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# A Neuroethological Approach to Song Behavior and Perception in European Starlings: Interrelationships Among Testosterone, Neuroanatomy, Immediate Early Gene Expression, and Immune Function

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GREGORY F. BALL,\* KEITH W. SOCKMAN,<sup>†</sup>  
DEBORAH L. DUFFY,<sup>‡</sup> and TIMOTHY Q. GENTNER<sup>§</sup>

\*DEPARTMENT OF PSYCHOLOGICAL AND BRAIN SCIENCES  
JOHNS HOPKINS UNIVERSITY, BALTIMORE, MARYLAND 21218, USA

<sup>†</sup>DEPARTMENT OF BIOLOGY, UNIVERSITY OF NORTH CAROLINA  
CHAPEL HILL, NORTH CAROLINA 27599, USA

<sup>‡</sup>CENTER FOR THE INTERACTION OF ANIMALS AND SOCIETY  
SCHOOL OF VETERINARY MEDICINE, UNIVERSITY OF PENNSYLVANIA  
PHILADELPHIA, PENNSYLVANIA 19104, USA

<sup>§</sup>DEPARTMENT OF PSYCHOLOGY, UNIVERSITY OF CALIFORNIA, SAN DIEGO  
LA JOLLA, CALIFORNIA 92093, USA

## I. INTRODUCTION: SONG, EUROPEAN STARLINGS, AND THE NEUROETHOLOGICAL APPROACH

It is not unusual to hear commentators on research trends in animal behavior lament current intellectual divisions in the field. During the past 25 years it can be argued that investigators in this field can be divided into those who focus on the adaptive significance and evolution of behavior (sometimes called ultimate causation) and those who focus on the study of sensory and physiological mechanisms controlling the production and ontogeny of behavior (sometimes called proximate causation; Dewsbury, 1992, 1999; Sherman, 1988). The concern of course is that the field is splitting into operationally specialized camps with little interaction between them. However, a more encouraging development that is also emerging is the cross-fertilization between these complementary approaches and the resultant new integrative views of the causes of behavior (Drickamer, 1998). The goal of this chapter is to review studies on the

interrelationships among endocrine state, brain mechanisms for song perception and production, and immunocompetence and relate these to the question of female choice based on male song in European starlings (*Sturnus vulgaris*). Our desire is to illustrate how by studying a single species from multiple viewpoints one can start to make connections among the major physiological communications systems, namely the nervous system, the endocrine system, and the immune system. This approach is needed in order to address how behavior is controlled and concomitantly how these physiological systems that regulate behavioral output have themselves been shaped by natural and sexual selection.

We adopt an explicitly neuroethological perspective in this research program. What do we mean exactly by this label? Neuroethology is of course a variant of ethology. An ethological research program starts with a view that animals are best understood when studied in their natural context and it encompasses the four questions about animal behavior that Tinbergen observed from the core of ethology (Hinde, 1982; Tinbergen, 1963). These questions consist of two related to proximate causes, that is, immediate causation and development and two questions related to ultimate causes, that is, adaptive significance and evolution (Hinde, 1982; Tinbergen, 1963; but see also Dewsbury, 1999). A neuroethological approach stresses an investigation of neural and physiological mechanisms that might control naturally occurring behavior. This approach can be distinguished from more general neuroscience approaches because it sets as its goal the understanding of the causes of behavior when produced under natural conditions and embraces the study of a wide range of species (Camhi, 1984; Gentner and Ball, 2005). Instead of starting with the goal to identify a model system of a human disease process, one starts with the premise that understanding the causes of complex naturally occurring behavior is interesting by itself. Adopting a neuroethological approach while being aware of questions of evolutionary function is not without potential pitfalls. Bolhuis and MacPhail (Bolhuis, 2005; Bolhuis and MacPhail, 2001) in particular have argued that confounding ultimate and proximate causes can lead one to erroneous notions about behavioral mechanisms. In this chapter we will try to illustrate that knowledge about the adaptive significance of behavior can be an important aide in guiding neuroethological investigations. When considering a complex learned behavior, such as birdsong, it is challenging to decide what aspects of the stimulus are most salient to the birds and are therefore the ones that investigators should concentrate on in sensory and neural studies. As will be illustrated in this chapter, work on aspects of song important in mate choice in starlings has been very helpful in guiding our studies of sensory responses to song and even for an analysis of the neural correlates of song production.

Another goal of this chapter is to highlight the range of methods that can be applied to the study of behavior. Although many neuroethological studies rely primarily on electrophysiological methods to investigate the structure and function of the nervous system, we argue that the entire range of neuroscience methods can and should be marshaled with this approach. The study of song in a species, such as the European starling, is particularly amenable to such a multimethod analysis. European (or Common) starlings have a huge native distribution in Eurasia but they have also been introduced in many areas of the world including North America, South Africa, and New Zealand (Feare, 1984). Thus, many scientists worldwide have access to this species and over time a substantial number of questions have been investigated about the control of song in these birds based on the use of a wide range of methods. We will begin by considering our some basic facts about song behavior.

## II. DESCRIPTION OF EUROPEAN STARLING SONG AND ITS FUNCTION

### A. FUNDAMENTALS OF STARLING SONG

Song in European starlings is long and complex and can include imitations of sounds from other species (Eens, 1997 for a review). It is often produced at a low amplitude, and field naturalists in the past have typically had the impression that it is rather unorganized with little overt structure (Feare, 1984). Detailed acoustic analyses in the 1980s and 1990s, however, revealed a clear underlying structure to starling song (Adret-Hausberger and Jenkins, 1988; Chaiken *et al.*, 1993; Eens *et al.*, 1989, 1991a; Mountjoy and Lemon, 1995) albeit one that is harder to discern than that of other well-studied songbirds. The nomenclature used to describe starling song in this paper follows the guidelines advocated by Eens (1997). Starling song is usually organized into long bouts that may be a minute or longer in duration. These bouts contain shorter phrases or motifs that can be repeated and are generally 0.5–1.0 sec in length. A complete song bout has four acoustically distinct sections (Fig. 1). The first section consists of relatively pure-toned whistles. The second section includes complex “warble” motifs of low amplitude and heterospecific motifs if the individual has copied any. Motifs in the third section are characterized by the presence of rapid, biphonated, click trains or “rattles.” Finally, motifs in the last section are characterized by high-frequency and high-amplitude components, typically the loudest part of the song (Eens, 1997; Fig. 1). Song production is often accompanied by wing movements, the rattle motifs with wing flicks, and the

