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Multiple ornaments and sexual selection in a passerine bird: the Serin.

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“Perguntam-nos muitas vezes: para o que serve o que estás a investigar? Ficamos por vezes atrapalhados porque sentimos que devemos uma resposta mas não sabemos bem o que responder ou como responder. A verdade é que investigamos porque queremos saber, porque a pergunta está ali, à nossa frente, como que a provocar-nos.”

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Abstract

A wide range of animals have more than one sexual ornament and much attention has been devoted to understand how they evolved. Concerning the quality content of ornaments, three main theories were proposed, the multiple message hypotheses, the redundant signal hypothesis and the unreliable signal hypothesis.

Frequently, birds exhibit simultaneously, a large variety of acoustic and visual ornaments, providing a multiplicity of signals with informative content. The Serin, *Serinus serinus*, is a small monogamous sexually dimorphic passerine bird, where males court females singing and exhibiting their colouration. It has been shown that song and colouration are involved in mate choice in serins, as females showed preference for higher song frequencies and for more colourful males. In order to understand if these traits signals the same qualities of individuals and the extent to which ornament expression reflects individual condition and behaviour, we studied the relationship between song and colouration and indicators of male quality: body condition, body size, immune response (PHA-P) and physiological response to stress (breath rate).

Our results show that colouration expression is significantly related to body condition, as saturation relates negatively to body condition index. Song measurements do not predict any quality trait that we assessed, however minimum song frequency is marginally negatively related to body size and breath rate. Moreover, age is related to body condition and size.

We conclude that the expression of multiple ornaments in the serin may deliver information on different aspects of male quality, meeting the multiple message hypotheses. It is also clear that song and colouration measurements are not related in the expression of quality traits, invalidating the redundant signal hypotheses. Irrespective of these results, more research needs to be accomplished in order to achieve a better understanding on which male traits the multiple ornaments are signalling in the serin.

Keywords: Multiple ornaments, *Serinus serinus*, song, colouration, quality traits.

Resumo

Variadas espécies caracterizam-se por possuírem ornamentos sexuais múltiplos, e muita atenção tem sido prestada de maneira a compreender de que forma é que os mesmos evoluiram. Tendo em conta o conteúdo de qualidade dos ornamentos, foram propostas três principais teorias, a teoria da mensagem múltipla, a teoria do sinal redundante e a teoria do sinal não confiável

Frequentemente as aves exibem em simultâneo uma grande variedade de ornamentos acústicos e visuais, através dos quais podem fornecer uma multiplicidade de sinais com conteúdo informativo. A milheirinha, *Serinus serinus*, é um pequeno passeriforme, monogâmico e sexualmente dimórfico, em que os machos cortejam as fêmeas, cantanto e exibindo a sua coloração. Tem sido demonstrado que o canto e a coloração estão envolvidos na escolha de par na milheirinha, em que as fêmeas preferem machos mais coloridos e que produzem canções com frequências mais elevadas. De maneira a compreender se estes traços sinalizam as mesma qualidades e até que ponto a expressão de ornamentos reflecte condição e até mesmos comportamentos foi estudada a relação entre o canto e a coloração com indicadores de qualidade: condição corporal, tamanho corporal, resposta imunitária (PHA-P) e resposta fisiológica ao stress (taxa respiratória).

Os nossos resultados demonstram que a expressão da coloração tem uma relação significante com a condição corporal, em que a saturação se relaciona negativamente com o índice de condição corporal. Por sua vez os parêmetros de canto não expressam nenhum dos traços de qualidade que avaliámos, no entanto a frequencia mínima da canção está marignalmente relacionada, negativamente, com o tamanho corporal e com a taxa respiratória. Para além disto, a idade está relacionada com a condição e tamanho.

Assim, concluímos que na milheirinha a expressão de ornamentos múltiplos pode fornecer informação sobre diferentes aspectos de qualidade do macho, o que vai de encontro com a hipótese da mensagem múltipla. É também claro que os parâmetros de canto e coloração não estão relacionados na expressão de traços de qualidade, invalidando assim a hipótese da mensagem redundante. Independentemente destes resultados, é necessária a elaboração de mais estudos de modo a se obter uma melhor compreensão ao nível de que traços, nos machos, os ornamentos múltiplos estão a sinalizar na milheirinha.

Palavras-chave: Ornamentos múltiplos, *Serinus serinus*, canto, coloração, traços de qualidade.

Chapter 1

Introduction

For hundreds of years, humans observed, explored, were curious, and wrote about animal life and behaviour in order to understand its diversity and complexity. Reproduction displays, communication routes, feeding and social behaviour are just a few issues that fascinated and still fascinate humans.

Since Darwin initial work on the development of sexual selection theory (Darwin 1871) other models and hypotheses related with sexual selection have been formulated and tested. Interest about structures apparently disadvantageous and at the same time beautiful, peculiar and even bizarre, but that somehow remained throughout generations, led to an intense research and pursue for the understanding how communication routes are used by animals (Andersson 1994). Besides the great work developed until now, a lot remains unknown and a challenge for several. Biologically areas, particularly crossing behavioural ecology with evolutionary biology, neurobiology, genetics, physiology and molecular biology.

Communication

Communication is an essential aspect of the animal life and plays a central role in animal societies (Bradbury & Vehrencamp 1998). The behaviour of an animal changes in accordance with the information obtained from other animals and the environment. At the same time that information is being received, some other can be sent, and is this dynamic that will affect and modify the behaviour (Bradbury & Vehrencamp). Given this, communication can be defined as the act of transmission and sharing of information, between at least two individuals, one that sends and other that receives, enabling the receiver to make a decision (Catchpole & Slater 2008). In order to communication to occur, it must be intentional and beneficial for the individual that sends it. Also, the receiver can answer or not to it choosing the best for itself (Figure 1) (Bradbury & Vehrencamp 1998; Searcy & Nowicki 2005). Non-intentional signals are called cues and the perception by the receiver is not beneficial for the sender (Bradbury & Vehrencamp 1998). In a communicational system the signal is considered the carrier of information (Bradbury & Vehrencamp 1998), and signals are considered to be some aspects of behaviour, physiology and morphology that were maintained over time, and which are beneficial for the carrier and conveyed to other individuals (Searcy & Nowicki 2005).

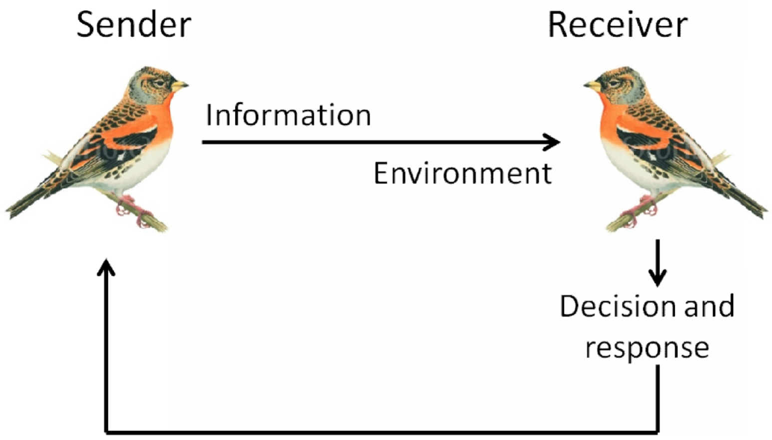

Figure 1 - The process of communication. Adapted from Bradbury & Vehrencamp (1998) (www.fotolibra.com/gallery/31609/brambling-bird-illustration-of-a/).

Signals evolve in the way that they are able to modify the receiver's behaviour favouring the sender. Signals can both be honest or deceitful in the delivery of information. Honest signals presuppose that the sender and the receiver enjoy from the positive value of information, whereas deceit is related with positive value only for the sender. In this way, deceit is considered to be intentional delivery of inaccurate information by a sender.

In general, animals convey several types of messages to each other that are the result of a combination of visual, acoustic or chemical signals (Marler & Slabbekorn 2004), which deliver information about identity, status and mood, intention and discoveries in the environment (Bradbury & Vehrencamp 1998). In all sexual species, reproduction cannot occur without exchange of information (Bradbury & Vehrencamp 1998), moreover some sexual signals evolved to a very peculiar and specific form encompassing beautiful mixtures of colour, song and behaviour displays.

Sexual Selection

It was Charles Darwin (1871), in his pioneer work, *The Descent of Man, and Selection in Relation to Sex*, who first delivered a new concept of selection, a selection exclusively concerning reproduction. With this book, Darwin was able to set the foundation for all modern work on sexual selection, however it was in his previous book, *The origin of species* (1859) when he first proposed the sexual selection hypothesis. It took more than one century before scientists got convinced that Darwin's theory of sexual selection was plausible and testable and, nowadays the principal ideas and arguments in this area are still based on his theory (Andersson & Isawa 1996). Darwin (1871) defined sexual selection as the differential and non-random reproduction between individuals, as a result of competition for access to a mate. This means that this kind of selection would depend on the advantage which certain individuals have over others of the same sex and species, exclusively concerning reproduction. This theory was based on the fact that some structures, apparently disadvantageous to the individual survival, were selected and evolved through time. The evolution of this kind of structures apparently do not seem to fit well within the concept of natural selection, also proposed by Darwin (1859), which comprehends the preservation of favourable variations and the rejection of less favoured variation. This took Darwin to prposed what he believed was a different kind of selection, sexual selection, but that we know now is just a special case of natural selection.

Sexual selection has two main categories, intrasexual and intersexual selection. Intrasexual selection is referred as the competition between individuals of the same sex for a mate of the opposite sex, whereas intersexual selection is related to the choice of a mate by an individual of the opposite sex (Darwin 1871). Usually intrasexual selection is defined as the competition between males and intersexual selection as the choice by the female for a male, but this is a generalization and it is known that the opposite can also happen (Amundsen *et al.* 1997; Amundsen & Forsgren 2001; Baldauf *et al.* 2011; Cheney *et al.* 2012). The red deer ornaments were the structures that boost Darwin's ideas on sexual selection, where contest competition for a mate favours threat displays like large body size, antlers and roaring (Andersson 1994). Following the same line, elephant seals, *Mirounga* spp., are one of the most sex dimorphic species, weighting more than three times as much as females and just like the red deer are an outstanding example of intrasexual selection. This dimorphism occurs essentially due to competition over a mate by male contests and endurance rivalry, where large male body size seems to be favoured by sexual competition affecting mating success (Andersson 1994). On the other hand, one of the most studied species in this context is the guppy, *Poecilia reticulate*, where selection seems to occur mainly by intersexual selection, and studies show that conspicuous male colouration is preferred by females (Andersson 1994). The same occurs with the peacock, *Pavo cristatus*, where female choice falls on the males with highest number of eye-spots on the tail (Petrie *et al.* 1991).

Mechanisms of mate choice and female preference

The evolution of male ornaments not directly related with fighting ability is generally attributed to intersexual selection, focusing on female preference (Andersson 1994). Several models were proposed to explain the evolution of female preference and consequently the origin, function and evolution of male ornaments in this context. Fisher (1930) proposed, with the runaway hypothesis, a coevolution between trait and preference, which involves indirect genetic benefits, *i.e.*, selection will favour females with preference for a determined attractive trait whilst, their sons and daughters, respectively, will express the trait and the preference. This mechanism is characterized by its arbitrariness and consequently by its instability, since even when at first an ornament seems to express a reliable signal, it is possible that it loses the indicative value continuing to be selected (Iwasa & Pomiankowski 1994; Iwasa & Pomiankowski 1999). Later on, Ryan (1990) predicted that the evolution of ornaments would evolve by a pre-existing sensory bias concerning females. In this case, females would respond to supernormal stimulus choosing over the considered normal stimulation. This both hypothesis are related to arbitrary preferences (Buchholz 1995), but were proposed other models on which the main concerning is quality (Andersson 1994).

