have no effect on decisions on reproductive investment in terms of clutch size.

369 One general, adaptive interpretation of the lack of the effect of predation on egg hormone
370 concentrations and clutch size is that predation risk during egg laying is a poor predictor of
371 predation risk that the offspring will experience after hatching. This is the case because local
372 predation risk may vary widely between laying and hatching, which occurs at least one month
373 later. In fact, our model species differs from several of the species where the effect of
374 predation of clutch size and egg composition has been tested in that local predation by the
375 predators that we used in our experiment (Cockrem and Silverin, 2002; Doligez and Clobert
376 2003; Saino et al. 2005; Eggers et al. 2006; Fontaine and Martin 2006; Coslovsky et al. 2011;
377 Pitk et al. 2012). Even a minimal (i.e. 1 egg) reduction in clutch size may entail a
378 considerable seasonal fitness cost in a species where maximal clutch size is three eggs. This is
379 particularly true in our study colony where large post-natal mortality typically occurs (our
380 personal observation since 2005). In fact, third-laid chicks often function as a back-up for
381 their larger, older siblings. Any reduction in clutch size in response to predation risk upon
382 laying would therefore be prevented by temporal stochasticity in predation risk and large
383 fitness cost of reduction of clutch size.

384 The interpretation that stochastic temporal variation in predation risk reduces the scope for
385 adaptive flexibility in reproductive decisions is partly contradicted by the observation that
386 experimental treatment had a highly significant, intense effect on egg mass, which was
387 considerably larger in the predator-exposed than in the control group. The predictions on the
388 effect of predators on egg mass were equivocal and dependent on the assumptions on the
389 effect of large egg mass on post-natal performance under high predation risk (Cresswell
390 2008). We interpret the observation of the positive effect of exposure to predators on egg
391 mass as suggestive that mothers enhanced post-natal survival prospects of their offspring by
392 increasing their size and reserves, which could promote viability in case post-natal parental
393 attendance is reduced by the presence of predators, by reducing offspring need of food and

394 thus their postural and vocal solicitation behaviour to their parents, and perhaps also by
395 enhancing their cognitive and motor skills at hatching. In addition, because size at hatching
396 strongly predicts subsequent growth rates (Krist 2011), by laying large eggs parents may also
397 reduce the duration of the period when chicks are most vulnerable to predation, at least by
398 aerial predators. Yet an alternative interpretation is that larger eggs are less exposed to the risk
399 of cooling when the presence of predators forces parents to leave the nest unattended and
400 suspend incubation (e.g. Rhymer 1988). In summary, we suggest that stochasticity in local
401 predation risk may have shrunk the scope for adaptive reduction of clutch size, which can
402 entail considerable seasonal fitness costs, but may also have prompted laying mothers to
403 adopt energetically relatively cheap strategies of incremental investment to boost offspring
404 performance.

405 Thus, we showed that exposure to increased predation risk has no detectable effect on the
406 concentration of egg steroid hormones and on clutch size, whereas it has a marked positive
407 effect on egg size in the yellow-legged gull, possibly to boost performance in the post-natal
408 stages. These findings are partly inconsistent with previous evidence on the positive effect of
409 predation risk on egg corticosterone concentration and on the negative effect on clutch size.
410 We suggest that the effects of predation risk on reproductive decisions vary idiosyncratically
411 among species possibly depending on predictability of predation risk and also developmental
412 mode.

413

414

415

416 **Compliance with ethical standards**. The study was carried out under the permission of the
417 Parco Regionale del Delta del Po (#388, 20 January 2016), and it was undertaken under the
418 combined prescriptions of Art. 4 (1) and Art. 7 (5) of the Italian law 157/1992, which
419 regulates studies on wild bird species. All applicable institutional and/or national guidelines
420 for the care and use of animals were followed.

421

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Table 1. Linear mixed models of corticosterone and testosterone concentration, of clutch size and of egg mass in relation to treatment (exposure to predators or control). Laying date was included as a covariate. Egg mass was also included as a covariate in models of corticosterone and testosterone concentrations. Sub-colony was included as a random effect in all models. Nest identity was included as a random effect and laying order as a fixed effect factor in the model of egg mass. χ^2 refers to the likelihood ratio test comparing two models including or, respectively, excluding the random effect of sub-colony. Two-way interaction terms were removed from all models as their effect was statistically non-significant.