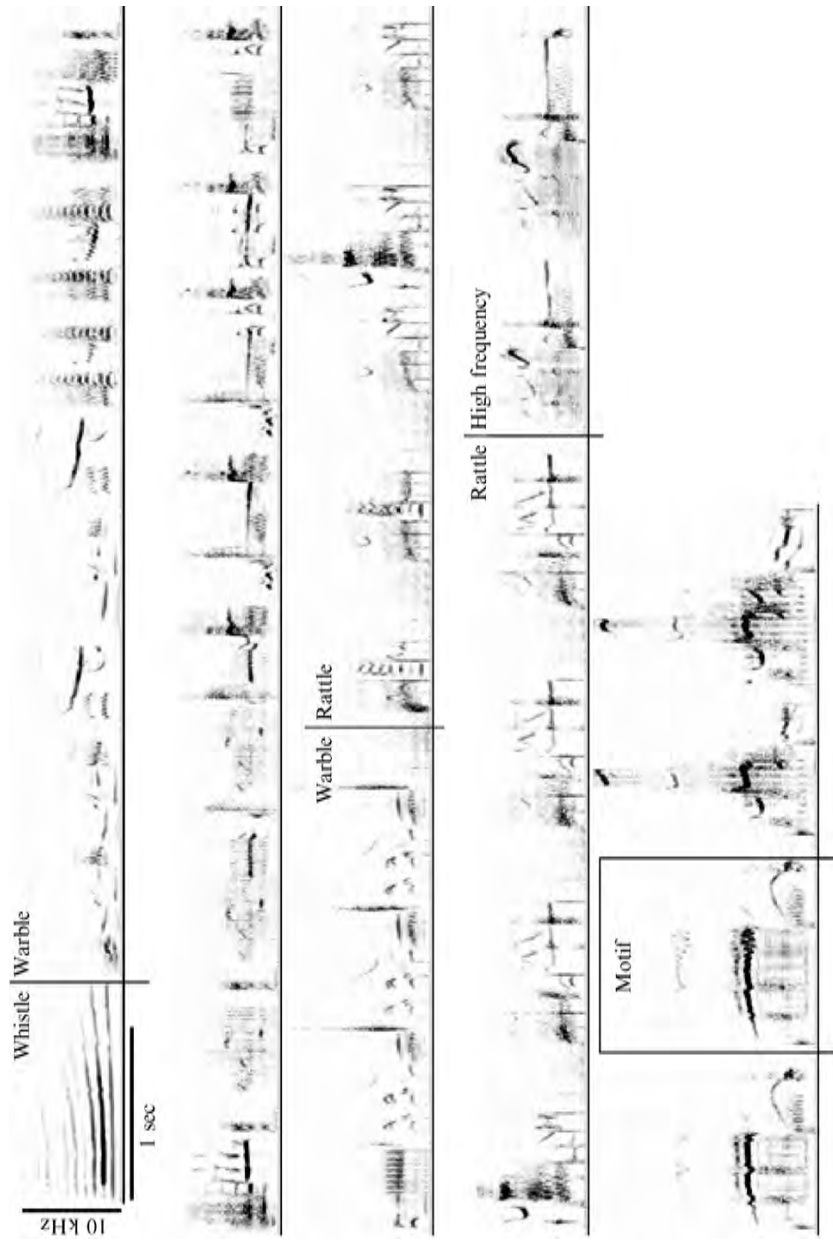


FIG. 1. Sonogram of a single song from one male starling showing the patterning of frequency spectrum power as a function of time. For clarity the sonogram is broken into separate rows. One motif in the last row is outlined in the square. Divisions between whistle, warble, rattle, and high-frequency motif types are denoted by the solid black bars in rows one, three, and four.

high-frequency motifs at the end of the song by wing waving (Bohner and Veit, 1993).

#### B. FUNCTION OF SONG IN EUROPEAN STARLINGS

Several lines of evidence support the contention that male starling song is important for female mate choice. Male starlings paired with a female will increase their rate of singing just prior to copulation (Eens and Pinxten, 1990) and stop singing only after their mate's clutch is complete, whereas unpaired males continue singing throughout the breeding season (Kluyver, 1935 in Eens, 1997). In fact, male song output is closely related to different stages in the female breeding cycle. There is a rapid decrease in male singing activity after pairing (Eens *et al.*, 1994; Hindmarsh, 1984), and then an increase 2–4 days prior to egg laying, after which song rates remain elevated until the end of the laying period before nearly ceasing altogether (Eens *et al.*, 1994). Likewise, male song rate is negatively correlated with the date of clutch initiation (Mountjoy and Lemon, 1996; Wright and Cuthill, 1992). Moreover, the postpairing period of high song output coincides with the presumed fertile period for female starlings (Birkhead *et al.*, 1987) and (albeit roughly) with the male's most rigorous period of mate guarding (Pinxten *et al.*, 1987). In the field, copulations between starlings are almost always preceded by bouts of male song (Eens and Pinxten, 1995; Eens *et al.*, 1989; Mountjoy and Lemon, 1996) and when presented with a conspecific female, unmated captive male starlings sing many more song bouts, than when confronted with a conspecific male (Eens *et al.*, 1993). The number of songs sung in the nest box also increases significantly with the introduction of a female, both for captive male starlings (Eens *et al.*, 1993) and those in the field (Eens *et al.*, 1991b). Average song bout length is positively correlated with the number of young per male (Eens *et al.*, 1991). In the field, there is a significant negative correlation between repertoire size and the delay between male nestbox occupation and clutch initiation, even when nest-site preference is controlled (Mountjoy and Lemon, 1996). Moreover, although males that mate earlier tend to be older, measures of male condition and gross morphology do not reveal affects on the timing of mating. Male body mass and tarsus lengths are not significantly correlated with initial pairing dates (Eens *et al.*, 1991b). Similarly, male body mass, tarsus length, culmen length, wing length, and the length of the iridescence on a male's hackle feathers are not significantly correlated with the timing of clutch initiation (Mountjoy and Lemon, 1996). Among males, both repertoire size and song bout length are directly correlated with age and mating success (Eens *et al.*, 1991b). Finally, in the laboratory, female behavioral preferences can be directly controlled by varying the mean

length of male song bouts. That is, females will spend more time listening to long male song bouts than to shorter ones, and will preferentially track the position of the longer bouts coming from different locations (Gentner and Hulse, 2000a).

### III. SONG CONTROL CIRCUIT AND THE NEUROENDOCRINE CONTROL OF SONG

#### A. NEURAL CONTROL OF SONG BEHAVIOR

European starlings and other members of the songbirds (suborder Passeres or Oscines) have evolved a suite of neural specializations in association with their sophisticated vocal abilities, which facilitate the learning, production, and perception of song (Brenowitz *et al.*, 1997; Farries, 2004; Jarvis, 2004). The best studied of these specializations is the song control system, an interconnected circuit of telencephalic, diencephalic, mesencephalic, and myelencephalic nuclei that regulate the learning and production of song. In this section we will provide a succinct review of the structure and function of the song system and related auditory pathways. Most of the work on the song system is based on studies of zebra finches (*Taeniopygia guttata*) and canaries (*Serinus canaria*). Although some species-specific specializations may exist, starlings share much of the same cytoarchitecture and neurochemistry described in these other species (Ball, 1990; Ball *et al.*, 1988; Bernard *et al.*, 1993) consistent with common neuroanatomical principles of song system organization.

The song control circuit can be divided into two main parts: the more caudal motor pathway and the more rostral anterior forebrain pathway (AFP; Fig. 2). The primary motor pathway, in order of descending projection, is made up of the nucleus HVC (used as a proper name), the nucleus robustus arcopallialis (RA), the dorsomedial portion of the intercollicularis, medullary nuclei that modulate respiratory motor neurons (Wild, 1994, 2004), and the tracheosyringal portion of the hypoglossal nucleus (nXIIIts) that controls muscles of the syrinx, the avian vocal organ (Nottebohm *et al.*, 1976, 1982; Reinke and Wild, 1998; Wild, 1993a,b; see Reiner *et al.*, 2004 for the current, recently revised nomenclature of the avian brain). HVC appears to be unique to songbirds (Ball, 1994; Brenowitz, 1997; Kroodsma and Konishi, 1991; Nottebohm, 1980). Immediate early gene (IEG) studies (Jarvis and Nottebohm, 1997; Kimpo and Doupe, 1997), lesion data (Nottebohm *et al.*, 1976; Simpson and Vicario, 1990), and electrophysiological recordings (Yu and Margoliash,