An indicator of quality presupposes honesty, and Zahavi (1975), with the handicap principle, considered that a handicap, with associated costs of production and maintenance, would be the target of female choice, since it represents a proof of survival capability and genetic quality. The quality is measured by the cost to the carrier, and depending on the cost type it can be a signal of physical, nutritional or immune condition or even motivational state or defence capacity (Zahavi & Zahavi 1997). On the other hand, Hamilton and Zuk (1982) suggested that females chose take into account the health condition and resistance to diseases. They particularized the case of parasites, inferring that resistance is reflected in male traits and may be target of female preference. Given this, females prefer to mate with males that demonstrate higher genetic quality in terms of resistance and survival, obtaining better genes to their offspring. After the Hamilton and Zuk studies, the immune system began to be a central issue, and in this context Folstad and Karter (1992) suggested the immunocompetence handicap hypothesis. This is considered a particular case of Zahavi's (1975) handicap hypothesis, assuming that the expression of secondary sexual ornaments can signal individual immunocompetence, which is directly linked to the relation between the level of circulating androgens and immunity response.

In conclusion, sexual dimorphism, aspects of plumage, several morphological and behaviour traits, such as complex song or elaborate courtships are all possible secondary sexual characters and targets of sexual selection (Shutler & Weatherhead 1990). Many species can have more than one elaborated sexual selected ornament, and those can be honest indicators of individual quality since it implies costs of production or maintenance for the bearer (Zahavi 1975).

Females may benefit from mating with high-quality males because they can provide direct benefits, such as good breeding territory and paternal care, or indirect benefits, like good genes viability and attractiveness of their offspring (Andersson 1994; Iwasa & Pomiankowski 1999). Besides, females can have costs such as ornamentation evaluation, wherein the increase in the number of ornaments to evaluate, on one hand, implies the increase of costs, and on the other hand involves de decrease of gains in terms of information (Iwasa & Pomiankowski 1994); however, Candolin (2003) denoted that in some cases the presence of multiple ornaments can decrease the evaluation costs.

In birds, song and colouration are the most common signals of sexual selection, both can be targets of intra and intersexual selection, and have an informative content.

Song

Singing is one of the most common forms of communication used by birds. Song can be an innate trait or it can be learnt. Innate singing is typical of non-passerines and suboscines, is genetically determined and characterized by its simplicity and stereotyped structure (Slater 1997). On the other hand, oscines passerines learn and record complex song elements (Kroodsma & Byers 1991), where learning occurs essentially in the first stages of life.

In song it is important to distinguish between vocalisations or calls and song itself. Vocalisations are usually assigned to specific situations like when signalling danger, they can be performed throughout all year by both sexes and are structurally simple and short in terms of duration (Catchpole & Slater 2008). Songs are more complex and longer. Conceptually they can be divided into elements that form syllables that in turn form phrases (Catchpole & Slater 2008). Singing behaviour is essentially performed by males in the breeding season (Catchpole & Slater 2008), although females can also sing, for example, in tropical species (e.g. de Silva *et al.* 2004; Grafe & Bitz 2004; Sethi *et al.* 2012). In general, song can provide reliable information about several aspects like species identity, geographic origin, motivational state, reproductive situation, intention and individual identity (Catchpole & Slater 2008). It is assumed that song had evolved through two main selective pressures, intrasexual and intersexual selection. At this level, concerning intrasexual functions, song plays a role of territory defence and male exclusion, and concerning intersexual functions it is considered mate stimulation and attraction (Catchpole & Slater 2008). It is important to note that mate stimulation has physiological and behavioural implications that can affect the follicle growth, production of plasma luteinizing hormone (LH), nest building and egg laying (Kroodsma 1976; Mota 1999). Bird song, is supposed to provide a reliable indicator of male quality due to the fact that the development of brain nuclei that constitutes song learning occurs when young birds are energetically and nutritionally challenged (Nowicki *et al.* 1998). Moreover, birdsong depends on the ability to coordinate the diverse muscles of the vocal system, indeed respiratory muscles, by forcing the air to pass through the vocal organ, the syrinx, are providing energy for the song production and this process involves complex muscular contractions (Suthers *et al.* 1999; Gil & Gahr 2002).

Song has been considered a multifaceted signal with several attributes, which, over other measurements, repertoire size, song rate and duration are the most studied in the context of sexual selection (Catchpole & Slater 2008). It was demonstrated that these attributes or traits can involve different motor and neuromuscular necessities (Suthers *et al.* 1999; Suthers & Margoliash 2002), which make them potential quality indicators and thus targets of sexual selection (reviewed in Gil & Gahr 2002). Gil and Slater (2000), suggest that repertoire size is an indicator of male quality in the willow warbler, *Phylloscopus trochilus*, and target of female mate choice, moreover offspring's of these males have a greater probability of being recruited into the population. In other study, the reproductive success of the water pipits, *Anthus spinoletta*, seems to be related to a specific song element, the "snarr", which is a dominance signal in males, but not directly selected by female choice (Rehsteiner *et al.* 1998). Furthermore, in the serin, *Serinus serinus*, in a mate choice experiment, it was demonstrated that females have a clear preference for high frequencies, which can also eventually be a quality indicator signal (Cardoso *et al.* 2007). Given that, in relation to mate choice, one can affirm that preference for a particular song can have a certain impact on females and her offspring (Andersson 1994; Bradbury & Vehrencamp 1998).

Colouration

Colour perception

Vertebrates have the ability to see in a certain range of the spectrum of light and the majority can even form distinct and focused images, due to the structure of the eye (Yokoyama & Yokoyama 1996). The perception of light and colour vision is generally controlled by different photoreceptor cells, whose most relevant property consists on a photosensitive molecule that can be sensitive to different wavelengths of light (Yokoyama & Yokoyama 1996; Cuthill 2006). There are two types of photoreceptor cells, rods that function on dim light and cones that are responsible for colour vision. Colouration is particularly important in territorial and courtship displays, and is usually associated with movements and postures that enhance colour (Fogden & Fogden 1974). By comparison to humans, birds have a wider spectral range that includes the visible human spectrum and the ultraviolet spectrum (320-700 nm). They actually have more cone types in their retina, they are tetrachromatic contrasting with human trichromaticity, and possess coloured oil droplets that filter the light entering the cones. Simple differences in photoreceptors number or sensitivity lead to a differential colour perception (Cuthill 2006). Bird vision is based on four single cone types sensitive to the ultra-violet range (UVS), short wavelengths (SWS), medium wavelengths (MWS) and long wavelengths (LWS), and one type of double-cones (DB) that overlaps both MWS and LWS (Figure 2). Single cones are involved in chromatic tasks and double-cones to achromatic or non-colour tasks, also referred as brightness (Cuthill 2006).

Several studies have been focusing on the avian ultraviolet vision. Although some pigments produce additional UV reflectance peaks, it is known that structural colours are the ones predominantly expressed in the UV range. Adding to the fact that birds' spectral range includes the ultraviolet spectrum, it is likely that UV has also an important role of communication and sexual selection (Guilford & Harvey 1998). In fact, several studies with birds demonstrated the important role UV plays on mate choice, like in starlings (*Sturnus vulgaris*, Bennett *et al.* 1997), bluethroats (*Luscinia s. svecica*, Johnsen *et al.* 1998), budgerigars (*Melopsittacus undulates*, Pearn *et al.* 2001) and black-capped chickadees (*Poecille atricapillus*, Woodcock *et al.* 2005). Furthermore, Alonso-Alvarez *et al*. (2004) in a study with blue tits, *Parus caeruleus ultramarinus*, gathered evidence that UV signals affect the outcome of aggressive interactions between males, suggesting that these results are the first experimental evidence on UV acting on intrasexual selection.

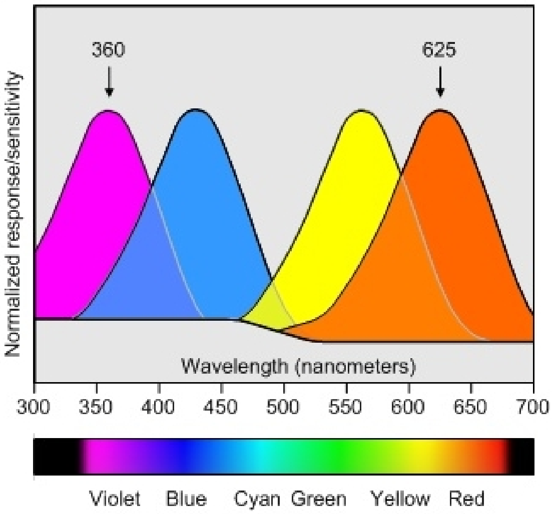

Figure 2 – Birds tetrachromaticity. Bird colour vision is based on four single cone types sensitive to UVS (violet), SWS (blue), MWS (yellow) and LWS (orange/red). DC, which is not represented in the figure, provides broad spectral sensitivity, overlapping MWS (yellow) and LWS (orang/red) (http://www.diycalculator.com).

Colour expression

In birds, colouration is the result of the expression of several chemical pigments (pigmentary colours), and physical interactions of light waves with biological structures (structural colours), and both of them contribute to the phenotype (Prum 2006). Structural colours are an important component of the plumage, eyes and skin and many of the most outstanding colours are produced by a combination of these with pigmentary colours (Prum 2006). In animals, pigments can be deposited in fat, skin, eyes or in contiguous structures like feathers (Olson & Owens 1998). The most prevalent pigments in the avian integument are melanins and carotenoids. Melanins are the most abundant and widespread pigments in birds and other animals. They promote several patterns that can be seen in plants, fungi and animals, and among vertebrates it is seen in all of the main types of integumentary structures (McGraw 2006b). The expression of black, brown, grey and rufous colours, just like buff shades, is dependent on melanins (McGraw 2006b). They are produced by the body and are related to aspects of condition (reviewed in Jawor & Breitwisch 2004). On the other hand the expression of yellow, orange and red colours is essentially due to carotenoids that confer colour in almost all invertebrate phyla, are common in all vertebrate classes (McGraw 2006a). Carotenoids have been involved in studies concerning signalling and sexual selection. Since vertebrates are not able to synthesize carotenoids de novo, the unique source of these pigments is through diet, directly consuming algae, fungi, plant parts and even ingesting preys, like insects, crustaceans or vertebrates that had also acquired carotenoids through their diet (McGraw 2006a). Because of that, carotenoid availability in nature can be a limiting factor (Andersson 1994). Moreover, some species are able to metabolically modify carotenoids structure after ingestion

(Stradi *et al.* 1995). Consequently, colouration can be constrained by physical condition (Pagani-Núñez & Senar 2011), since metabolic ability is probably related with body condition in general, instead of foraging ability and carotenoid availability, once those capabilities can change with age (McGraw 2006a). Taking into account the previous assumptions, the connection between diet and health makes carotenoid-based colouration, an attractive type of signal to the understanding the honesty-reinforcing mechanisms that cause costly but yet beneficial ornamental traits (McGraw 2006a). They perform a remarkable diversity of physiological roles in animals and provide a series of reliable sexual signals, like foraging efficiency, nutritional condition, body quality, body condition and immune defence (Blount 2004; McGraw 2006a). The first experimental evidence relating carotenoids and mate preference was performed with house finches, *Haemorhous mexicanus,* where females preferred males with redder plumage (Hill 1990). In European bitterlings, *Rhodeus amarus*, carotenoid-based colouration is a predictor of spermatozoa number and signals male fertilization efficiency, being a target of direct female choice (Smith *et al.* 2014).