	χ^2	F	df	P	coefficient (SE)
Corticosterone concentration in first eggs					
Sub-colony	6.11			0.013	
Treatment		0.12	1,41	0.732	
Laying date		6.24	1,41	0.017	-0.093 (0.037)
Egg mass		0.16	1,41	0.691	0.003 (0.008)
Testosterone concentration in first eggs					
Sub-colony	0.00			>0.999	
Treatment		0.01	1,42	0.907	
Laying date		0.57	1,42	0.454	0.526 (0.695)
Egg mass		0.42	1,42	0.523	-0.087 (0.136)
Clutch size					
Sub-colony	0.00			>0.999	
Treatment		0.04	1,63	0.837	
Laying date		0.07	1,63	0.787	-0.013 (0.048)
Egg mass					
Sub-colony	0.00			>0.999	
Nest identity	88.26		1	<0.001	
Treatment		10.75	1,122	0.001	
Laying order		21.35	2,122	<0.001	
Laying date		3.30	1,122	0.072	-0.588 (0.324)

620 **Legend to Figures**

621 Figure 1. Corticosterone and testosterone concentrations in the first laid eggs from control
622 nests or nests that were exposed to predators. Sample sizes (number of eggs) are reported.

623

624 Figure 2. Relationship between testosterone and corticosterone concentrations in the yolk of
625 control and predator-exposed eggs. The regression lines for controls (dashed) and predator-
626 exposed (continuous) eggs are presented. The slopes of the regression lines did not differ
627 between the two groups.

628

629 Figure 3. Mass of the eggs from control nests or nests that were exposed to predators. Sample
630 sizes (number of eggs) are reported. The eggs from nests exposed to predators were
631 significantly heavier than those from control nests.

632

633 Figure 1

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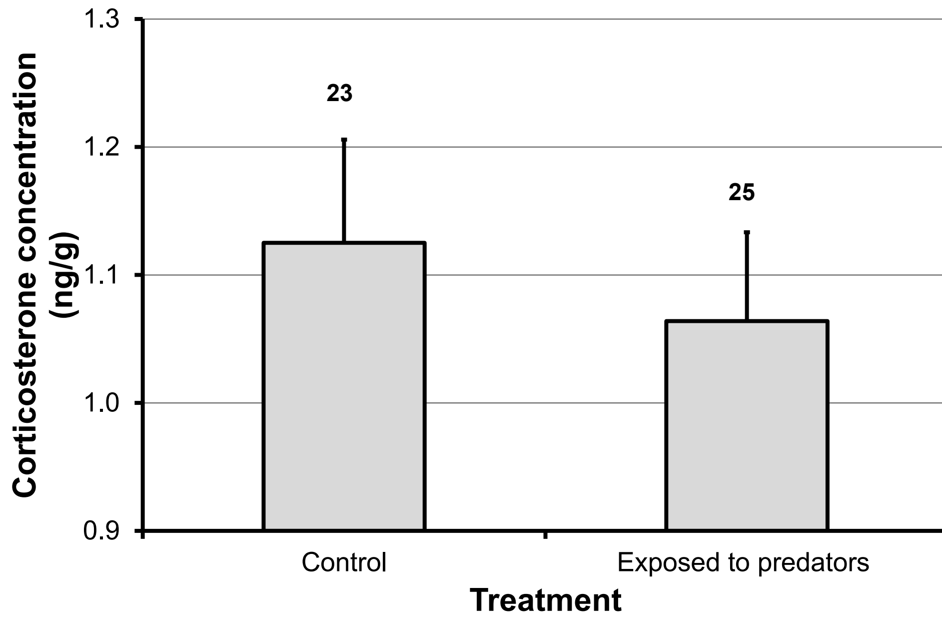