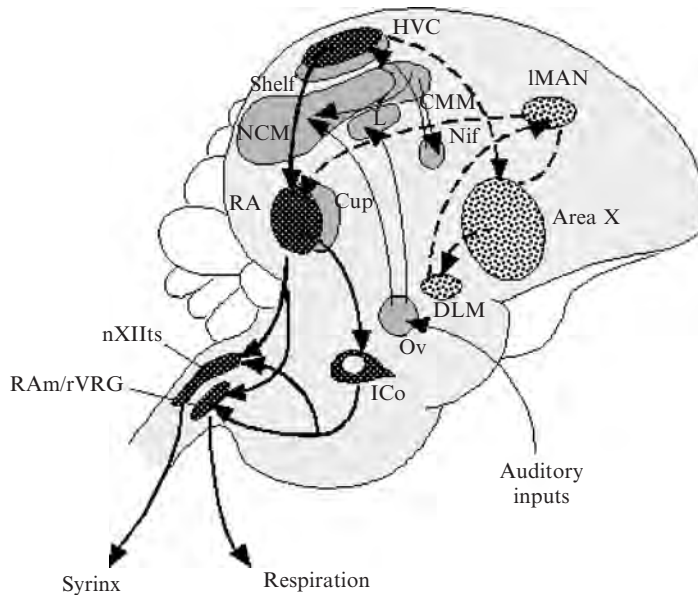


FIG. 2. Schematic representation of a sagittal view of the song control system of songbirds. It consists of at least two basic pathways. One pathway, essential for song production, involves a projection from nucleus HVC (initially misnamed the hyperstriatum ventrale, pars caudale and now known as the acronym only) to the nucleus robustus arcopallialis (RA) that in turn projects to both the nucleus intercollicularis (ICo) and the tracheosyringeal division of the nucleus of the XIth cranial nerve (nXIIts). Efferent projections from motor neurons in this brainstem nucleus innervate the vocal production organ, the syrinx. ICo and RA also innervate medullary structures that coordinate song production with respiration. HVC also connects with RA through a more circuitous route. This anterior forebrain pathway consists of a projection from HVC to area X of the medial striatum that in turn projects to the medial portion of the dorsolateral nucleus of the anterior thalamus (DLM). DLM projects to the lateral portion of the nucleus magnocellularis of the anterior nidopallium (IMAN) that in turn projects to RA. In contrast to the more posterior pathway that is needed for song production, the anterior forebrain pathway is involved in song learning, maintenance, and various forms of sensory feedback on song production. Some of the auditory inputs to the song system are also illustrated. Nucleus ovoidalis (Ov) of the thalamus projects to telencephalic auditory areas such as field L (L) and the caudal and medial nidopallium (NCM). These in turn project to other auditory areas adjacent and connected to the song system such as the caudal ventral hyperstriatum, Nif, and the shelf near HVC and the RA cup. See text for further details.

1996) consistently implicate the primary motor pathway (HVC→RA→ICo→nXIIts) in song production.

Neurons from HVC also innervate the anterior forebrain pathway. HVC projects to area X of the medial striatum (homologue of the caudate/putamen), which in turn projects to the medial dorsolateral thalamic

nucleus (DLM). DLM projects to the lateral magnocellular nucleus of the anterior nidopallium (IMAN), and IMAN projects (predominantly) to RA. This pathway is organized as follows  $HVC \rightarrow X \rightarrow DLM \rightarrow IMAN \rightarrow RA$  (Fig. 2; see Doupe *et al.*, 2005 for a review). Thus there are two pathways from HVC to RA. The caudal pathway is essential for song production, and the more indirect AFP appears to function in song learning (see Bottjer and Johnson, 1997; Doupe *et al.*, 2004 for reviews) and in the maintenance of stereotypic adult song (Benton *et al.*, 1998). Lesions to nuclei within the AFP do not immediately effect adult song production (Bottjer *et al.*, 1984; Scharff and Nottebohm, 1991; Sohrabji *et al.*, 1990).

Explicit functions of the AFP have been slower to yield to investigators than those of the primary motor pathway. One hypothesis is that the AFP carries (or computes) an error signal between the song that the bird is trying to produce and that which it actually does produce (Brainard, 2004). Blocking auditory feedback by deafening (Nordeen and Nordeen, 1992) or distorting it via delayed feedback (Leonardo and Konishi, 1999) results in a decline in the quality of song production. Moreover, the negative effects of deafening are largely reversed by subsequent lesions to IMAN (Brainard and Doupe, 2000), perhaps because the effects of a putative error correction signal provided by the AFP are blocked by the lesion. Consistent with this idea, song-evoked microstimulation of IMAN disrupts ongoing song, again suggesting that the AFP mediates the ongoing song maintenance (Kao *et al.*, 2005), perhaps in a context-dependent fashion (Hessler and Doupe, 1999a,b; Jarvis *et al.*, 1998).

During ontogeny and in adulthood song is profoundly regulated by hearing. Thus, it is important to understand the anatomy of the auditory inputs to the song system. The basic plan of the passerine auditory system follows a general reptile–bird pattern of connection (Carr, 1992; Carr and Code, 2000; Ulinski and Margoliash, 1990). The auditory nerve projects to the cochlear nuclei magnocellularis and angularis. These nuclei project in turn to second order olivary nuclei, to the lemniscal nuclei, and contralaterally to the central nucleus of the nucleus mesencephalicus lateralis dorsalis (MLd), the avian analogue of the inferior colliculus. Efferent fibers from the central nucleus of the MLd target primarily the medial portion of the dorsal thalamus, the nucleus ovoidalis (Ov; Karten, 1968). The caudal medial portion of the avian telencephalon is composed of five cytoarchitectonic subregions—L1, L2a, L2b, L3, and L—called the field L complex (Fortune and Margoliash, 1992). The field L complex is the primary telencephalic target for auditory information arriving via several parallel pathways from the Ov complex in the thalamus. The subregions of field L are densely interconnected and project to the caudal and medial nidopallium (NCM) and reciprocally to the lateral portions of the caudal mesopallium



(CLM). The NCM and CLM share reciprocal connections with the caudal medial mesopallium (CMM; Fig. 2).

In European starlings, neurons throughout the Ov and the auditory telencephalon show tonotopic organization (Bigalke-Kunz *et al.*, 1987; Leppelsack and Schwartzkopff, 1972; Rubsamen and Dorrscheidt, 1986). In starlings, roughly 11 different regions can be identified on the basis of the direction of the tonotopic gradient and tuning curve bandwidth (Capsius and Leppelsack, 1999; Häusler, 1996) with similar patterns observed in zebra finches (Gehr *et al.*, 2000). Neurons in L1 and L3 have lower response rates to tone bursts than those in L2 and show greater selectivity to species-specific vocalizations (Bonke *et al.*, 1979; Leppelsack and Voigt, 1976; Müller and Leppelsack, 1985; Theunissen and Doupe, 1998). This selectivity is borne out by the complexity of the spectrotemporal receptive fields (STRFs) for many neurons within field L. More reliable estimates of the STRF are derived from responses to conspecific vocalizations than tone pips (Schäfer *et al.*, 1992; Theunissen *et al.*, 2000). This general pattern of increasing response selectivity from field L2 to the higher order areas continues into NCM and CM (Grace *et al.*, 2003; Müller and Leppelsack, 1985; Sen *et al.*, 2001), suggesting that these regions are involved in the extraction of complex features. Early data from white-crowned sparrows are consistent with this in showing a small subset of neurons in the NCM that are selective for specific directions of frequency modulation (FM) in a common trill element of conspecific song (Leppelsack, 1983). Single neuron recordings from operantly trained starlings (Gentner and Margoliash, 2003) implicate CMM in the representation of complex acoustic features in behaviorally relevant conspecific songs.

Neurons in NCM are broadly responsive to conspecific stimuli and respond to the repeated presentation of conspecific song in a stimulus-specific manner (Chew *et al.*, 1995; Stripling *et al.*, 1997). The repeated presentation of a single conspecific song elicits a rapid modulation in the initial firing rate of NCM neurons (Stripling *et al.*, 1997). If the same song is repeated on the order of 200 times, this initial modulation of the firing rate is no longer observed when that same song is presented on subsequent trials. This is true even though the initial response modulation can still be observed for other conspecific songs (Chew *et al.*, 1995; Stripling *et al.*, 1997). These stimulus-specific changes in the response properties of NCM neurons have led to the hypothesis that NCM may contribute to individual vocal recognition (Chew *et al.*, 1996). Consistent with this idea, many neurons in NCM (and CM) show a rapid upregulation of the IEG *zenk* in response to the presentation of conspecific songs (Mello *et al.*, 1992) that is tuned to the acoustics of particular conspecific song syllables (Ribeiro *et al.*, 1998). The genomic response also habituates to the repeated presentation

of the same conspecific song (Mello *et al.*, 1995) and is elevated during specific components of the vocal-recognition task described earlier in starlings (Gentner *et al.*, 2004). The mammalian homologue to *zenk* is required for expression of late long-term potentiation (LTP) and long-term memories in mice (Jones *et al.*, 2001). These results suggest that *zenk* (the avian homologue of and an acronym for *zif-268*, *egr-1*, *NGFI-A*, and *Krox-24*) expression in NCM and cHV may be related to learning about conspecific songs and implicates these structures in concomitant processes.