Besides melanins and carotenoids, there are at least another five classes of uncommon pigments, porphyrin, pterin, psittacofulvin, flavin and undescribed pigments, responsible for colour that were detected in feathers, bare parts, shells and egg yolk (McGraw 2006c).

Colour measurement

Human eye is not a reliable quantifier of colouration in birds. To understand the signal value of bird colours we need to assess avian's perception of colours. Specific techniques for measuring colour in a quantitative way were adopted, being the most used now reflectance spectrophotometry. This technique can be achieved using a spectrophotometer to directly calculate reflectance spectra, in the perceptive range of bird vision. Data derived from spectrophotometry can be analysed by a variety of methods (Montgomerie 2006). One of the most used methods for colour analysis is the tristimulus colour variables (hue, saturation and brightness), which is based on the human visual system (e.g. Johnsen 1998). However it is unclear how close it is to the avian visual systems (see Evan *et al.* 2010). Nowadays, the visual models are preferred, since they allow the calculation of the photon catch of the cone recepetors of the species or group under study, providing an avian visual perspective. Visual models also take into acount irradiance spectra of ambient light and the spectral properties of incident light (Montgomerie 2006; Evans *et al.* 2012).

Multiple sexual ornaments

A wide range of animals have more than one sexual ornament, and the evolution for this multiple state is commonly attributed to pressures related to female mate choice and male-male competition (Darwin 1871; Andersson 1994). However, there are alternative explanations like spatially or temporally variable signal selection that may allow a stable coexistence between handicap signals (Marchetti 1998; Andersson *et al.* 2002). Indeed, it is predicted that females would evolve preference for a single ornament because, as said before, assessing multiple signals can be costly, and accordingly with this theory, multiple sexual ornaments should be evolutionary unstable (Moller & Pomiankowski 1993; Schluter & Price 1993; Iwasa & Pomiankowski 1994). Nevertheless, this has been proven to be a questionable matter, since the use of multiple signals may strongly influence on the strength of sexual selection and on the maintenance of variation (Candolin 2003). Therefore, there are several proposed hypotheses in order to explain the existence of multiple ornaments (see below), although the reason why some species possess more than one sexual ornament, and why do not invest in only one remains unknown (Candolin 2003). Some investigators consider that quantifying both male displays signals and a range of ecological and genetic factors is the way to increase the knowledge about multiple ornaments (Dongen & Mulder 2009).

The proposed hypothesis explaining the existence of multiple ornaments can be divided in two categories, those concerning the quality content and those concerning other issues besides quality content, which are of uncertain general application (reviewed by Candolin 2003) (Table I). Concerning the quality content, Moller and Pomiankowski (1993) suggested three different approaches, 1) the multiple message hypothesis, that comprehends that different ornaments indicate different quality aspects; 2) the redundant signal hypothesis, defending that different ornaments in combination are condition indicators; and 3) the unreliable signal hypothesis, which assumes that ornaments are not reliable indicators of overall condition, only maintained for an uncostly production and weak target by females preference. Beyond quality content, there are other possibilities to consider: 4) the transfer hypothesis, which assumes that multiple ornament is an unstable and transitory situation, and since ornaments have all identical value the constraint of an ornament leads to the elaboration of the other (Darwin 1871; Gilliard 1956); 5) the hypothesis of Fisherian cues, where certain traits are advantageous not because they provide information about quality, but because they are attractive, and that thus can result in genetic benefits (Fisher 1930; Pomiankowski & Iwasa 1993); and 6) the species recognition hypothesis, that entails the interaction between species recognition and mate-quality recognition that modulate mate-choice decisions, is important for understanding the diversification of sexually selected traits (Pfennig 1998). Also, 7) the chase-away model of sexual selection which comprehends the permanence of ornaments by past selection patterns (Holland & Rice 1998); 8) or evolution of multiple ornaments is due to sensory bias (Endler & Basolo 1998); 9) the receiver psychology, considering that multicomponent signals are easier to detect and because of its complexity they provide more reliable information for receivers (Rowe 1999). Continuing, 10) the multiple receiver hypothesis, that argues that different ornaments aim different receivers (Andersson *et al.* 2002); and finally, 11) differences related to environment conditions or distance leads to the existence of different ornaments, once individuals pay attention to different traits in different conditions (reviewed by Candolin 2003).

Table I - Hypothesis explaining the existence of multiple ornaments

	Hypothesis	Explanation	References
Quality content	Multiple Message	different ornaments indicate different quality aspects	Moller & Pomiankowski (1993)
	Redundant Signal	different ornaments in combination are condition indicators	
	Unreliable Signal	ornaments are not reliable indicators of overall condition	
Other than quality content	Transfer Hypothesis	multiple ornament is an unstable and transitory situation, and the constraint of an ornament leads to the elaboration of the other	Darwin (18719 Gilliard (1956)
	Fisherian cues	certain traits are advantageous not because they provide information about quality, but because they are attractive, and can result in genetic benefits	Fisher (1930) Pomiankowski & Iwasa (1993)
	Species Recognition	the interaction between species recognition and mate-quality recognition is important for understanding the diversification of sexually selected traits	Pfennig (1998)
	Chase-away model of sexual selection	the permanence of ornaments by past selection patterns	Holland & Rice (1998)
	Sensory bias	evolution of multiple ornaments is due to sensory bias	Endler & Basolo (1998)
	Receiver Psychology	multicomponent signals are easier to detect and provide more reliable information for receivers	Rowe(1999)
	Multiple Receiver	different ornaments aim different receivers	Andersson *et al.* 2002
	Different environment conditions or distance	individuals pay attention to different traits in different conditions	reviewed by Candolin (2003)

In the last two decades, increasing theoretical and empirical work has been made, regarding the interactions between different types of signals. Kortet & Taskinen (2004) highlighted the case of the roache, *Rutilus rutilus*, a fish species which possess two different aspects of sexual signalling, where the frontal head tubercles that signal condition and parasite load, and the lateral tubercles that denote parasite resistance. Concerning birds there is the example of the Northern cardinal, *Cardinalis cardinalis*, which plumage colouration is sexually selected, and while the red chest is positively correlated with body size and negatively with nestling feeding rate, the red from the beak indicates body condition and the size of the black mask is related to reproductive success (Jawor & Breitwisch 2004). Both roache and cardinal, confirm the multiple message hypotheses, where different sexual selected ornaments have different meanings. In the case of the great bustard, *Otis tarda*, the two secondary sexual traits of males, whiskers and neck plumage colouration, have been proven to be reliable indicators of age and weight, suggesting that multiple ornaments functions as redundant signals (Alonso *et al.* 2010). In a different case, Rivera-Gutierrez *et al*. (2010) demonstrated that in great tit, *Parus major*, the analysis of several different song components meet several multiple ornaments hypotheses. While some song components express a redundant signal, others meet the multiple message hypotheses, moreover there are even evidence that some of the song traits may provide unreliable information. This last situation meets the Iwasa & Pomiankowski (1994) arguments that unreliable traits, especially Fisherian cues, are likely to evolve along with traits that indicate viability. On the other hand, Andersson *et al*. (2002) were responsible for a study with the red-collared widowbird, *Euplectes ardens,* where it was demonstrated that the red collar is selected by male competition and the tail length by female choice, concluding that these two sexual ornaments aim different receivers.

Frequently, birds exhibit simultaneously, a large variety of acoustic and visual ornaments, which can increase the complexity of decisions related with mate choice (Moller & Pomiankowski 1993). For example, song is detectable at major distances in comparison to colouration, and consequently the evaluation of these two different traits implies distinct sensory and cognitive capacities (Cardoso 2005). Many studies have shown that ornaments are reliable signals of individual quality, like Reid *et al*. (2005) that showed in song sparrows, *Melospiza melodia*, that song repertoire size indicates inbreeding and immunity, and Kristiansen *et al*. (2006), that in a study with *Larus marinus*, the great black-backed gull, demonstrated that carotenoid colouration reflects individual quality. Furthermore, because of differences in production, maintenance and flexibility of these two signals, they are likely to express different information or are used in different signalling contexts (Moller & Pomiankowski 1993). Costs and physiological limitations constrain the evolution of song and colouration, but these same costs and limitations are the basis for signals of quality of the individuals (Zahavi 1975).

There are only a few studies on the relationship between song and coloration as sexual multiple ornaments. In a comparative study concerning trade-offs across Trogonidae family, Ornelas *et al*. (2009), showed that the relationship between these two traits was not significant, demonstrating that these traits are uncorrelated. On the other hand, Taff *et al*. (2012), found that song and colouration are related to different components of male fitness in the yellowthroat, *Geothlypis trichas*, assuming that they may convey redundant, or very similar, information since both are related to experience and survival of the individuals, even though in different contexts. In a study among 41 taxa of cardueline finches, Badyaev *et al*. (2002), verified that song complexity was particularly negatively related with the expression of carotenoid-based plumage ornamentation and unrelated with melanin-based colouration. Moreover, this last study provides evidence on how individual condition and environmental variation is likely to affect the expression and coexistence of sexual ornaments, suggesting the accomplishment of within-species studies concerning this issue.

More studies are clearly needed regarding the relation between different types of sexual signals, such as song and colouration, in order to understand the extent of information provided by ornament expression provides (Jawor & Breitwisch 2004).

Study Species

General features

The study species, *Serinus serinus*, (Linnaeus, 1766), the European serin, is a cardueline finch that belongs to the family Fringillidae. The serin is a small monogamous passerine, with approximately 11.5 cm, possessing a short conic beak, forked tail and long wings. Gregarious, non-territorial, serins forage or migrate in small flocks. This species is sexually dimorphic, particularly in their plumage, where males and females have a similar plumage pattern but the intensity and quantity of yellow colouration is much more evident on the male, mainly on the head, throat, and breast, while female show more intense brown stripes and little yellow if any (Figure 3) (Cramp & Perrins 1994).

The breeding season occurs in late February and July (Mota 1995), and this marks the period when males become more aggressive with each other and start to sing more intensively away from the group. The male courts the female and the nesting site is chosen by the female. The couple performs several breeding attempts, never reusing nests, and produces broods with usually 4 eggs (Newton 1972). Usually no more than one is successful due to high nest predation by jays (Mota 1995). Incubation is carried out by females and lasts about 12 to 14 days, and the young can stay in the nest for 14-16 days (Newton 1972; Mota 1995).

Feeding is based on seeds and other plant parts and rarely by small invertebrates. Very common in Southern Europe, the habitat consists on open forest areas, gardens, cultivated and urban zones (Cramp & Perrins 1994). In Portugal, the species is very common and has a conservation status of Least Concern, according to the IUCN Red List.