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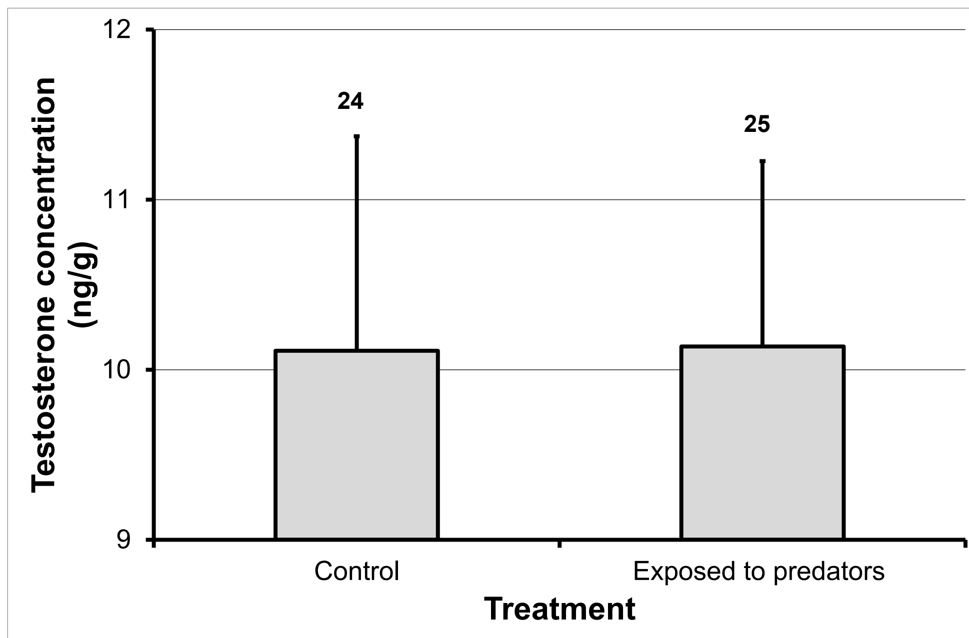

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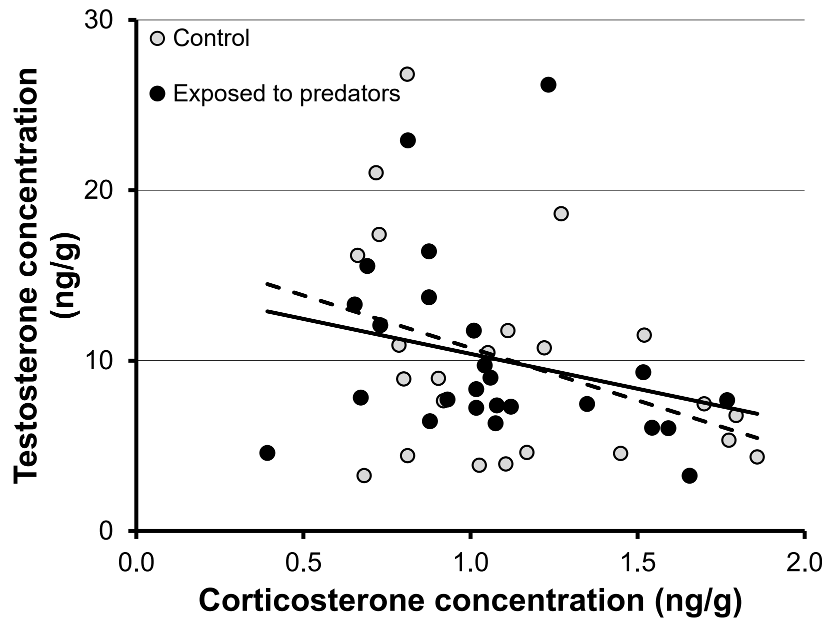

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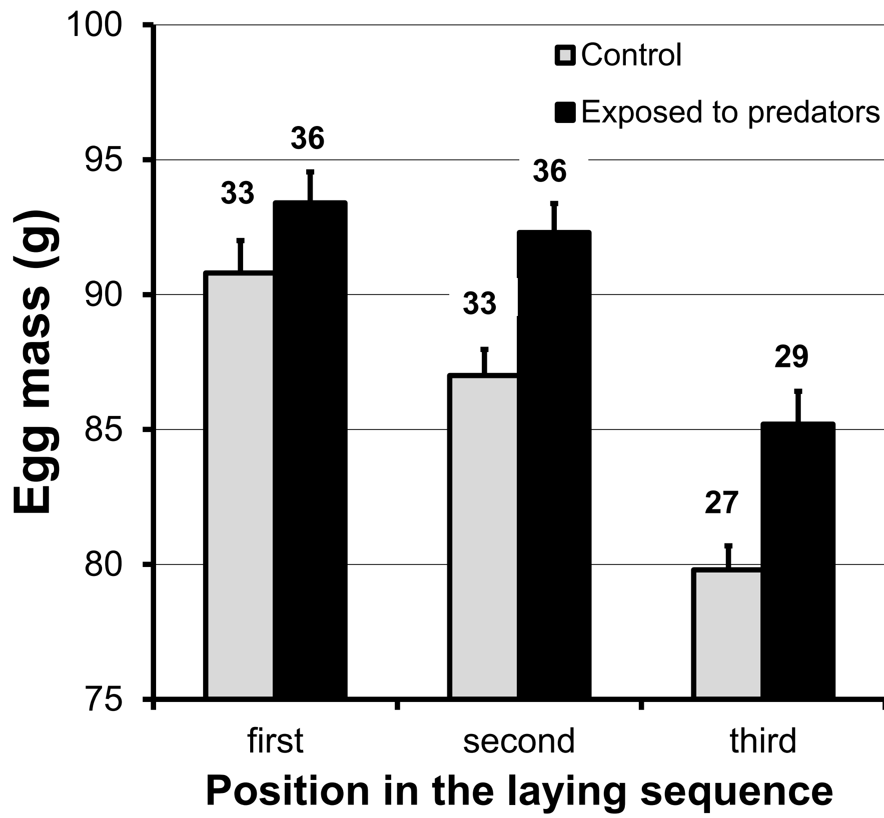