## B. HORMONAL CONTROL OF SONG BEHAVIOR

It is well known that there is a link of some sort between sex steroid hormone secretion and song behavior (see Ball, 1999; Harding, 2004; Schlinger, 1997 for reviews). This link was first posited based on field studies correlating seasonal changes in gonadal size and other aspects of endocrine physiology with changes in song behavior. These descriptive studies have been reviewed in some detail (Ball, 1999; Catchpole and Slater, 1995; Tramontin and Brenowitz, 2000). Therefore, only major features of these findings will be presented here in order to place the hormonal control of song in starlings in a broader context. Starlings are highly seasonal breeders (Ball and Bentley, 2000). Many north temperate zone male song birds sing at high rates in the spring as compared to other seasons (Cox, 1944; Slagsvold, 1977; see Catchpole and Slater, 1995 for a review). In these species seasonal differences in male song are correlated with dramatic seasonal increases and decreases in aspects of reproductive physiology such as gonadal size and plasma hormone concentrations (Dawson *et al.*, 2001; Wingfield and Farner, 1993). However, among these temperate zone birds, there is interspecific variability in the degree to which maximal rates of singing are observed outside the breeding period. For example, robins (*Erithacus rubecula*) living in northern Europe sing at relatively high rates throughout the year (Hoelzel, 1986) only pausing in July (Cox, 1944) while most other songbirds living in the same region do not. Starling song is not limited to their breeding season (Eens, 1997); for example, song can be heard quite commonly in the fall. Although it has not been quantified properly, song seems to be the least common in the late summer and early fall when the birds become photorefractory and molt (Feare, 1984). Birds, such as starlings, that sing outside the breeding season exhibit seasonal cycles in gonad size and endocrine secretions that are similar to other temperate zone species. Studies of select species that exhibit territorial song production in the autumn, such as song sparrows (*Melospiza melodia*) in the western United States, mockingbirds, and the European robin clearly suggest that song behavior in the fall can be elicited

by the appropriate stimulus in the absence of substantial concentrations of testosterone (T) of gonadal origin (Logan and Wingfield, 1990; Schwabl and Kriner, 1991; Wingfield, 1994; Wingfield and Hahn, 1994). In the case of the song sparrow, there is evidence that this autumnal singing involves estrogen acting in the brain even though the gonad is inactive (Soma *et al.*, 2000). The source of this neuroactive estrogen in this case does not appear to be from T of gonadal origin that is then locally metabolized (Soma *et al.*, 2000). It could be derived from the neural aromatization of a substrate (potentially dehydroepiandrosterone, DHEA) produced by the adrenals or synthesized *de novo* from cholesterol in the brain (i.e., a neurosteroid, Soma and Wingfield, 2001; Soma *et al.*, 2002, 2004). Overall, these data indicate that there is not necessarily a tight correlation between endocrine activity and song rate in all temperate zone species.

However, seasonal changes in reproductive physiology in these species may relate to changes in other aspects of song such as repertoire size or stereotypy. For example, seasonal changes in song repertoires have been observed in European starlings (Eens, 1997) and canaries (Nottebohm *et al.*, 1986) where the number of song types and other measures of song complexity may change. Additionally, seasonal changes in other measures of song, such as stereotypy, have been described in white-crowned sparrows (*Zonotrichia leucophrys*, Smith *et al.*, 1995) and song sparrows (Smith *et al.*, 1997).

A careful consideration of behavioral data from temperate zone songbirds suggests that although song output is positively correlated with various measures of reproductive physiology, including hormone concentration in the plasma, there is not necessarily a strong causal relationship between the two as is the case for sex steroids and certain reproductive behaviors such as lordosis in rats (Pfaff *et al.*, 1994), the bow coo display in male ring doves (Lehrman, 1965), or male-typical copulatory behaviors in Japanese quail (Balthazart *et al.*, 2004). Experimental studies on the effects of exogenous hormone administration or castration with hormone replacement on song have been performed on a relatively small number of species but these studies confirm this view of the relationship between steroids and song behavior. Administering exogenous T can clearly increase song rate (Hunt *et al.*, 1997; Nowicki and Ball, 1989). Several independent studies of zebra finches have shown that castration greatly reduces but does not eliminate male-typical song (Arnold, 1975; Harding *et al.*, 1983), whereas in red-winged blackbirds castration was reported to eliminate adult song production (Harding *et al.*, 1988). In the case of song sparrows in the western United States, castrated males were able to maintain fall territories and sang at high rates in response to territorial challenge in a manner that was indistinguishable from intact controls (Wingfield, 1994). The hormonal

control of song behavior therefore appears to be a clear case of a hormone-enhanced, rather than hormone-dependent behavior. Species-typical stimulus factors (the presence of a conspecific male and/or female as well as a nest site or a favorable environment) promote song production in males. The presence of gonadal steroids in the plasma can increase the probability and intensity of these behavioral responses to the appropriate stimulus but this presence is not essential for behavioral activation (Wingfield, 1994). One should therefore not be surprised by reports of substantial song production being observed in association with low steroid hormone concentrations in some cases. The stimulus factors releasing song can be so strong in some cases that high gonadal steroid concentrations are not necessary for song production to be observed.

In European starlings, T effects on song appear to be limited to certain social contexts. For example, castrated European starlings continue to express high basal rates of singing but fail to exhibit an increase in singing rate when presented with a female (Pinxten *et al.*, 2002). A female-induced increase in singing rate was, however, observed in castrates treated with T (Pinxten *et al.*, 2002). Breeding season song in starlings usually declines after mating. If male starlings are treated with T during the incubation period, there is a robust song rate increase while a similar treatment during the nestling-feeding period has little effect (De Ridder *et al.*, 2000). The authors interpret these findings as follows. In the population they studied in Belgium, during the incubation period, there are still a large number of reproductively active females for the males to direct their song at, while by the time the nestling feeding stage starts there are few receptive females available (De Ridder *et al.*, 2000). Thus, T was only effective in inducing an increase in song in breeding starlings when females available for mating were present. Finally, comparing the effects of a female on male song rate in starlings in the spring when T concentrations are high and the fall when they are low reveal an enhancing effect of a female only in the spring (Riters *et al.*, 2000). These data are all consistent with the notion that in starlings T is effective in enhancing song produced in response to the presence of a receptive female.

Although T does not appear to be necessary for the initiation of song production in all cases, it does appear to influence aspects of song quality such as stereotypy. For example, castration prevents the onset of crystallized (i.e., stereotyped) adult song in 1-year-old song and swamp sparrows singing for the first time in the spring (Marler *et al.*, 1988). On receiving T, the song rapidly crystallizes (Marler *et al.*, 1988). As mentioned previously, in both white-crowned sparrows and song sparrows, fall song in the presence of low concentrations of T is less stereotypic than spring song

produced in the presence of high concentrations of T (Smith *et al.*, 1995, 1997). Overall the data indicate that song can be produced (though at a low rate) in the presence of low concentrations of T, but that the stereotypic quality of the song is also regulated by the presence of T.

Finally, some steroid hormone replacement studies indicate that both androgenic and estrogenic metabolites of T are needed to fully restore high rates of singing (Harding *et al.*, 1983, 1988). In zebra finches it has been suggested that estrogenic metabolites selectively promote female-directed song (Walters *et al.*, 1991). Similarly, in canaries there are data suggesting that estradiol selectively activates syllables that are particularly attractive to females while other aspects of song are activated by androgenic metabolites (Fusani and Gahr, 2005). These studies indicate that there may be selective actions of the two primary metabolites of T on song behavior.

#### IV. PERCEPTION OF SONG IN STARLINGS

##### A. BEHAVIORAL EXPERIMENTS ON SONG RECOGNITION

As noted previously, one can consider starling song as a sequence of phrases or motifs, where each motif is an acoustically complex event. The number of unique motifs that a male starling can sing (i.e., his repertoire size) can be quite large, and consequently different song bouts from the same male are not necessarily composed of the same set of motifs. This broad acoustical variation in their song provides several potential cues that starlings might use when learning to recognize the songs of an individual conspecific and while maintaining that recognition over time. One straightforward recognition mechanism is the association of specific motifs with specific singers. Although some sharing of motifs does occur among captive males (Hausberger, 1997; Hausberger and Cousillas, 1995), the motif repertoires of different males living in the wild are generally unique (Chaiken *et al.*, 1993; Eens *et al.*, 1989). Thus, learning which males sing which motifs can provide discriminative cues for song classification.