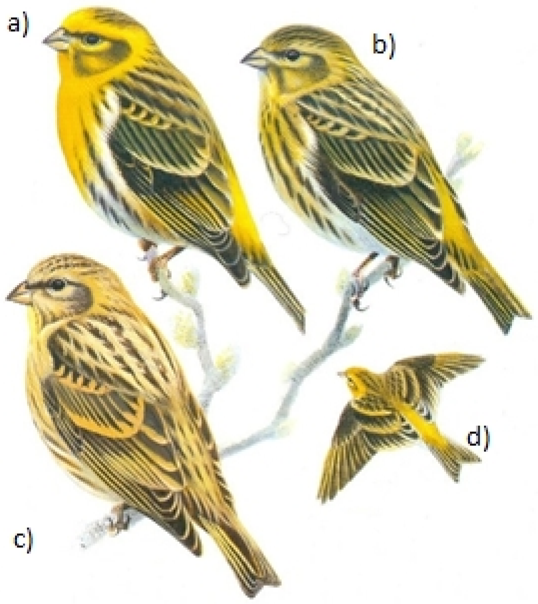

Figure 3 – *Serinus serinus* representation. a) adult male; b) adult female; c) juvenile; d) adult male in flight (adapted from Clement *et.al* 1993)

Song

In the serin only the male sings, and the song is characterized by its fast rates, enormous complexity and high frequencies. Actually, it is found to be one of the most complex and with the highest frequencies of the genus (Cardoso & Mota 2007), moreover, taking into account body weight, the serin song is considerably higher than expected (see Wallaschlager 1979; Mota & Cardoso 2001). It is known that producing high frequencies requires a major muscular contraction, which suggests that singing at high frequencies is a quality indicator, in the case of the serin, of male quality (Suthers *et al.* 1999). In fact, Cardoso *et al*. (2007) demonstrated that females showed preference for high frequency songs in the serin, which supports the previous idea. However, it was not revealed any correlation between body size and spectral song parameters (Cardoso *et al.* 2008). The repertoire size is considered to be large, with 40-87 syllables and is delivered in a rigid way with few structural modifications (Mota & Cardoso 2001), presenting no significant changes with age (Mamede & Mota 2012).

The serin song is composed by two different syntaxes, the repetitive syntax (trills), and the sequential syntax, that is predominant (Figure 4). In the repetitive syntax or trills, occurs the repetition of the same syllable a variable number of times, and usually takes place in the beginning of the song but it can also be found in the middle. The sequential syntax, comprehends the production of syllables in a very organized and determined order (Mota & Cardoso 2001). Male serins sings intensively through all breeding season, beginning some time before reproduction and pairing, and ending only when breeding attempts cease (Mota 1995).

The main functions of song in this species are female mate choice (Cardoso *et al.* 2007), reproductive stimulation (Mota 1999; Mota & Depraz 2004), and there are indications it will also in function in male-male aggression (Mota 1999; Cardoso *et al.* 2007). In this context, mate guarding is not considered a direct function of song, but it has been registered a major number of extra-pair copulations attempts in couples that the male preformed less song flights, and this infers some meaning to song in relation to mate guarding (Mota 1999; Mota & Depraz 2004).

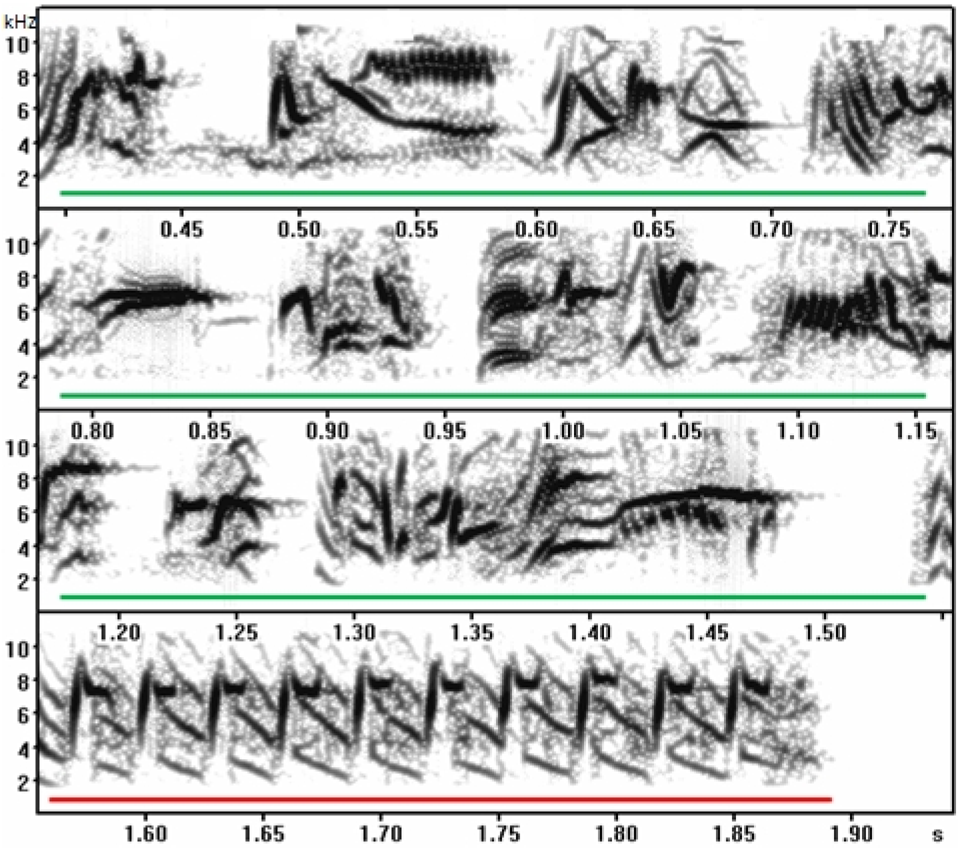

Figure 4 – Spectrogram of part of a serin song. The green line represents the sequential mode that is predominant, and the red line the repetitive mode, with one trill.

Colouration

The typical yellow colouration of the serin is due to the expression of carotenoids, essentially those named “canary xanthophylls” and lutein (Stradi *et al.* 1995). As a dimorphic species they are expressed in males, although some females also have some signs of yellow. Yellow colouration expression is affected by carotenoid intake through diet. Consequently it is expected that more colourful males had a larger carotenoid intake. Studies have already proven that serin females have a sexual preference for males with a more saturated yellow colour, *i.e.*, a major percentage of carotenoids (Leitão *et al.* 2014). Furthermore, in a manipulative diet experience, individuals that were also supplemented with carotenoids enhanced their plumage saturation and also exhibited a higher immune response, inferring about carotenoide-based colouration plumage as an honest quality signal in this species (Trigo *personal communication*). It is also known that UV wavelengths are an important component of visual information and relevant to female mate choice, since UV blocking reduced male attractiveness (Leitão *et al.* 2014).

Objectives

The Serin is a species with two major signalling traits involved in sexual selection, song and colouration, being a strong candidate for testing hypothesis concerning the evolution of multiple sexual ornaments. The main objective of this study it is to clarify the presence of multiple ornaments in male serins, checking for the type of linkage between them and the characteristics that they are signalling. This will be assessed in light of the three proposed hypothesis for the evolution of multiple ornaments in relation to quality content, multiple message, redundant signal and unreliable signal hypothesis. Thus, we aim to understand if 1) song and colouration signal different information content; 2) song and colouration are expressing a redundant signal; and 3) song and colouration do not signal any specific traits. Taking into account the roles and studies about song and colouration signalling, in order to accomplish this objectives it is intended to determine which song and colouration variables are associated with individual quality traits. The four quality traits considered for the present study were body condition, body size, immune response and response to stress.

Concluding, the working hypothesis then are to assess if, 1) the expression of song and colouration reflect individual body condition; 2) the expression of song and colouration reflect body size; 3) the expression of song and colouration reflect immune response and 4) the expression of song and colouration reflect the intensity of a physiological response to stress.

Chapter 2

Material and Methods

Birds were captured using mist nets, in November 2013 and from January to March 2014, in cultivated and uncultivated areas around Coimbra, specifically, Pereira (40°10'48.15"N-8°35'2.63"W), Anobra (40°8'54.72"N-8°31'41.00"W) and Arzila (40°11'0.04"N-8°33'4.99"W). After capture birds were transported immediately to an indoor aviary in the laboratory. The experiments were carried out between March and May 2014, in the Laboratory of Ethology of the University of Coimbra, Portugal. They were then, sexed based on plumage and banded with numbered black plastic rings (A. C. Hughes), for posterior individual identification. During the experiments, the birds were kept in single sex group cages, to a maximum of five individuals per cage, in the aviary of the Department of Life Sciences. The individuals were kept at room temperature with natural and regulated photoperiod, with *ad libitum* access to a commercial seed mixture and tap water. Individuals were release in the end of the experiments.

All values are reported as mean ± standard error (SE).

Morphometry

Morphometric parameters were taken a few days after capture. The measurements taken were weight, measured with a pesola, (±0.5g), wing length measured with a ruler (±1mm), tarsus length, tail length and width and depth of the beak measured with a calliper (±0.01mm). Age, first year or older than first year, was determined as described by Svensson (1992). All measurements have high repeatability ($0.64 \leq r \leq 0.91$; $4.6 \leq F_{(18,18)} \leq 22.26$; $0.001 < p \leq 0.002$).

An index of individual body condition was obtained as the residuals of a linear regression of body weight on tarsus length (Brown 1996), which we denominate body mass index (BMI). In order to be relevant to song recordings analysis, the measure of body weight was taken closer to song recordings. Principal Component Analysis (PCA) was performed on wing length and beak width and depth, in order to produce a body size index taken from the first component (PC1). PC1 explained 45% of the variation in body size, where wing length loaded negatively on the component (-0.075), while beak width and depth loaded positively (0.825 and 0.817 respectively).

Song

Stimuli preparation

In order to stimulate male serins to sing individually in captivity, *i.e.*, isolated acoustically and visually from conspecifics, we prepared song stimuli for playbacks. These stimuli were based on serin's subsong recorded in the winter of 2013 and from previous years. We tested a sub-sample of males for active singing in conditions of captivity and isolation, which would permit good recording conditions, in the spring of 2013. Males were subjected to a series of different stimuli in order to determine which were more efficient in eliciting male singing. This small preliminary experiment showed that male subsong was the best stimulus. Stimuli recording for playbacks was performed with a portable digital recorder Marantz Professional PMD661 MKII (Marantz, Kanagawa, Japan), connected to a directional long shot microphone Sennheiser MKH 70 P48 (Sennheiser, Wedermark-Wennenbostel, Germany), with a wind protection. All recordings of more than one minute of consecutive singing of subsong would be considered for stimuli production. They were extracted directly from the sound files, of solo individuals or in groups. Selected recordings were converted to a 24000 Hz frequency and manipulated in Avisoft-SASLab Pro (Version 5.2.06, R.Specht, Glienicke, Germany), in order to normalize the volume (90%) and to eliminate low-frequency noise applying a high-pass filter at 1.5 kHz, when necessary. In whole, it was used one stimulus, with amplitude at 1 meter set at 58 dB, using a Tenma RS-232 72-860A Sound Level Meter (Tenma, Taipei, Taiwan), that was emitted in wav format. The unique purpose of stimuli production was to motivate birds to sing, without constituting a defying signal, which was accomplished by exposing them to a non-structured singing (subsong).