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Chapter 9

Synthesis and concluding remarks

The present thesis investigated prenatal maternal effects mediated by egg quality in the yellow-legged gull. The main purpose of this work was deepening the knowledge about the consequences of variation of maternally-transferred compounds on the offspring. I mainly adopted an experimental approach by which I directly manipulated the biochemical composition of unincubated eggs, injecting yolk with a physiological dose of a specific maternal compound. I thus analysed multiple phenotypic traits in both gull embryos and newly hatched chicks.

Antioxidant-mediated maternal effects

Chapters 2 and **3** deal with the potential effects of vitamin E on oxidative status and somatic growth during embryonic development and soon after hatching. In the study focused on embryos at late prenatal stages (**Chapter 2**), the supplementation of vitamin E promoted embryonic growth increasing body mass, as expected, but independently of laying order. Since the concentration of vitamin E in the yellow-legged gull decreases with the laying sequence, a more marked positive effect on growth of embryos from third-laid eggs was expected (see Parolini et al., 2015). However, during pre-hatching development, egg vitamin E concentration may be sub-optimal for somatic growth of all embryos, independently of laying order. Moreover, the physiological dose of vitamin E increased total antioxidant capacity (TAC) in residual yolk but, contrary to the expectations, it did not affect TAC, the amount of pro-oxidant molecules (TOS) and oxidative damage to lipids and proteins in both liver and brain. An interpretation is that the concentration of vitamin E transferred by mothers to the eggs efficiently contrasts the occurrence of oxidative damage, and thus the supplemental amount of vitamin E may be allocated to boost embryonic somatic growth. According to results presented in Chapter 2, in newly hatched chicks (**Chapter 3**), vitamin E treatment increased TAC but did not affect oxidative damage to proteins and lipids, although it was found to reduce the concentration of pro-oxidant molecules. These effects on TAC and TOS were independent of egg laying order, suggesting that all chicks benefited from vitamin E supplementation, contrary to what was observed in a previous study, where vitamin E administration positively affected morphological traits of chicks from third laid-eggs (see Parolini et al., 2015). Finally, despite the supplementation of vitamin E ameliorated oxidative status (TAC and TOS), it did not reduce telomere attrition. Because no study to date has investigated the effect of prenatal antioxidants on telomere length at the end of the embryonic stage, these finding are the first evidence showing that vitamin E has little influence on telomere dynamics in early life stages.

The study presented in **Chapter 4** aimed at investigating the relationships occurring among different egg antioxidants stored in the yolk, liver and brain of embryos, and the consequences of the experimental increase of vitamin E concentration on the other antioxidant distributions as well as their effects on multiple embryo phenotypic traits. The results showed that embryo growth was positively associated with the concentration of antioxidants and TAC, and negatively predicted by markers of oxidative status (i.e. TOS, lipid peroxidation and protein carbonylation), as expected. Moreover, antioxidant concentrations were positively correlated both within and between the yolk, and liver and brain, suggesting that some mothers have access to relatively large amounts of antioxidants, which are deposited in large concentrations in the yolk and, consequently, distributed in embryo organs (Costantini and Verhust, 2009). Alternatively, mothers must balance the antioxidant concentrations by allocating them in amounts that reciprocally positively correlate in order to optimally accomplish antioxidant functions. Moreover, markers of oxidative status were only partly correlated within organs and not correlated between organs, implying that oxidative damage in a particular organ does not predict oxidative damage in another organ. In addition, total antioxidant capacity was positively associated with the concertation of antioxidants in the yolk, liver and brain but it was not correlated with TOS, lipid peroxidation and protein carbonylation, suggesting that large amounts of antioxidants were not necessarily associated with low oxidative damage. Finally, the experimental increase of vitamin E did not affect the relationship between other antioxidants or oxidative status markers and vitamin E concentration, the covariation between other antioxidants and oxidative status markers, and the relationship between embryo morphology or oxidative status and other antioxidants. These findings suggest that under natural selection regime, physiological supplementation of one major antioxidant in egg yolk has minor, if any, effects on other components of the antioxidant defence system and their effects on embryo phenotypic traits.