As shown in Fig. 3, data from operant studies in starlings indicate that song recognition is based at the level of the motif. Starlings trained by operant conditioning procedures recognize individual conspecifics by one set of songs and can readily generalize correct recognition to novel songs from the same singers (Gentner and Hulse, 1998). However, recognition falls to chance when these novel song bouts have no motifs in common with the training songs (Gentner and Hulse, 2000b). Likewise, starlings trained to discriminate among pairs of motifs will reverse the discrimination when

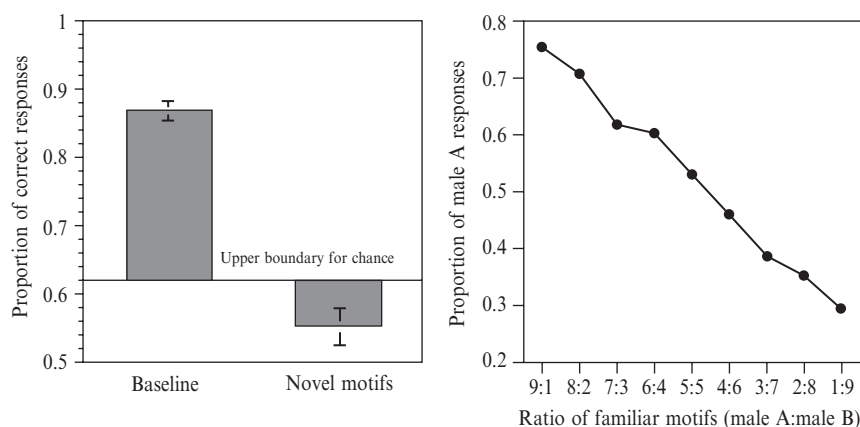


FIG. 3. Song recognition behavior in starlings based on an operant task. The bar graph on the left illustrates the proportion of correct responses made to familiar (baseline) and novel motifs. In the left panel data are shown consisting of response to chimeric songs composed of familiar motifs from two different singers.

transferred to the same motif sung by the opposite individual and perform at chance when transferred to novel motifs sung by the training singers. This failure to generalize correct recognition to songs composed of novel motifs, or to single novel motifs, is inconsistent with the use of particular voice characteristics for vocal recognition. Instead, the data suggest that starlings learn to recognize the songs of individual conspecifics by attending to information contained at (or below) the level of the motif. They appear to associate distinct sets of motifs (or variant motif features) with individual singers.

If starlings learn to recognize individuals by the sets of unique motifs that they sing, then once learned, it should be possible to control recognition systematically by varying the proportions of motifs in a given bout that come from two “vocally familiar” males. That is, recognition behavior ought to follow the proportional distribution of motifs from two vocally familiar males rather than the presence or absence of single diagnostic motifs from either male. The behavioral data confirm this prediction by showing that when starlings are compelled to classify conspecific songs, they do so by memorizing large numbers of unique song components (i.e., motifs) and then by organizing subsets of these motifs into separate classes (Gentner and Hulse, 2000b). As a cognitive recognition strategy, classifying songs according to their component (motif) structure represents a straightforward method of dealing with these complex acoustic signals.

Because individual starlings tend to possess unique motif repertoires, disjoint sets of motifs will generally correspond to individual identity. Therefore, attending to the motif structure captures a significant portion of the individual variation in the signal, albeit at the expense of a large memory.

The behavioral data suggest several hypotheses regarding the neural mechanisms underlying the recognition of natural (i.e., high-dimensional) acoustic events. First, the functionality of motifs as auditory objects in recognition behavior implies their explicit representation in the central nervous system. That is, the response functions of single neurons or of populations of neurons in appropriate forebrain auditory regions should reflect the segmentation of song at the level of the motif. Second, because recognition behavior requires the *learned* association between sets of motifs and singers, motif representations (or the representations of sub-motif features that correspond to unique motifs) should reflect the behavioral relevance of specific motifs. That is, there should be a bias for representations of familiar motifs. Third, the representational mechanisms and capacity (i.e., memory) of the system should permit the acquisition of very large numbers of acoustically complex, natural objects (motifs).

## B. FEMALE SONG PREFERENCES IN A MATE-CHOICE CONTEXT

In a mate-choice context, female European starlings prefer male song organized into long bouts over male song organized into short bouts, even when the total duration of song does not differ between the long- and short-bout exposure (Gentner and Hulse, 2000a). This preference for long-bout over short-bout song is apparently independent of the length of the song itself, but instead is related to some other song component associated with song length, such as its motif repetition rate or stereotypy. Thus, females are able to parse differences between long- and short-bout songs independently of the song length and probably relative to highly complex spectrotemporal components of the song associated with its bout length. Complex behavioral preferences such as these require a nervous system with variable sensitivities to these complex stimuli and capable of precipitating motor programs that give rise to a mate-choice decision (Tinbergen, 1950). Nonetheless, despite the powerful selective forces likely driving mate-choice preferences, we still know relatively little about the neural processing of cues that release such preference behavior (Wilczynski and Ryan, 1992).

## V. PHYSIOLOGICAL RESPONSES TO SONG IN STARLINGS

### A. IMMEDIATE EARLY GENE (IEG) EXPRESSION

#### 1. *Neural Induction of IEGs*

Developments in molecular neuroscience have provided methods that are useful in understanding the neural underpinnings of complex behavior. One such advance has been the identification and characterization of specific genes encoding transcription factors and other types of proteins which are rapidly induced in the brain in response to a variety of stimuli (Goelet *et al.*, 1986; Hoffman *et al.*, 1993; Morgan and Curran, 1989). These so-called IEGs are by definition the earliest or first genomic response to an inducing stimulus and they therefore can be operationally defined as those that are induced in the presence of protein synthesis inhibitors because they do not require any other gene to be transcribed in order to be active (Clayton, 2000). The mapping of IEG expression in the brain associated with the occurrence of a behavior or in response to behavioral stimuli has been a particularly powerful method for mapping functional neural circuits (Clayton, 2000; Hoffman *et al.*, 1993; Mello, 2002).

Several lines of evidence suggest close associations between IEG activation and activity at the presynaptic neuron responding to the behaviorally relevant stimulus (see Guzowski, 2002 for a brief but detailed review of the signal transduction events leading up to IEG activation). Exactly what sort of activity drives IEG activation is not always known for many of the genes in this category. The induction of an action potential in the IEG-expressing neuron is one event that can trigger gene activation (Mello, 2004) but it is not essential for gene expression to occur (Clayton, 2000; Jarvis, 2004). If the neuron depolarizes or if neurotransmitter binding results in a graded potential above some presumed threshold, intracellular second messengers, such as cyclic AMP or calcium ions either entering the cell or released from intracellular stores, activate cellular phosphatases, as well as kinases, such as protein kinases C and A,  $\alpha$ -calmodulin protein kinase II, and the tyrosine kinases (Bozon *et al.*, 2003). Among their wide range of functions, these enzymes then activate various constitutive regulatory transcription factors, such as cyclic AMP response element-binding protein (CREB), which initiate a cascade of neuronal transcription events, some of which include transcription of IEGs. Importantly, the degree to which this intracellular signal transduction cascade is capable of ultimately giving rise to an IEG response is highly sensitive to neuronal experience and can be fine-tuned by noradrenergic (Cirelli and Tononi, 2004; Yamada *et al.*, 1999), dopaminergic, cholinergic, and cannabinoid (Whitney *et al.*, 2003) neuromodulatory input.