Song recording

A total of 21 individuals were recorded individually in a sound-attenuation chamber, in the laboratory, lined with foam and sound treatment panels (Cinema Round 120.7 Premium, Vicoustic, Paços de Ferreira, Portugal), to maximize acoustic efficiency and avoid echo and reverberation. Recordings were carried out in March and April. Males were stimulated with a subsong playback, followed by 10 minutes of silence, during 07:30 hours. Spontaneous solo singing of each bird was recorded during that time. For acclimatization to the room, males were housed in the acoustic chamber in the evening of the previous day of recordings. They were provided with *ad libitum* to food and water. In the following day, the recordings took place from 08.00 am to 03.30 pm, with the same recording equipment used for stimuli recordings (see above). The digital recorder was connected to a directional microphone placed in the centre of the chamber and just above the only perch in the room. The stimulus was emitted, through a Sony Walkman digital media player NWZ-E453 (Sony, Tokyo, Japan) with 3.35 GB, and a loudspeaker Sony SRS-A57, placed in a tripod 87cm from the floor in a corner of the chamber. Moreover, the loudspeaker was directed to the perch, at about 1 meter of distance from the microphone (Figure 5). The volume of both the digital media player and the loudspeaker was set so that the normalized playback sounded realistic in the test room, as perceived by the human ear. The floor was covered with sawdust to avoid floor reverberation and to be suitable for the birds. Individuals that did not sing in the first round were subjected to a second round, after the first ended. Overall, 5 individuals did not sing in any of the rounds, 8 individuals sung in the first round and 8 in the second.

In order to calibrate all the recordings before analysis, a calibrated sound with a standardized frequency, amplitude and volume was emitted and recorded with the same equipment, at the end of each recording session. The corresponding sound pressure level (SPL), at the level of the microphone, was recorded using a sonometer.

All song recordings were also video recorded with a time lapse of 20 seconds between them. The distance and orientation of the subject to the recording device was controlled by the microphone position and video images.

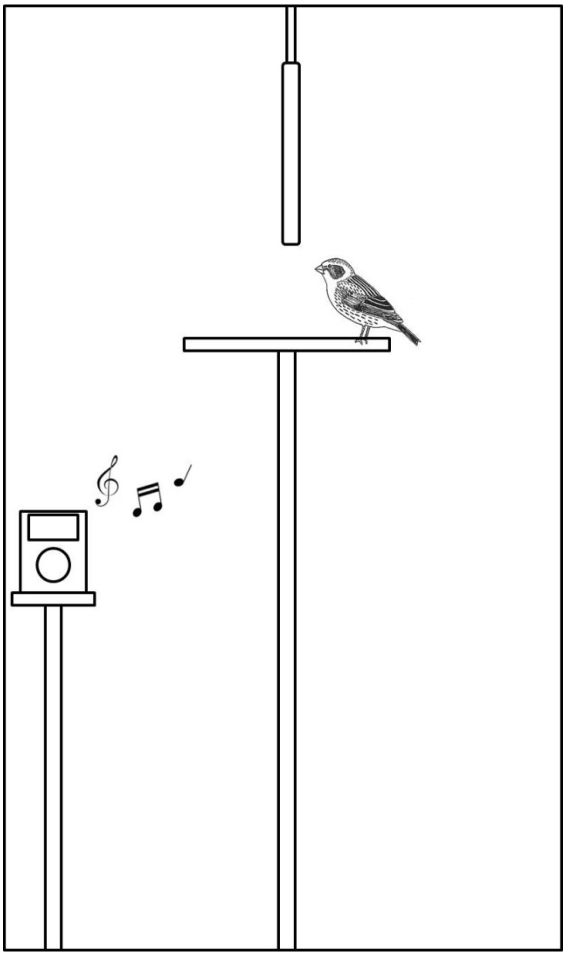

Figure 5 – Representation of the song recording setup. Frontal plan of the acoustic chamber (2.30 x 1.12 x 1.36 m), where serin's song was recorded. The loudspeaker is at 0.87m from the floor, and oriented to the perch at 1m from the microphone. The perch is at 1.46m high placed in the centre of the chamber. The microphone is just above the perch at 0.50 m distance.

Song analyses

We analysed the song performances of 16 serins, in a total of 120 hours of recordings and 320 songs, with Avisoft-SASLab Pro (Version 5.2.06, R.Specht, Glienicke, Germany). Each recording was converted to a 24000 Hz frequency and it was applied a high-pass filter at 1.5 kHz and a low-pass filter at 11 kHz, since serins have a frequency range that varies between 2.02 kHz and 10.74 kHz (Mota & Cardoso 2001). All sound files were calibrated before analysis using the reference song SPL previously registered. All song spectrograms were made with the following parameter specifications: Hamming window, FFT length=256, frame size=50%, and overlap=93.75%, resulting in a 244 Hz bandwidth, 94 Hz frequency resolution and 4.1ms time resolution. Twenty songs were selected per bird, fifteen were analysed for song measurements and five for syllable measurements. Song measurements were taken from the longest songs produced by each individual, randomly selected from the entire sound file, with an average duration of 6.1±0.87s. Syllable measurements were taken from songs with average duration of 3s (3.02±0.04), which is considered to be the average size of songs in the serin (Mota & Cardoso 2001). All songs and syllables were labelled by hand with a marker cursor. From each song it was extracted the values of peak frequency, maximum and minimum frequency, peak amplitude and peak amplitude of the sequential and repetitive modes. From syllables it was extracted the syllable duration and also the duration of the respective song. Frequency is the number of cycles per second, indicating how high or low pitched the sound, it is measured in thousands of cycles per second or kilohertz (kHz), as peak frequency is the frequency of highest intensity (or volume). Amplitude represents the height or volume of the sound waves, measured in decibels (dB). All frequency measurements were registered using the power spectrum of the spectrogram with a -24 dB threshold, while the other measurements were obtained by the automatic parameters measurement tool. The variables considered for analysis are the mean of the measures per individual.

Moreover, the proportion between syllable duration on song duration was used to define the sound to silence ratio, which consists on the proportion of sound in relation to the real duration of the song.

Colour assessment

Male serins colouration was measured in 19 males, in April, right after the tests on song recording. A spectrophotometer Ocean Optics USB4000 (Ocean Optics, Dunedin, FL, USA), was used with a light source with two lamps of deuterium and halogen (Mikropack Mini-DT-2-GS, UV-VIS-NIR Lightsource), that emitted light covering the range of 300nm and 700nm, which includes the range of vision of passerines (320nm and 700nm) (Cuthill 2006). Measurements were taken with Y shaped optical fibre (optical cable OceanOptics R400-7 UV/VIS). The probe was attached to a rigid protection that excluded external light and kept the probe at a fixed distance of 3mm and perpendicular to the surface of the feathers. The sampled area was of 28mm^2 approximately. All measurements of the spectrum are expressed in the proportion of light relative to a white standard (Ocean Optics, WS-1-SS White Standard). Three random measurements of four areas of yellow (crown, throat, breast and abdomen) were made and the average was considered to the analysis. The values resulted from spectrophotometry were used to calculate cone quantum catches for achromatic (double-cone) and chromatic (UVS, SWS, MWS, LWS ratio), in accordance with a model of avian colour vision, that corresponds with the current understanding of avian visual processing (Osorio & Vorobyev 2005; Evans *et al.* 2010; Evans *et al.* 2012), obtaining an avian visual perspective (Figure 6).

Double-cone is the estimated quantum catch of the double-cone, calculated using data from the blue tit, because of limited availability of double-cone sensitivity data for other species, including the serin. Because the natural context of the individuals is important and the environment is expected to be variable, we used the standard daylight (D65) as a measure of the incident lighting spectrum (Delhey *et al.* 2010; Evans *et al.* 2012). SWS ratio was a measure proposed by Evans et al 2010 which is particularly adequate for species with carotenoid colouration, since it compares the quantum cone catch for the SWS single-cone to the mean quantum cone catch of the other 3 single-cones UVS, MWS, and LWS:

SWS ratio = 3^{-1}(UVS*MWS*LWS)/SWS

As the serin has tethracromatic and UVS vision, it was also chosen the blue tit to model serin’s vision to be in accordance with the previous delineations, and also because this species is commonly taken as a representative of UVS vision (Håstad *et al.* 2005). The resulting variable was logarithmically transformed in order to represent normal distribution. Previous studies have demonstrated that both plumage reflectance indices are substantially independent (Evans et al 2010), where double-cone is assumed to process brightness (Osorio & Vorobyev 2005; Hart et al 2000), as SWS ratio reflects saturation (chroma) (Evans et al 2010). For this propose, it was used *pavo*, an R package for the analysis, visualization and organization of spectral data (Maia *et al.* 2013), ran in R (ver. 3.1.0, R Developement Core Team), with RStudio interface.

For further analysis, a PCA was performed on both double-cone variables and SWS ratio. PC1 double-cone explained 37% of the variation in brightness, where abdomen values loaded negatively on the component (-0.279), while crown, throat and breast loaded positively (0.852, 0.749 and 0.330

respectively). SWS ratio PC1 explained 47% of the variation in saturation, where all variables, abdomen, crown, throat and breast, loaded positively on the component (0.640, 0.834, 0.202 and 0.864 respectively).

It was also measured the extent of yellow in the breast by capturing an image of the ventral side of the individuals, against a metric scale background (millimetre paper), with repeatability ($r=0.42$, $F_{(18,18)}=2.54$, $p=0.04$). Photographs were taken in a photography room, using a Canon EOS 350D digital camera with flashlight. Through an image processing software, ImageJ (imagej.nih.gov/ij/), it was delineated a contour fitting the yellow extent of the breast. Area was automatically computed, in an average of 3 measures, given in cm^2.

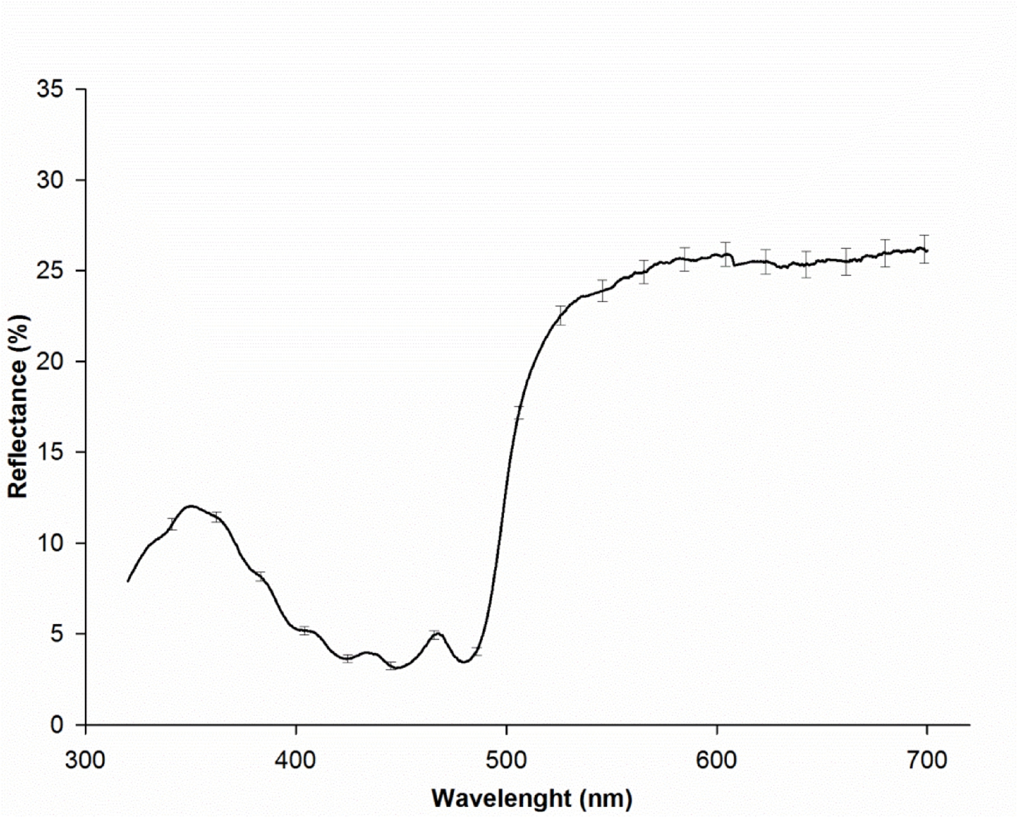

Figure 6 – Reflectance spectrum from the yellow carotenoid-based plumage of the serin. Mean ± SE of data average from abdomen, crown, throat and breast (N=19).