Hormone-mediated maternal effects

Chapter 5 concerns the prenatal effects of elevated testosterone level on development and oxidative status of gull embryos. Testosterone supplementation was found to promote somatic growth, increasing body mass and tarsus length, and reduce residual yolk mass, suggesting that testosterone may enhance body size by accelerating yolk absorption. Despite embryos from testosterone-eggs showed larger body size, they had a smaller brain size compared to controls; these contrasting effects suggest that testosterone may mediate a developmental trade-off between allocation to somatic growth and energetically demanding growth of brain at the embryonic stage. Moreover, brain size of control embryos from the second-laid eggs was

smaller than that of siblings from the first- and third-laid eggs while no such variation according to laying order was observed among embryos from testosterone-eggs. These findings were paralleled by variation in brain TOS. Control embryos from second-laid eggs had lower TOS compared to their siblings whereas TOS of embryos from testosterone-eggs did not differ depending on laying order. Therefore, testosterone may enhance brain growth of embryos from second-laid eggs, causing an increase in the concentration of pro-oxidant molecules. This interpretation is supported by evidence showing that testosterone is anabolic and an increase in growth rate generally results in high production of oxidizing molecules. Finally, brain size was found to differ between sexes, being larger in males than in female embryos. Sexual dimorphism in brain size and structure has been observed in several *taxa* (Gahr, 1994; Jacobs, 1996) but information generally refers to adulthood. Therefore, these findings are the first evidence of sexual brain dimorphism at embryonic stage in any vertebrate species in the wild.

In the study presented in **Chapter 6**, where the consequences of testosterone supplementation were scrutinized on hatchling morphology, testosterone appeared to negatively affect somatic growth depending on laying order, with a reduction in body mass of chicks from the first-laid eggs. This result is partly consistent with a previous study where a dose of testosterone twice as large as that used here impaired somatic growth of the chicks from second- and third-laid eggs, at 4 days after hatching (Rubolini et al., 2006). The different effect of the supplemental dose may depend on the amount of testosterone injected and on the age of hatchlings. In this study, I also analysed the potential organizational effects of testosterone on the direction and the consistency of behavioural lateralization. Contrary to the expectations, no evidence emerged for an effect of testosterone supplementation on the direction of lateralization in begging and escape behaviours. However, individual-level consistency in lateral preference in escape behaviour was increased by the treatment, suggesting that the physiological dose of testosterone positively affects the persistence of behavioural patterns (Andrew, 1972; Andrew and Rogers, 1972; Klein and Andrew, 1986; Andrew and Jones, 1992). Lastly, a strong lateralization at individual-level occurred in begging behaviour, independently of treatment, while neither sex-dependent nor population-level lateralization were observed in both behavioural tasks.

Chapter 7 deals with the effects caused by a physiological increase in yolk corticosterone concentration on the direction of postnatal behavioural lateralization. The results showed that the supplementation did not affect the direction of lateralization in reverting to prone position response. However, corticosterone treatment was found to reduce the frequency of begging with a subtle effect on rightward pecks. These results combined suggest that corticosterone may cause leftward lateralization by reducing the absolute number of pecks on either side and also

weakly effect on the rightward response. I speculated that corticosterone exposure during the prenatal period interferes with the development of the structural asymmetry of the visual system (Manns and Güntürkün, 1999; Rogers and Deng, 2005; Manns and Ströckens, 2014), leading to an impairment in the ability to perform pecks at the right side, which increases the tendency for leftward lateralization. These findings are the first evidence suggesting an organizational effect of corticosterone on lateralization during prenatal stage, although they are not conclusive due to the weak observed effect.