IEGs fall into two categories. One is the effector IEGs, which include *Arc*, *narp*, and *homer*, among others. The other is the inducible- or regulatory-transcription-factor IEGs such as *c-fos* and *zenk* (Mello *et al.*, 1992). Lanahan and Worley (1998) determined (with the use of subtractive hybridization techniques) that approximately 30–40 genes make up the total IEG response in the hippocampus of rats, approximately 10–15 of which encode inducible transcription factors and the rest of which encode effector IEGs. A reasonable hypothesis is that a similar suite of genes makes up the total IEG response in other areas of the brain and in other species, but this has not yet been determined.

The effects of these newly transcribed IEGs have been among the more vexing problems in the study of IEGs, and, to be sure, these effects are likely to be varied, multifaceted, and heavily dependent on context, condition, and experience. For example, the stimulus-evoked expression of a particular inducible transcription factor does not necessarily result in the transcription of a particular gene. This is the case in part because of the extraordinary vagaries of eukaryotic gene transcription and its dependence on a suite of potentially interacting proteins present in the cell at any given time, which itself may depend on the cell's recent synaptic experience. Moreover, the mechanism by which an IEG exerts its function depends on whether it is an effector or inducible transcription factor. Nonetheless, some generalities are emerging about IEG function. Effector genes function in diverse ways, from regulating cellular growth, intracellular signaling, and metabolism to synaptic remodeling and other cell-structure changes. Inducible transcription factors may largely regulate the transcription of delayed effector genes having similar functions to those of immediate effector genes but possibly playing more of a role in modulating the potential of synapses to express experience-dependent plasticity, such as LTP or depression, sometimes referred to as metaplasticity (Fischer *et al.*, 1997). Induction of IEGs has been associated with a variety of cellular processes linked to the formation, consolidation, and retrieval of memories as well as to other cognitive processes thought to be mediated by long-term neuronal plasticity (Bozon *et al.*, 2002, 2003; Clayton, 2000; Guzowski, 2002; Tischmeyer and Grimm, 1999). For example, disruption of the *Zif268* IEG transcription factor targeted at the dentate gyrus of the mouse hippocampus prevents late LTP and impairs performance in tasks requiring long-term memory but does not impair early LTP or performance in tasks requiring short-term memory (Jones *et al.*, 2001). Lack of central nervous system *c-fos* in adult mice impairs hippocampus-dependent spatial and associative learning tasks, and this impairment is likely due to impairment of NMDA receptor-dependent LTP formation (Fleischmann *et al.*, 2003). Still, despite these broad general effects of some IEGs,

a detailed understanding of the mechanisms giving rise to their function has yet to emerge. Moreover, although most research on IEG function has focused on synaptic plasticity, IEGs might serve a spectrum of other functions not closely related to neural plasticity.

Notwithstanding these gaps in knowledge, the role of IEG quantification in response to behaviorally relevant stimuli in neuroethological research should not be underestimated. Specifically, with very little knowledge of the downstream effects of IEG induction, quantification of IEG induction has been instrumental in identifying and localizing brain regions sensitive to the stimulus and thus for functional mapping of neural circuitry involved in precipitating complex, experience-dependent behavior (Mello, 2002). This role has been particularly visible in studies of the songbird auditory forebrain and its responses to behaviorally relevant acoustic stimuli sometimes directly related to the task of mate choice in females.

## 2. *IEG Induction in Songbirds*

Across multiple taxa, a variety of nuclei within the avian brain specifically expresses IEGs in association with a number of behavioral states, from appetitive and consummatory components of sexual behavior in Japanese quail (Tlemçani *et al.*, 2000) to onset of incubation behavior in ring doves (Sharp *et al.*, 1996) and broody behavior in Japanese quail (Ruscio and Adkins-Regan, 2004) to homing behavior in pigeons (Shimizu *et al.*, 2004). Perhaps in no other avian system has the analysis of IEG expression provided such insight on brain–behavior relationships as it has in songbirds (see Ball and Balthazart, 2001; Ball and Gentner, 1998; Clayton, 2000; Mello, 2002, 2004; Mello *et al.*, 2004; Ribeiro and Mello, 2000 for reviews). Quantification of IEG expression in songbird brains has played an important role in identifying brain areas in songbirds that function in processing complex acoustic stimuli related to behavior. As noted previously, the importance of auditory areas, such as CMM and NCM, for the processing of conspecific song was discovered by virtue of the massive *zenk* induction described in these areas specifically in response to conspecific song exposure (Mello and Clayton, 1994; Mello *et al.*, 1992). Electrophysiological and tract-tracing studies have confirmed the role of these areas as an acoustic processing hierarchy fundamental in the behavioral responses to species-specific song (Chew *et al.*, 1995; Stripling *et al.*, 1997; Vates *et al.*, 1996). The work on IEG expression and song perception has led to important new studies about the locus of the memory needed for song learning (Bolhuis *et al.*, 2000). Two IEGs, *zenk* and *fos*, are expressed in nuclei in the motor production pathway for song, including HVC and RA, specifically in association with song production, even in deafened birds that sing (Jarvis and Nottebohm, 1997; Kimpo and Doupe, 1997).

This latter observation indicates that these gene responses in the song system associated with song production are not related to hearing song. The number of songs produced tends to be positively correlated with the number of cells expressing IEGs (Jarvis and Nottebohm, 1997; Kimpo and Doupe, 1997), although European starlings also exhibit a positive correlation between the protein product of the IEG *c-fos* and singing rate in HVC and RA (Heimovics and Ritters, 2005). Such positive correlations between *fos* expression and singing were also observed in brain areas related to the motivation to sing such as the preoptic medial nucleus (POM) and the ventral tegmental area (VTA). However, in this study singing during the nonbreeding season was assessed and compared with singing in a breeding context (i.e., in the spring in the presence of a female) and positive correlations between *fos* expression and song rate were only apparent in the breeding season but not in the nonbreeding season (Heimovics and Ritters, 2005). Based on all these findings related to song production and perception, the application of IEG quantification has since extended beyond the songbirds and resulted in the discovery of homologous song nuclei in other avian orders such as parrots (Jarvis and Mello, 2000) and hummingbirds (Jarvis *et al.*, 2000).

IEG expression in the songbird CMM and NCM is very sensitive to conspecific song, with song-induced expression evident after as little as a 2-sec duration of song (the length of a single song in the zebra finch; Kruse *et al.*, 2000) and in the presence of substantial levels of background noise (Vignal *et al.*, 2004). The IEG response to conspecific song requires experience with song during the young bird's primary period of song acquisition, between 20 and 30 days of age in a zebra finch (Jin and Clayton, 1997). One interpretation of this finding is that IEG induction during the sensitive period for song learning may therefore play an important role in this learning. Studies in zebra finches have found that songs that were most accurately copied during the sensitive period for song learning were the most effective in inducing *zenk* expression in NCM (Bolhuis *et al.*, 2000, 2001; Terpstra *et al.*, 2004). These data have been used to argue that *zenk* induction in NCM is an important step in the formation of memories of tutor song and that NCM may be an important site for the localization of these memories (Bolhuis *et al.*, 2000, 2001; Terpstra *et al.*, 2004). Although the songbird auditory forebrain expresses ZENK and FOS after an individual is exposed to any of a number of acoustic stimuli, including those which would seemingly be of little relevance to the individual's natural history, one hallmark of the CMM and NCM is the stimulus specificity that yields a differential IEG response (Bailey and Wade, 2003; Bolhuis and Eda-Fujiwara, 2003; Clayton, 2000; Mello, 2002, 2004). For example, the type of conspecific vocalization and the sex of the individual exposed to the

vocalization affect distributions of IEG expression patterns. In canaries, IEG induction patterns in NCM are tonotopically organized with the NCM areas of greatest sensitivity corresponding to the frequency of the whistle in the male's song (Ribeiro *et al.*, 1998). In zebra finches, some degree of hemispheric lateralization emerges in the NCM ZENK response to song (Lieshoff *et al.*, 2004). In black-capped chickadees, both the fee-bee song and chick-a-dee call elevate ZENK induction in the CMM and NCM, but dorsal NCM is more sensitive to song-induced than to call-induced ZENK induction, whereas caudal portions of the auditory forebrain show equal sensitivity (Phillmore *et al.*, 2003). Males exhibited greater vocalization-induced ZENK expression than females in this species, although no sex differences in song-induced NCM ZENK induction have been found in European starlings (Duffy *et al.*, 1999).