Immune response

As mentioned before, immune function can be a measure of individual quality and condition. Phytohaemagglutinin (PHA) skin-swelling is one of the most used immune challenges in studies of immunocompetence in birds (Biard *et al.* 2009). This challenge consists on the subcutaneous injection of a plant (*Phaseolus vulgaris*) lectin provoking thickness of the skin, which reflects the combined responses of T-cells, cytokines and inflammatory cells (Davison *et al.* 1996), which involves both innate and adaptive components of immunity (Martin et al, Tella et al 2008). PHA first induces an acute response, approximately 4 hours after injection, and then a delayed type hypersensitivity response, that as a peak 18 hours after injection and usually lasts up 36 hours, given this, the skin swelling is generally measured 24 hours after injection (Biard *et al.* 2009).

All subjects (n=17) were injected, in the right wing patagium, with 20µl PHA (PHA-P L8754, Sigma) dissolved in PBS, on 29th April in the morning, between 09.00 am and 12.00 am (Smits *et al.* 1999). With a calliper (±0.01mm), we measured the thickness of the injection site just before injection, and 24 hours later it was measured again in the same site, where a swelling was formed. The index of the immune response is given by the subtraction of the averages of three thickness measures pre-injection to the values post-injection, measurements were taken by a single researcher. Two individuals were excluded from analysis for measurement irregularities (n=15).

The resulting variable was logarithmically transformed in order to represent normal distribution.

Response to stress

Breath rate (BR) is considered to be a physiological response to stress (Carere & van Oers 2004), and eventually can predict individual quality. In this study breath rate was quantified by handling stress. Since breath rate has been shown to be higher right after capture (Carere & van Oers 2004), subjects were kept 5 minutes in a cotton bag, after capture in the cage, in order to normalize their respiratory rate. After respiratory normalization, breath rate was measured as the number of chest movements during 30s (n=17), registered with a mechanical tally counter and a countdown stop watch (Torné-Noguera *et al.* 2014), while firmly keeping the bird in the hand. Two trials were made in different days for more accurate results, 5th May and 12th May, between 09.00 am and 12.00 am. The measurements were consistent revealing high repeatability (r=0.82, $F_{(16,16)}$=10.287, p<0.001). The index used was the average of the 2 measurements.

Statistical analysis

Data was analysed using SPSS software (ver. 22.00, IBM SPSS Statistics®, Armonk, New York, USA) for Windows. Values are reported as mean ± SE and all probabilities are two-tailed. Al variables presented homoscedasticity, and it was performed a logarithmically transformation on the data that was not normally distributed before inclusion into statistical models, except for maximum frequency of the song. Hence, correlation matrices were constructed between song variables and colouration variables for overall visualization of the data

(Appendix I – A and B, respectively). Samples sizes vary among statistical tests because some measurements were missing in some individuals.

The rationale for the analyses was that signals such as song and colouration can be predictors of the condition of animals if they actually are signalling individuals' condition. Thus, to assess which song and colouration variables would best predict the four individual quality traits referred before (body condition, body size, immune response and response to stress), four distinct stepwise backward multiple linear regression analysis were performed, where colour, song and age variables were considered as predictors. Collinearity between the predictor variables was previously assessed, building correlation matrices, and variables significantly correlated in pairs were removed. The potential predictor variables used in each of the four models were: peak song frequency and minimum song frequency (for song), breast extension, PC1 SWS score, PC1 DC score (for colour) and age ($p \leq 0.05$ for inclusion). Data for amplitude variables was excluded from the analysis because of high variability between measurements (Appendix I – C). Through visual inspection of the recorded images, it is possible to infer that individuals sing at different distances, in different positions and orientation of the microphone, which implies large differences in amplitude values, invalidating the data. By performing a backward procedure, it was possible to assess if combined variables, which individually would not be included in the model, could predict quality traits.

In all tests the level of statistical significance was 0.05.

Chapter 3

Results

All the models of the backward stepwise regressions, assuming body condition as the dependent variable, were significant. An inspection through all the models suggests that age has an important role in predicting body condition, once it is a significant predictor in the majority of the models (Table II, Figure 7). However, the most significant model that demonstrates which are the variables with more predictive power, highlights the colouration variables (F=7.605, p=0.015).

Table II – Significant predictors of *Serinus serinus* body condition. Significant variables backward stepwise multiple linear regression models predicting body condition.

Model	Predictors	β'	p	Model		
				R^2	F	p
1	Age PC1 SWS ratio	-0.471 -0.671	0.045 0.013	0.732	4.092	0.029
2	Age PC1 SWS ratio	-0.490 -0.645	0.043 0.017	0.512	4.145	0.027
3	Age PC1 SWS ratio	-0.498 -0.463	0.049 0.048	0.445	4.005	0.030
4	PC1 SWS ratio	-0.567	0.017	0.393	4.232	0.029
5	PC1 SWS ratio	-0.613	0.012	0.333	4.739	0.028
6	PC1 SWS ratio	-0.593	0.015	0.306	7.605	0.015

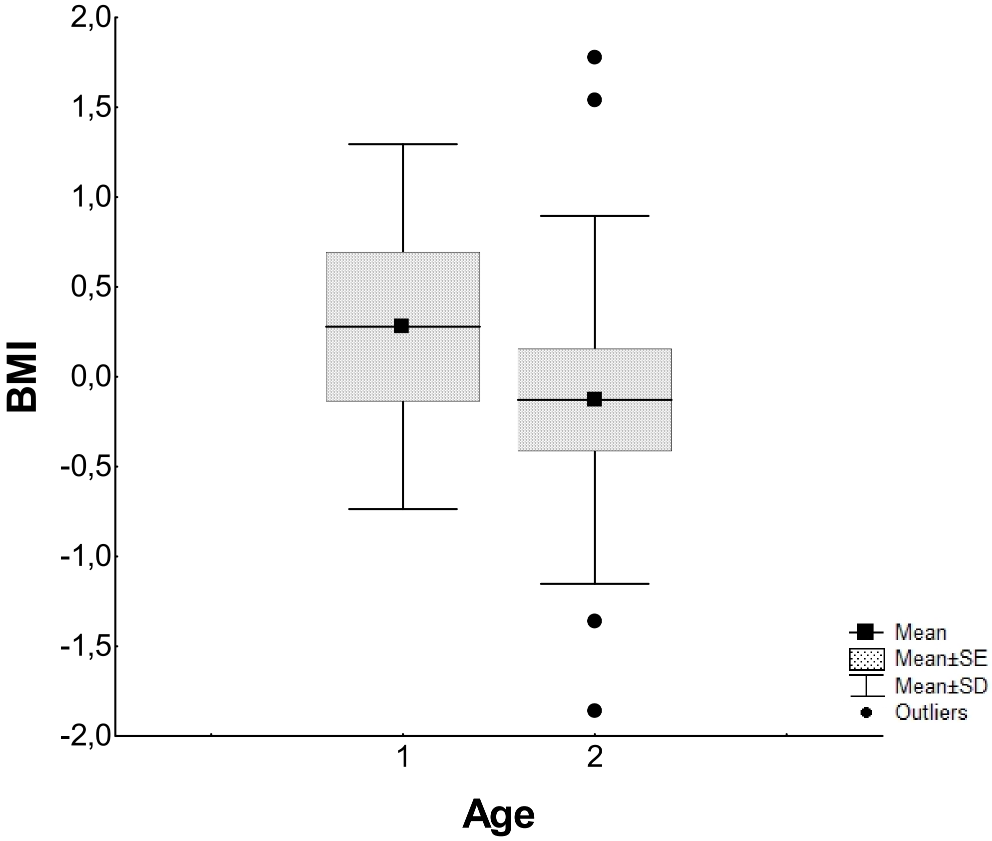

Figure 7 – Body condition (BMI) values for first year individuals (1) and adults (2) in *Serinus serinus.* The first plot represents first year individuals (mean=0.28, SE=0.38, SD=0.93), and the second plot represents older than first year individuals (mean=-0.13, SE=0.27, SD=0.98).

Given that, colouration expression is intricately associated with body condition in the serin, as male saturation (PC1 SWS ratio) is significantly negatively related with body mass index (p=0.015) (Figure 8, Table III).

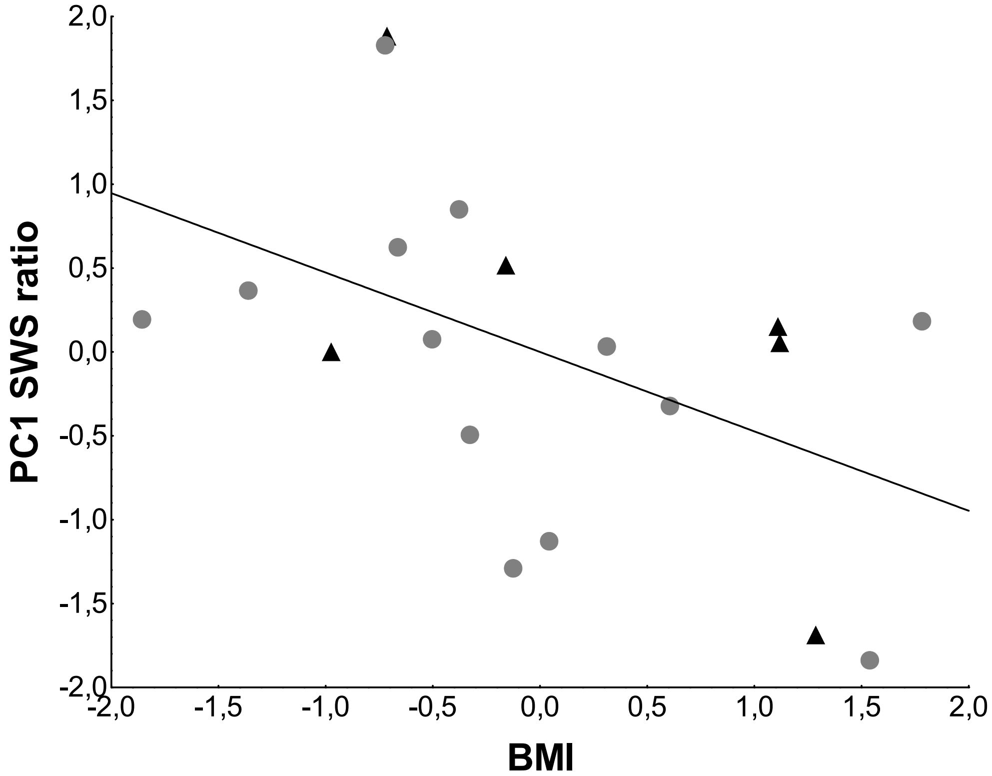

Figure 8 – Relation between plumage saturation (PC1 SWS ratio) and body condition (BMI) in *Serinus serinus.* The line represents the regression line, the black markers represent first year individuals and grey markers older than first year individuals.

Age is a significant predictor of body size (Figure 9, Table III) in a model that is marginally significant (F=3.773, p=0.051). Also minimum song frequency enters in the model, and although is not significant (p=0.079) it seems to show an effect on body size.