Finally, the work presented in **Chapter 8** aimed at examining non-consumptive effects of predation on egg concentration of steroid hormones, clutch size and egg mass. Although an increase in corticosterone and testosterone levels was expected (e.g. Saino et al., 2005; Coslovsky and Richner, 2011), concentrations of both hormones did not differ between eggs from mothers exposed to the predators and control ones. However, corticosterone concentration in the eggs declined along the breeding season and depended on environmental effects, as suggested by variation among sub-colonies. In addition, despite testosterone level was not increase by predation, it negatively correlated with corticosterone concentration, independently of experimental treatment. Finally, while clutch size was not affected by experimental treatment, egg mass was markedly enhanced by exposure to increased predation risk. One adaptive interpretation of the lack of effect of predation on egg composition and clutch size is that predation risk during egg laying is a poor predictor of predation risk experienced by the offspring soon after hatching. Moreover, a reduction in clutch size may entail a considerable seasonal fitness cost in a species where maximal clutch size is three eggs. The positive effect of predation risk on egg mass may be interpreted as an active modulation by mothers, which laid large eggs containing a great amount of resources and from which usually hatch large chicks that growth fast, to reduce offspring food solicitation and the duration of the period when chicks are most vulnerable to predation.

Concluding remarks

The evidence emerged from this thesis showed that maternal transmission to the eggs may entail substantial offspring costs and benefits, which may vary according to laying order, in relation to phenotypic traits as well as depending on ontogenetic stage.

In fact, vitamin E supplementation had a general positive influence on different phenotypic traits depending on pre- and postnatal period. It positively affected somatic growth during embryonic development, while ameliorating oxidative status soon after hatching. The effects

caused by an increase in yolk testosterone concentration were contrasting and varied in intensity depending on offspring phenotypic traits and developmental stages, suggesting that testosterone may enforce either trade-offs between multiple traits at prenatal period or in the same trait at different ontogenetic stages. Finally, the results showing the influences of variation in testosterone and corticosterone concentrations on the consistency and the direction of lateralization add information to the scant knowledge about the organizational effects of steroid hormones on lateralization development, although the underlying mechanisms remain to be elucidated.

To conclude, the studies presented in this thesis provide novel findings on the consequences of maternal effects mediated by egg quality during both pre- and postnatal periods, supporting the general idea that variation in egg components during early life stage may significantly affect offspring phenotype and fitness in bird species.

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Acknowledgements

I would like to express my gratitude to my supervisor Prof. Nicola Saino for the precious support during the past three years and for helping me to publish my researches in high-level international scientific journals. During these years, he often provided me forceful and invaluable teachings that stretch well beyond the scientific enquiry.

Special gratitude goes to Dr. Marco Parolini, an astounding researcher and a friend, for tutoring me in different situations, from field and lab work to statistical analyses, and for transmitting me his knowledge and passion; without Marco my PhD would have been much harder and less pleasant.

I am sincerely grateful to Dr. Adrian Covaci, Giulia Poma and Saskia Defossé, for their willingness to host me in Antwerp, for teaching me new lab techniques and providing helpful suggestions.

I also warmly thank to the colleagues I worked with, Prof. Diego Rubolini, Andrea Romano, Alessandra Costanzo, Stefano Podofillini, who gave me very much appreciated support. I would also like to thank Manuela Caprioli for the precious assistance with the lab and field work.

I am thankful to Gaia Bazzi and Margherita Corti, colleagues who became dear friends during these years, and with whom I spent special moments.

I would like to thank all the students who have greatly helped me in collecting data in both the field and the lab: Davide Genovesi, Eleonora Agostani, Sara Di Giacomo, Alfredo Schiavon, Simona Secomandi and Silvia Carboni.

Finally, I thank my family and my dear friend Giulia, who both supported me during these years.

PhD Student Final Report

UNIVERSITÀ DEGLI STUDI DI MILANO

PhD Environmental Sciences

PhD Course in Environmental Sciences

PhD Student Final Report

PhD Student:	Cristina Daniela Possenti
PhD Course Cycle:	XXX
Scientific Tutor:	Prof. Nicola SAINO
Scientific Co-Tutor:	Dr. Marco PAROLINI
Thesis project title:	Maternal effects mediated by egg quality in the yellow-legged gull (*Larus michahellis*)
Project performed at:	University of Milan; Department of Environmental Science and Policy; Laboratory of Behavioural and Evolutionary Ecology

Research Period Abroad			
January-February 2017	Toxicological Center, Department of Pharmaceutical Sciences, University of Antwerp, Belgium	Local Supervisor: Dr. Adrian COVACI	Research Project: Effects of Triclosan on yellow-legged gull embryos