The strength of this IEG expression increases with increasing relevance or novelty of the stimulus and itself can depend on prior experience. For example, ZENK and FOS induction in the CMM and NCM is selective for conspecific over heterospecific song (Bailey *et al.*, 2002; Mello *et al.*, 1992a) and as noted previously for songs better learned during early developmental stages over those not as well learned (Bolhuis *et al.*, 2000, 2001; Terpstra *et al.*, 2004). Isolation from song early in life results in reduced song-induced ZENK induction during the adult phase (Hernandez and MacDougall-Shackleton, 2004), whereas repeated exposure to the same song results in habituation of the ZENK response in the NCM, which is rescued with exposure to a novel song (Chew *et al.*, 1995; Jarvis *et al.*, 1995; Mello *et al.*, 1995). Due to the participation of the IEGs ZENK and FOS in synaptic remodeling in other systems, it is likely these areas of the songbird auditory forebrain, specifically CMM and NCM, undergo the experience-dependent plasticity that is necessary for long-term memory formation, memory consolidation, and memory retrieval (Mello, 2002).

Because male songbirds produce song, in part, to attract mates, females are behaviorally sensitive to song exposure, and one might therefore hypothesize that quantification of differential IEG induction in the female brain would reveal neural systems involved in female perception of song. Therefore, of particular interest was the discovery of ZENK and FOS induction in the CMM and NCM of females that was greater after exposure to male conspecific song than after male heterospecific song (Bailey *et al.*, 2002; Duffy *et al.*, 1999; Mello *et al.*, 1992a), suggesting that IEG induction in the auditory forebrain might be involved in song-mediated recognition of a mate of the appropriate species.

Females of a diversity of animal species show behavioral responses to males that suggest a degree of perceptual sensitivity substantially greater than what would be required for mere species recognition. Females of

many species discern high-quality from low-quality males based on phenotypic variation among males that presumably serves as an honest signal of their quality. In birds, vocal phenotypic features are thought to be one such signal. Females tend to choose mates based on variation in song type (Catchpole and Slater, 1995; Searcy, 1992; Searcy and Yasukawa, 1996), which may serve as an honest signal of male quality (Gil and Gahr, 2002). A large body of research on songbird species suggest that at least one dimension of song variability highly salient to choosy females involves song complexity as measured, for example, by repertoire size or by motif stereotypy (Searcy, 1992) which can be limited by nutritional constraints during development (Buchanan *et al.*, 2003b; Nowicki *et al.*, 2002a,b).

In the field of mate choice, which concerns the processes whereby individuals choose between different prospective mates based on their phenotypic characters, the study of bird song and related IEG induction has also played a useful role. In fact, avian IEG studies have revealed a level of perceptual discrimination in the female auditory forebrain that is sufficient in some cases to resolve quality differences between males of a single species. For example, in the non-Oscine budgerigar (*Melopsittacus undulatus*), NCM ZENK induction increases with the complexity of male song to which they are exposed (Eda-Fujiwara *et al.*, 2003). In an oscine, the mountain white-crowned sparrow (*Zonotrichia leucophrys oriantha*), females prefer in a mate-choice context song of their local, natal dialect to song of a foreign dialect (MacDougall-Shackleton *et al.*, 2001), and CMM and NCM ZENK expression is greater in females exposed to local than in those exposed to a foreign dialect (Maney *et al.*, 2003). In another oscine, the canary, females prefer male songs that contain a particular syllable type, and, when females are exposed to songs with this syllable type, ZENK induction in their auditory forebrain increases relative to that in females exposed to songs without this syllable type (Leitner *et al.*, 2005). But, is this auditory forebrain IEG induction part of a signaling pathway that ultimately gives rise to the mate-choice decision, or is this association between the IEG response and mate choice merely correlational? It is possible that the induction is related to sensory processing but that there is not a causal connection between the IEG induction and the behavioral result. Until we can manipulate IEG induction in a controlled fashion, perhaps via anti-sense oligonucleotide methods or small interfering RNA we may not be able to determine the answer to this question definitively. However, evidence in white-crowned sparrows provides further support of the tight association between ZENK induction and mate preference. Specifically, ZENK induction in both the CMM and NCM of females correlates positively with the level of sexual receptivity to a particular song type, as measured by the female's vocal behavior and copulatory solicitation

behavior (Maney *et al.*, 2003). Thus, there is a strong association between the strength of the preference and the level of ZENK expression, suggesting the presence of a functional tie.

### 3. *IEG Induction and Mate Choice in European Starlings*

The first demonstration that conspecific song variation directly relevant to mate choice affects IEG induction was found in the European starling (Gentner *et al.*, 2001). Female starlings, which prefer long-bout over short-bout song, have greater ventral NCM (NCMv) ZENK induction in response to long-bout than short-bout songs, even when the total song exposure does not vary (Fig. 4). Because the NCM and CMM share reciprocal projections (Vates *et al.*, 1996), it was not surprising to find from a second study that this ZENK response bias toward long-bout song also occurs in the CMM and dorsal NCM (NCMd) (Sockman *et al.*, 2002). However, there is evidence that CMM and NCM process different aspects of song recognition (Gentner *et al.*, 2004) and may release different female responses to song (Maney *et al.*, 2003).

These results demonstrated a neural-response correlate to a mate-choice preference and raised the question as to how this neural response bias toward the behaviorally favored cue arises. There could well be a complex interplay between genetically influenced predispositions to respond more robustly to particular songs and experience with song that might shape these IEG response biases. There is still much we do not know, but an insight concerning this problem comes from two recently published studies on the role of recent adult song type experience on the IEG response bias in the female starling auditory forebrain. We detail these studies later, but first provide some background on some fundamental concepts in mate choice and the potential role of the prevalent song culture in shaping behavioral preferences and neural response biases that presumably give rise to such preferences.

When discussing mate choice, one can often make generalizations about preferences as if they are relatively fixed in populations and therefore might not be modulated by experience (Andersson, 1994). However, the strong directional selection for trait expression predicted by such a perspective is difficult to reconcile with the many observations of substantial between-population, between-individual, and even within-individual variation in expression of sexually selected traits (Badyaev and Qvarnström, 2002). For a sender's traits to communicate to the receiver an honest signal of the sender's quality, expression of the traits must be subject to constraint and therefore incur some cost in order for this device to remain stable as an effective communication mechanism (Grafen, 1990). As a sexually selected