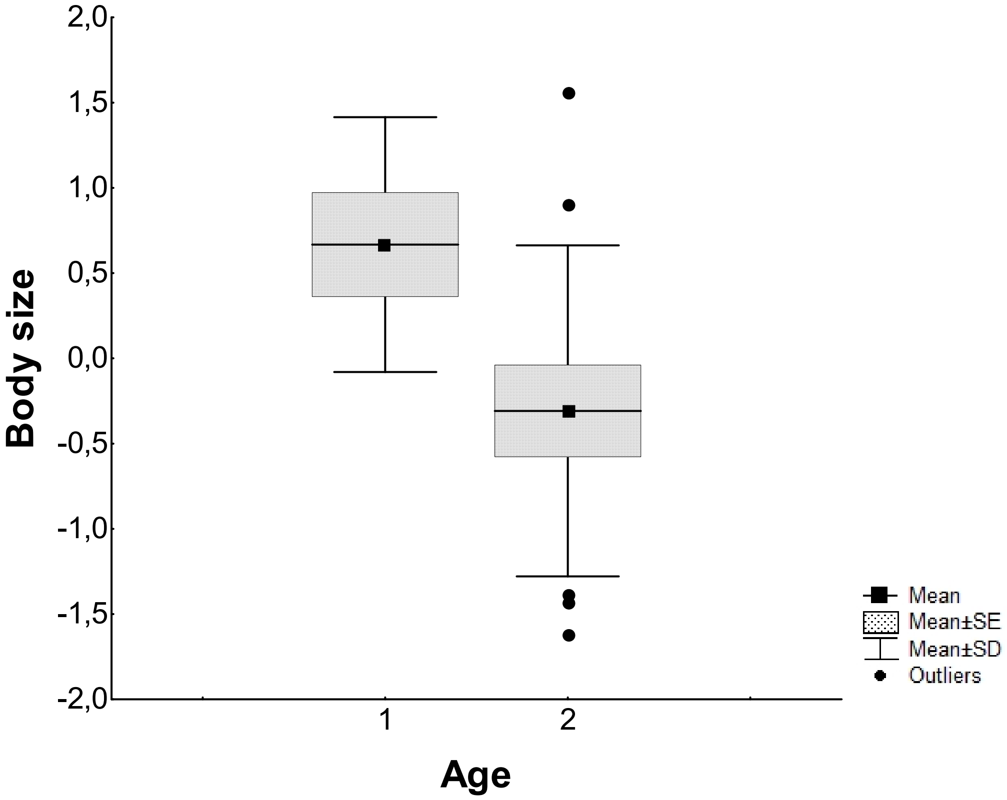

Figure 9 - Body size values for first year individuals (1) and adults (2) in *Serinus serinus*. The first plot represents first year individuals (1) (mean=0.67, SE=0.28, SD=0.68), and the second plot represents older than first year individuals (2) (mean=-0.31, SE=0.26, SD=0.93).

For immune response, mediated by the PHA-P immune challenge, none of the song and colouration independent variables entered in the multiple linear regression are significant predictors (Table III). Also, none of the variables showed to be a significant predictor of the physiological response to stress mediated by breath rate. However, minimum song frequency appears to have some relation with the parameter, since the song variable was marginal to significance (F=3.551, P=0.084) (Table III).

Table III – Backward stepwise regression models which best predict *Serinus serinus* individual quality. Backward stepwise multiple linear regression models predicting individual quality traits (body condition, body size, immune response and physiological response to stress). Only the most significant model for each trait is presented.

Dependent variable	Predictors	β'	p	Model		
				R^2	F	p
I. Body condition	PC1 SWS ratio	-0.593	0.015	0.306	7.605	0.015
II. Body size	Minimum frequency Age	-0.429 -0.519	0.079 0.038	0.367	3.773	0.051
III. PHA-P	*none*					
IV. Breath rate	Minimum frequency	-0.478	0.084	0.228	3.551	0.084

Chapter 4

Discussion

Colouration expression is significantly related to body condition, as saturation relates negatively to body condition index. Song measurements do not predict any quality trait, however minimum song frequency is marginally negatively related to body size and breath rate. Moreover, age is related to body condition and size. These results suggest that the expression of multiple ornaments in the serin may deliver information on different aspects of male quality.

Body condition

As predicted, colouration expression is intricately associated with body condition in the serin. It is known that carotenoid-based-colouration is generally an honest signal of individual health and quality, since it is considered a condition-dependent trait providing information about the condition of the bearer (Hill 2006). In a companion study on northern cardinals, using the same body condition index, bill colour predicted current body condition, with birds with redder bills in better condition (Jawor & Breitwisch 2004), furthermore, Doutrelant *et. al* (2013) also demonstrated that indices of colouration were positively linked to estimates of condition in a study with Atlantic puffins, *Fratercula arctica*, and blacklegged kittiwakes, *Rissa tridactyla*, where redder gapes (both species) and bill (puffins) are indicators of better condition. Given this, it was expected that individuals with more conspicuous colours would be in better body condition, however, results reported here demonstrate the opposite scenario. The findings demonstrate that in male serin plumage saturation is negatively related with body condition, *i.e.*, individuals with lower values of body condition presented more saturated colouration. Body condition indices are supposed to reflect various aspects of the animal’s quality including health, nutritional status, and fat content (Brown 1996), but, it is known that body fat can vary considerably in a season within individuals. Thus, body condition indices must be carefully used (Labocha & Hayes 2012). There are circumstances, however, where heavier weight is not an advantage, and is likely to be a burden. Flying birds have to deal with a very strong constraint: they have to fly; and being heavier means spending more energy to travel. If for migratory birds, accumulation of energy before migration is very important, that is not the case in non-migratory birds. Thus, there must be an optimum weight for a flying bird that balances the need for body reserves and muscle tissue with the best flight capacity and manoeuvrability. In studies on starlings, males reduced their angle of ascent at take-off (Witter *et al.* 1994), and in females both speed and angle of ascent were reduced with weight increases (Lee *et al.* 1996). Furthermore, flight performance and the ability for fast take-off are crucial in determining whether a small bird will survive an attack by a predator (Witter & Cuthill 1993). Actually, an increased predation risk due to reduced flight ability with enlarged fat reserves has been predicted in several theoretical papers (McNamara & Houston 1990; Bednekoff 1996). Considering flight performance and increased body mass, will result in more energy losses in order to accomplish flight. An indication that weight might be a strong constraint on flight is the interesting study performed by Andrews *et al.* (2009) on 74 birds of the order Passeriformes, which showed a relation between genome size and nucleus/cell size, and also between genome size and wing loading index, supporting the hypothesis that that flying birds possess a smaller genome size. Flight performance has indirect implications on serins breeding behaviour, which makes weight such an important trait to consider. They court female both singing and showing their colouration, typically from tall exposed trees and from overhead wires, performing regularly song flights over and beyond the territory (Cramp & Perrins 1994). And more important, they must fly for considerable distances in order to feed (Mota 1995).

Alternatively, increases in body weight might also be related to metabolic efficiency (Hill 2006). It is important to consider that metabolic efficiency and foraging abilities vary between individuals, causing discrepancy in body mass. It can occur that some individuals necessarily have to increase food intake in order to compensate for metabolic failure. It is also known that foraging ability can change with age (Hill & Montgomerie 1994; McGraw 2006). In fact, carotenoid traits might be indicators of foraging efficiency because only good foragers would be able to acquire more carotenoids than their less efficient conspecifics, and therefore express more intense colouration (Hill & Montgomerie 1994). However, the ability to synthesize these carotenoids may be more closely related to the birds' general body condition than to their ability to locate and ingest carotenoids (Hartley & Kennedy 2004; Hill & Johnson 2012). In the case of the serin, it could be suggested that because birds were provided with food *ad libitum* in captivity that might have affected their weight. But, all weight measurements taken throughout the season, starting on capture, had a strong repeatability.

In previous studies it was shown that body condition relates with age in the serin, and suggested that older individuals are in better condition (Conroy *et al.* 2002), which is the opposite from that described in this study, were younger individuals appear in better body condition. However, we had a small sample and quite unbalanced age groups: only 6 individuals in 19 were first year. This renders inferences on age complicated, with our data.

Body size

It was expected that age would be related with body size in the serin, however it was not predictable that this would be a negative relation. Older individuals are reported here as possessing lower values of body size, and first year individuals higher values, which is not consistent with observations and growing rates. For the previous pointed reasons, inferences on age must be carefully considered.

There were no significant results relating song measurements and body size. Only, a tendency for minimum song frequency to be negatively related with body size was found. In many species the frequency of vocalisations reveals information on body size or mass (Bradbury & Vehrencamp 1998; Amorim & Hawkins 2005; Searcy & Nowicki 2005; Mager *et al.* 2007). The notion is that larger organisms involve larger vocal organs and vocal tracts that produce and emit lower frequency sounds more efficiently (Bradbury & Vehrencamp 1998). This study shows a negative relationship between minimum song frequency and body size in the serin, so that larger individuals have tendency to produce songs with lower minimum frequencies. Although not significant, this tendency goes against Cardoso *et al.* (2008) findings, where none of the frequency traits in serins song was related to body size. However, for several times it was reported negative associations between frequency and body size in many animal species, including birds (Ryan & Brenowitz 1985; Bennet-Clark 1998), which is consistent with the present results. A review of the literature on the relationship between the frequency of avian vocalisations and body size showed that simpler vocalisations, like calls, have a more significant relation with body size than more variable vocalisations, like songs (Patel *et al.* 2010). Taking this into account it might be interesting to consider for analysis serin's simpler vocalisations then songs. However, Cardoso *et al.* (2008) argued that in this species there is not an obvious way to receivers assess the body size of singing males from the frequency of their songs. There are two important facts to consider here. Firstly, in the Cardoso *et al. (2008)* study it was used a larger sample size the used sampled size is evidentially larger, n= 48. Secondly, all the individuals were adults with well-structured songs, while in our sample of 19 individuals a part of them was first year. It is also important to attention to the fact that individuals can change the frequency of their vocalisations due to variation in motivational states and cultural learning (Osiejuk *et al.* 2005). Furthermore, theory predicts that when differences in size among adult conspecifics are so small that finding a relation between size and frequency is particularly challenging (Fletcher 2004).

There are other song parameters, like amplitude, repertoire size or trill rate that should be analysed in this context. It was our intention to have analysed them in our study. For reasons already referred, it was not possible to consider amplitude measurements for the majority of birds, in spite of the controlled conditions. It was also not possible to analyse the repertoire size of each bird, because it requires very long analysis.

The vocal amplitude of a song element is given by the air pressure in the bird's air sacs, which is assembled by the abdominal expiratory muscles (Oberweger & Goller 2001; Brumm & Todt 2002). As the size of these muscles is limited by the size of the bird's body, it is possible that amplitude acts as an honest signal of body size. The potential of song amplitude as an indicator of individual quality or condition is real when the great inter-individual variation in this signal is considered, as it has been shown in several bird species (Brumm & Todt 2002; Brumm & Slater 2006). However, song amplitude is affected by a multiple of factors, such as background noise, (Brumm & Slabbekoorn 2005) wind, environmental reverberation and attenuation, temperature, social context and distance to the sender, and orientation (Brumm & Todt 2002; Brumm & Slater 2006). Only in very controlled situations is possible to obtain objective absolute values of amplitude, which was seldom achieved. However, some studies have shown that song amplitude can provide information about body size in birds, but only when challenged (e.g. Brumm & Todt 2002).