List of Scientific Publications

Possenti, C.D., Secomandi, S., Schiavon, A., Caprioli, M., Rubolini, D., Romano, A., Saino, N., Parolini, M. Independent and combined effects of pro- and anti-oxidants on yellow-legged gull chicks growth and oxidative status. Submitted to: *Journal of Experimental Biology*

Parolini, M., Iacobuzio, R., **Possenti, C.D.**, Bassano, B., Pennati, R., Saino, N. Carotenoid-based coloration signals antioxidant defenses in the brown trout (*Salmo trutta*). Submitted to: *Hydrobiologia*

Parolini, M., **Possenti, C.D.**, Romano, A., Caprioli, M., Rubolini, D., Saino, N. Telomere length, egg testosterone levels and oxidative status in gull hatchlings. Submitted to: *Biology Letters*

Possenti, C.D., Bentz, A.B., Romano, A., Parolini, M., Caprioli, M., Rubolini, D., Navara, K., Saino N. Predation risk affects egg mass but not egg steroid hormone concentrations in yellow-legged gulls. Submitted to: *The Science of Nature*

Possenti, C.D., Parolini, M., Romano, A., Caprioli, M., Rubolini, D., Saino, N. (2018). Effect of yolk corticosterone on begging in the yellow-legged gull. *Hormones and Behaviour,* 97, 121-127

Parolini, M., Khoriauli, L., **Possenti, C.D.**, Colombo, G., Caprioli, M., Santagostino, M., Nergaze, S., Milzani, A., Giulotto, E., Saino, N. (2017). Yolk vitamin E prevents oxidative damage in gull hatchlings. *Royal Society Open Science*, 4, 170098

Parolini, M., **Possenti, C.D.**, Karadas, F., Colombo, G., Romano, M., Caprioli, M., Dalle-Donne, I., Rubolini, D., Milzani, A., Saino, N. (2017). Yolk vitamin E positively affects prenatal growth but not oxidative status in yellow-legged gull embryos. *Current Zoology* https://doi.org/10.1093/cz/zox037

Parolini, M., **Possenti C.D.**, Saino, N. (2017). The yellow-legged gull *Larus michahellis* (Charadriiformes, Laridae) as a model species in ecotoxicology: application in monitoring and toxicity assessment of environmental pollutants. Book chapter in: *Ecotoxicology and Genotoxicology: Non-traditional Terrestrial Models. pp 269-288 Royal Society of Chemistry.*

Romano, A., **Possenti, C.D.**, Caprioli, M., Gatti, E., Gianfranceschi, L., Rubolini, D., Saino, N., Parolini, M. (2017). Circadian genes polymorphism and breeding phenology in a resident bird, the yellow-legged gull. *Journal of Zoology* DOI:10.1111/jzo.12501

Parolini M., Colombo, G., Valsecchi, S., Mazzoni, M**., Possenti, C.D.**, Caprioli, M., Dalle-Donne, I., Milzani, A., Saino, N., Rubolini, D. (2016). Potential toxicity of environmentally relevant perfluorooctane sulfonate (PFOS) concentrations to yellow-legged gull *Larus michahellis* embryos. *Environmental Science and Pollution Research* 23, 426-437

Possenti, C.D., Romano, A., Caprioli, M, Rubolini, D., Spiezio, C., Saino, N., Parolini, M. (2016). Yolk testosterone affects growth and promotes individual-level behavioural lateralization of yellow-legged gull chicks. *Hormones and Behavior* 80, 58-67

Romano, A., De Giorgio, B., Parolini, M., Favero, C., **Possenti, C.D.**, Iodice, S., Caprioli, M., Rubolini, D., Ambrosini, R., Gianfranceschi, L., Saino, N., Bollati, V. (2016). Methylation of the circadian *Clock* gene in the offspring of a free-living passerine bird increases with maternal and individual exposure to PM_{10}. *Environmental Pollution* 220, 29-37

Costanzo, A., Parolini, M., Bazzi, G., Khoriauli, L., Santagostino, M., **Possenti, C.D.**, Romano, A., Nergadze, S.G., Rubolini, D., Giulotto, E., Saino, N. (2016). Brood size, telomere length, and parent-offspring color signaling in barn swallows. *Behavioral Ecology* arw147

Bazzi, G., Cecere, J.G., Caprioli, M., Gatti, E., Gianfranceschi, L., Podofillini, S., **Possenti C.D.**, Ambrosini, R., Saino, N., Spina, F., Rubolini, D. (2016). *Clock* gene polymorphism, migratory behaviour and geographic distribution: a comparative study of trans-Saharan migratory birds. *Molecular Ecology* 25, 6077-6091