In many bird species repertoire size was correlated with different aspects of male quality. For instances, it was shown that it provided information about reproductive success (e.g. Hasselquist *et al.* 1996), age (e.g. Kiefer *et al.* 2009), social status (e.g. Spencer *et al.* 2004), and also condition (Kipper *et al.* 2006). In relation to body size, for example, Hesler *et al.* (2012) reported that in the Eurasian blackbird, *Turdus merula*, larger males have larger repertoires. Moreover, a study on the common nightingale, *Luscinia megarhynchos*, demonstrated that individuals with larger repertoires have longer wings and higher body mass (Kipper *et al.* 2006). It is known that serins have a relatively large song repertoire, with considerable inter-individual variation: 40-87 syllables (Mota & Cardoso 2001).

Song traits of physically challenging signals such as trills can reflect the signaller quality. Indeed, trill performance has been shown to encode information such as age (Hasselquist *et al.* 1996; Ballentine 2009; de Kort *et al.* 2009), body condition (Weiss *et al.* 2012), or morphological characteristics (Ballentine 2009; de Kort *et al.* 2009; Derryberry 2009). According to (Podos 1997), a motor constraint on vocal performance implies a trade-off between trill rate and frequency bandwidth, which results in production of syllables near the performance limit of the individual. Positive association between trill performance and body size suggested that trills can function as an indicator of male quality, for example, in a study with the Java sparrow, *Lonchura oryzibora*, it was demonstrated that trill performance is positively associated to body size (Kagawa & Soma 2013). It is also important to refer that there are a number of studies emphasizing the importance of beak morphology in trill performance and structures (Podos 2001; Ballentine 2009; Sockman 2009), which can also be predictive of body size.

Immune response

It our study none of song and colouration measurements related with immune response, mediated by PHA challenge. It was expected that at some extent colouration would relate to immunocompetence, but the small sample size and the absence of carotenoid manipulation probably lead to these results. As mentioned before, carotenoids have important physiological properties that make them valuable for self-maintenance functions. They play important roles in the nervous system, and are related with immunoregulation and immunostimulation, lymphocyte proliferation, free radical scavenging and detoxification and also have antioxidant functions (reviewed by Moller *et al.* 2000). It is probable that individuals exhibiting more colourful carotenoid-based ornaments may have greater responses to immune challenges, like in zebra finches, *Taeniopygia guttata*, where males with redder bills demonstrated to have higher levels of circulating carotenoids and also larger PHA responses (Blount *et al.* 2003), or the case of the greenfinches, *Carduelis chloris*, where males with brighter yellow breast feathers showed stronger immune response (Saks *et al.* 2003). Thus, this suggests that high-quality individuals are capable to both allocate carotenoids to signaling and to physiological needs (Olson & Owens 1998). PHA-induced skin swelling test has been intensively used due to its simplicity with little training requirements, and no harsh laboratory facilities (Smits *et al.* 1999). However, this test is widely used in vertebrates, particularly birds, showing trade-offs and correlations with individual condition and quality (Martin *et al.* 2006), the understanding in terms of immunocompetence is not totally clear (Biard *et al.* 2009). Indeed, it has been suggested by immune-ecology studies that besides PHA immune challenge, it would be useful to take into account other physiological parameters (see Biard *et al.* 2009). Nowadays, oxidative stress assessement in studies associating immunocompetence and carotenoids are increasing (Horak *et al.* 2006; Giraudeau *et al.* 2013). Oxidative stress is caused by the production of extremely reactive compounds and free radicals during immune response. These compounds are by products of normal metabolism and immune defences, which can cause extensive damage to nuclei acids, proteins and lipids, especially if antioxidant protection is scarce (Halliwell & Gutteridge 1999). In this light, one can affirm that oxidative stress is an accurate immunocompetence assessor and would be interesting to understand how it would relate with the expression of colour in the serin.

Response to stress

In the present study we found a tendency for individuals with higher breath rates to produce lower minimum song frequencies. Breath rate, the frequency of respiratory movements, is considered to be a measure of stress, providing information how an individual reacts facing a stressful situation, like a fearful stimuli, or novelties (Carere & van Oers 2004). In the present study I used breath rate as an indicator of handling stress. Handling stress is related with the rise in glucocorticoids and usually with fast rise in body temperature associated with tachycardia (Cabanac & Aizawa 2000), whereas breathing frequency is probably related with cardiovascular parameters (Carere & van Oers 2004). In fact, very little is known about the physiological ways that initiate the breath rate response and as far as I am concerned it is not often considered in studies of this nature. Breath rate was used in studies concerning personality measurements, also referred as behavioural syndromes (e.g. Carere & van Oers 2004) and to assess response differences to diverse habitat backgrounds (e.g. Torné-Noguera *et al.* 2014). A study on the Atlantic salmon (*Salmo salar*) showed a positive relationship between breath and metabolic rates (Millidine *et al.* 2008). But no evidences were found relating these two traits in birds, suggesting that much more information is needed to infer about this matter. The potential of this measurement concerning ornament expression needs yet to be explored.

Methodological aspects

The term “condition” is widely used in a variety of Biological sub-disciplines, but it is a poorly defined term. Considered to be an indicator of individual quality, a predictor of fitness, and even a predictor of population dynamics, condition is largely measured with estimates of energetic reserves of body fat (Hill 2011). Morphometric estimates of body condition are widely used in ornithological and ecological studies instead of direct measurements, and the residuals of body mass on a structural measure of body size is one of the most used index (Labocha & Hayes 2012). There are other body condition indices that can provide reliable information about individual quality, and among other factors choosing the best will depend of the species, season, sex and population studied. However, the majority have one thing in common, they are based on fat content (Labocha & Hayes 2012). In this study the body condition index was taken as the residuals of body mass on tarsus length, and although is a reliable body condition index it is admissible that other condition measurements rather than based on fat content are potentially signalled by ornament expression in the serin. We know that song and colouration in this species are sexually selected traits if they signal individual quality, for example, in a manipulative diet experience, individuals that were supplemented with carotenoids exhibited a higher immune response (Trigo *personal communication*).

Amplitude is a potential indicator of individual quality but it is a difficult measure to obtain. In this case we tried to control the subject position and distance by placing only one perch in the acoustic chamber and positioning the microphone just above it. All song recordings were also video recorded controlling for orientation. In video recordings I could observe that some individuals that had lower amplitude values were singing on the floor or hanging on the walls due to the texture of the material lining the sound treatment panels. This invalidated amplitude recordings. The best approach to surpass this situation would be retesting song recordings always controlling for distance, position and orientation and also, adding a bird model on the perch to stimulate birds to sing on it.

Final remarks and future work

The results from this study suggest that multiple ornaments in male serins may convey information on different aspects of male quality. Moller and Pomiankowski (1993) proposed three hypothesis in order to explain the coexistence of multiple ornaments: multiple message hypotheses, redundant signal hypotheses and unreliable signal hypothesis. Besides the variety of hypothesis concerning multiple ornaments, only these three are based on the quality content of the signals and are of certain general application (Candolin 2003), and subsequently considered for discussion.

Findings reported here meet the multiple ornament hypotheses, where the expression of a single colouration measurement predicts a unique quality trait. Male plumage saturation is negatively related with body condition, as song measurements are not significantly related with any of the considered quality traits. Figuerola and Senar (2007) findings that intermediated plumage brightness predicts survival, somewhat support this conclusion, as survival is also considered a quality trait (e.g. Rivera-Gutierrez *et al.* 2010; Simons *et al.* 2012). However, it is important to note that in the two studies different colouration assessment methodologies were used, which make it difficult to compare results. Specifically Figuerola and Senar (2007) used a colorimeter which does not account for UV and provides non-specified measures of brightness and chroma. Moreover, we used visual models for colouration analysis, as they used the tristimulus colour variables.

It is also clear that song and colouration measurements are not related in the expression of quality traits, invalidating the redundant signal hypotheses. Irrespective of these results, it cannot be excluded the possibility that colouration and song traits may signal alternate aspects of male quality in the serin beyond those measured in this study.

In future research on the expression of multiple ornaments in the serin, it is important to consider a larger study sample, as the effects are not too strong requiring larger samples to be assessed. I suggest that other quality traits must be assessed in this context, like oxidative stress. Oxidative stress provides accurate immunocompetence information and has been recently used in studies relating carotenoid availability. In fact, blood from all individuals was sampled and may be used for future oxidative stress analysis. Also, I think it would be very interesting to measure amplitude both in songs and in calls in order to assess performance differences between individuals. The setup developed in this study has good potential to do it, if some improvements are made.

In general, more research needs to be accomplished in order to achieve acuter conclusions on how the expression of multiple ornaments can predict male quality traits in the serin.

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Appendix

Appendix I – Material and Methods

(A) Correlation matrix of song measurements (N=16)

		Peak frequency	Minimum frequency	Maximum frequency	Silence to noise ratio
Peak frequency	Pearson Correlation		0.241	0.734**	0.466
	p		0.368	0.001	0.069
Minimum frequency	Pearson Correlation			0.221	0.501*
	p			0.412	0.048
Maximum frequency	Pearson Correlation				0.180
	p				0.505
Silence to noise ratio	Pearson Correlation				
	p				

(B) Correlation matrix of colour measurements (N=19)

		SWS abdomen	SWS throat	SWS crown	SWS breast	DC abdomen	DC throat	DC crown	DC breast
SWS abdomen	Pearson Correlation		0.042	0.297	0.365	0.142	0.324	0.523*	0.023
	p		0.863	0.216	0.124	0.563	0.175	0.022	0.927
SWS throat	Pearson Correlation			0.086	0.095	0.098	-0.156	-0.014	0.088
	p			0.728	0.699	0.690	0.523	0.955	0.721
SWS crown	Pearson Correlation				0.623**	0.078	0.196	0.681**	-0.110
	p				0.004	0.750	0.421	0.001	0.653
SWS breast	Pearson Correlation					-0.043	0.295	0.495*	0.325
	p					0.862	0.220	0.031	0.175

(B) Correlation matrix of colour measurements (N=19) (continuation)

		SWS abdomen	SWS throat	SWS crown	SWS breast	DC abdomen	DC throat	DC crown	DC breast
DC abdomen	Pearson Correlation	0.142	0.098	0.078	-0.043				
	p	0.563	0.690	0.750	0.862				
DC throat	Pearson Correlation	0.324	-0-156	0.196	295	-0.067			
	p	0.175	0.523	0.421	0.220	0.786			
DC crown	Pearson Correlation	0.523	-0.014	0.681**	0.495*	-0.113	0.402		
	p	0.022	0.955	0.001	0.031	0.644	0.088		
DC breast	Pearson Correlation	0.023	0.088	-0.110	0.325	0.044	-0.021	0.216	
	p	0.927	0.721	0.653	0.175	0.859	0.933	0.375	

(C) Amplitude measurements (dB). SM and RM represent the sequential mode and repetitive mode of the song, respectively.

Individual	Peak amplitude song	Peak amplitude SM	Peak amplitude RM
60	40.062	42.240	46.579
62	58.528	59.653	61.767
63	23.083	24.847	29.436
64	31.499	34.393	36.529
65	26.236	28.326	30.913
67	39.135	42.032	46.125
68	33.990	36.046	39.042
70	44.546	46.352	51.248
72	48.582	49.830	52.348
75	50.546	50.510	55.621
76	38.583	40.378	42.454
77	41.323	43.511	45.372
83	46.566	48.444	49.656
84	36.108	37.647	43.841
85	40.805	41.969	46.336
195	41.280	44.669	43.845