Possenti, C.D., Karadas, F., Colombo, G., Caprioli, M., Rubolini, D., Milzani, A., Dalle-Donne, I., Saino, N., Parolini, M. (2016). Antioxidants and embryo phenotype in there experimental evidence for strong integration of the antioxidant system? *Journal of Experimental Biology* 220, 615-624

Parolini, M., Romano, A:, **Possenti, C.D.**, Caprioli, M., Rubolini, D., Saino, N. (2016). Contrasting effects of increased yolk testosterone content on development and oxidative status in gull embryos. *Journal of Experimental Biology* 220, 625-633

Meeting and Congress Contributions

Possenti, C.D., Parolini, M., Milzani, A., Saino, N. Sub-optimal egg vitamin E concentration limits offspring growth in the yellow-legged gull. 1° Congresso Nazionale Congiunto S.It.E. (Società Italiana di Ecologia) - UZI (Unione Zoologica Italiana) - SIB (Società Italiana di Biogeografia): Milano, 30 agosto-2 settembre 2016

Parolini, M., **Possenti C.D.** Eggsposure: a promising approach to assess the toxicity of environmental pollutants. Poster vincitore ‘Premio Marchetti’. 1° Congresso Nazionale Congiunto S.It.E. (Società Italiana di Ecologia) - UZI (Unione Zoologica Italiana) - SIB (Società Italiana di Biogeografia): Milano, 30 agosto-2 settembre 2016

Parolini, M., **Possenti C.D.** Eggsposure: a suitable approach to investigate the toxicity of emerging pollutants in birds. 7a edizione Giornate di Studio - Ricerca e Applicazione di Metodologie Eco-tossicologiche: Livorno, 22-24 novembre 2016

Possenti C.D., Bazzi G., Caprioli M., Gianfranceschi L., Rubolini D., Saino N. The role of *Clock* and other candidate genes in the control of phenology in long-distance migrants. XVIII Convegno Italiano di Ornitologia: Caramanico Terme, 17-20 settembre 2015

Mazzoni M., Parolini M., **Possenti C.D.,** Saino N., Valsecchi S. Perfluoroalkyl acids in *Larus michahellis* from Comacchio lagoon. XI Incontro dei Dottorandi in Ecologia e Scienze dei Sistemi Acquatici: Roma, 17-19 settembre 2015

List of attended Seminars

DNA barcoding: a universal tool for biodiversity characterization: theoretical and practical aspects – Galiberti, A. May 2017

Environmental disasters and their ecological consequences. a short course on how to study them - Bonisoli-Alquati, A. December 2017

Do carbon based nanoparticles act as carrier for benzoαpirene. An investigation on *Danio rerio* embryos - Della Torre, C. February 2016

Evolution of prenatal sex allocation strategies in a sexually promiscuous passerine birds – Romano, A. March 2016

Genetic architecture and mapping of traits – Mueller, T.C. July 2016

Environmental DNA to understand biodiversity changes – Ficetola, F. November 2016

Amphibian population ecology an environmental changes – Green, M.D.November 2016

On symbionts arthropods and vector-borne diseases – Epis, S. November 2016

Gendercide symbionts...e altre storie di sesso, simbiosi e parassitismo – Bandi, C. February 2015

Simbiosi, parassitismo, e malattie infettive: un approccio evoluzionistico – Bandi, C. March 2015

Variabilità e cambiamenti climatici in Italia negli ultimi due secoli – Maugeri, M. March 2015

L’epigenetica ambientale come interfaccia tra ambiente ed espressione genica – Bollati, V. March 2015

Unraveling climate change effects on migration birds: a comparative approach – Rubolini, D. March 2015

Cave colonization by the first salamander (*Salamandra salamandra*): zoological, ecological and evolutionary insights – Manenti, R. November 2015

Ecology and Evolution at Chernobyl and Fukushima – Møller, A.P. December 2014

How to write a paper – Møller, A.P. December 2014

List of attended PhD Courses

February 2015. Tree rings as archives to understand past and present environmental conditions (Dr. Paolo Cherubini)

February-April 2015. English course organized by the PhD course in Environmental Sciences (Prof. Carson)

September-October 2015. Statistic course organized by the PhD course in Environmental Sciences (Prof. Ambrosini)

May-June 2016. Corso di matematica: ottimizzazione in più variabili (Dr. Paola Morando)