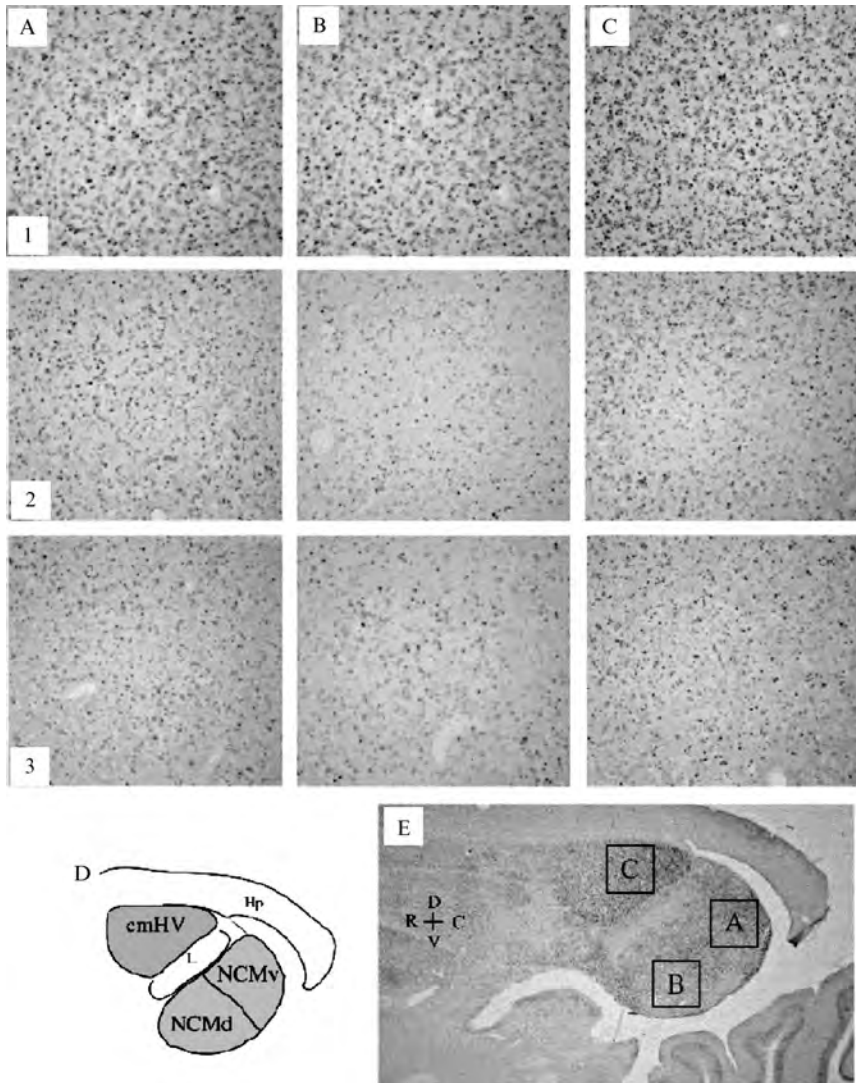


FIG. 4. Patterns of expression of the protein product of the IEG *zenk* in response to song organized in long bouts (panels in row 1), the same amount of song organized in short bouts (panels in row 2), and a short amount of song (panels in row 3). Note that expression is highest in response to long-bout song (row 1).

trait in some species, song production by songbirds can entail a variety of costs, including those associated with increased energy requirements (Eberhardt, 1994; Oberweger and Goller, 2001; Thomas *et al.*, 2003; Ward *et al.*, 2003), increased predation risk (Catchpole and Slater, 1995), and a reallocation of time also required for other activities, such as foraging (Gil and Gahr, 2002). It is not unreasonable to predict that the degree to which each of these costs can constrain song behavior might itself vary. For instance, increased energy requirements of relatively costly forms of song production may constrain expression of such forms relatively little when food is plentiful, resulting in a greater proportion of individuals expressing the trait than otherwise. Similarly, under heavy predation risk, the probable costs of some song types (i.e., those most likely to attract a predator) increase and may drive a greater proportion of individuals to express the less costly trait than expected under lighter predation pressure. Therefore, within a population, the proportion of individuals singing one song type may vary with environmental context, and females will be faced with variation in the proportion of preferred traits being expressed. Through mate sampling experience and variable population densities of sexually selected traits, individuals should therefore express some flexibility in the phenotypic threshold they set for their mate choice (Badyaev and Qvarnström, 2002; Jennions and Petrie, 1997; Wiegmann *et al.*, 1996). Otherwise, they pass some years without mating due to the inability of many males to meet high song quality demands.

Several studies provide evidence for such frequency-dependent choice behavior. In damselflies, males choose females of the more prevalent color morph (van Gossum *et al.*, 2001); and in wolf spiders, females likewise choose the male morph which is more familiar (i.e., prevalent; Hebets, 2003). Importantly, this process appears to occur in songbirds, as well. Female white-crowned sparrows show behavioral preferences for their natal song dialect unless, during preceding months, they experienced a more prevalent foreign dialect (MacDougall-Shackleton *et al.*, 2001). Female cowbirds (*Molothrus ater*) also exhibit mating preferences for the male song environment most recently experienced by the female, and it is likely that song was the salient component of the social environment releasing such preferences (Freeberg *et al.*, 1999). Similar findings have been reported in canaries (Nagle and Kreutzer, 1997a,b). In sum, because not every female can mate with a particular male phenotype when the phenotype is in short supply, females seem to adjust the threshold or criteria for mate preference, mate choice, or both.

A physiological mechanism for experience-dependent modulation of mate choice has not been determined. However, evidence was accumulated in female European starlings of two complementary neural systems, each



mediated by forebrain induction of separate IEGs that are made sensitive by experience with one of the ends of the behaviorally relevant axis of song variation (Sockman *et al.*, 2002, 2005). In these studies, pairs of photosensitive female starlings were exposed to 1 week of male song played 5.5 hr/day while maintained on a photoperiod of 11L:13D. Female pairs differed based on whether the week of song experience to which they were randomly assigned was composed entirely of long-bout or short-bout songs. This was our attempt to mimic and manipulate the song environment females might experience when sampling mates before making mate-choice decisions early in the spring.

Following this manipulation of the perceived song experience, we isolated each female of each pair individually for approximately 40 hr and then, while they were still isolated, exposed them to 30 min of song stimulus, sacrificed them 60 min later, and then collected their brains for sectioning and subsequent IEG quantification by immunocytochemistry. Specifically, we exposed one female of each pair to a 30-min long-bout stimulus (mean song length = 55.6 sec) and the other to a 30-min short-bout stimulus (mean song length = 25.4 sec). Importantly, the 1 week of experience songs and the 30 min of stimulus songs had been recorded from different males, meaning that the stimulus songs were always novel, even when the experience and stimulus categories were the same (e.g., long-bout experience followed by long-bout stimulus; Sockman *et al.*, 2005). In this manipulation, there were four groups of females: (1) long-bout experience long-bout stimulus, (2) long-bout experience short-bout stimulus, (3) short-bout experience long-bout stimulus, and (4) short-bout experience short-bout stimulus.

As previously demonstrated (Gentner *et al.*, 2001), we found that ZENK induction in the auditory forebrain of female starlings was greater after exposure to the 30-min long-bout than after exposure to the 30-min short-bout song stimulus (Sockman *et al.*, 2002, 2005). However, this ZENK response bias toward the long-bout stimulus was modulated by experience. That is, the 1-week long-bout experience enhanced this bias (i.e., greater ZENK in response to the long-bout than in response to the short-bout stimulus), whereas the 1-week short-bout experience attenuated it. So, based on these results, it appears that recent song-sampling experience during adulthood influences the neural response bias toward a preferred male trait important in female mate choice. The presence of experience-dependent mate-choice decisions suggests that such a system must exist, but this is the first identification of such a system in any species. More importantly, these findings demonstrate a surprising level of adult neuroplasticity, whereby responses to a recent adult cultural experience are modulated not by experience with the stimulus itself, but rather by

experience with the category of stimulus to which they are sensitive. That is, because the song sets comprising the experience and stimulus treatments were taken from different males, the songs varied across treatments in their spectrotemporal features while they retained their categorical distinctions as being long bout or short bout. In light of this treatment design, our data reveal an experience-dependent response plasticity spanning the category into which the stimulus is organised.

The auditory forebrain regions of CMM and NCM that are so sensitive to recent adult experience in their ZENK expression levels also express IEGs other than ZENK. For example, the auditory forebrain of zebra finches and canaries upregulates both of the heterodimerizing transcription factors FOS and JUN in response to conspecific song (Bailey and Wade, 2003; Bailey *et al.*, 2002; Bolhuis *et al.*, 2000, 2001; Nastiuk *et al.*, 1994). Moreover, song-induced expression of both ZENK and FOS correlates with how well an individual learned the stimulus song during development and varies with sex (Bailey and Wade, 2003; Bolhuis *et al.*, 2001). Such associations suggest a dynamic sensitivity of these IEG systems to modulation by experience. An environment conducive to song learning in young birds may modulate their future sensitivity to song stimuli, as reflected by the song-induced IEG induction in the auditory forebrain. However, explanations that do not require such individual plasticity are also possible. An elevated sensitivity to a particular song during development (as reflected in the magnitude of the IEG response to that song) may predispose an individual to learning that song better than individuals without an elevated sensitivity to the song. Our study on experience-dependent modulation of song-induced ZENK induction in the auditory forebrain of female starlings indicates, in fact, that within an individual, auditory neural responses to song are plastic and shaped by the individual's recent adult experience. But are other IEGs similar to ZENK in their song-induced sensitivity to recent adult experiences?

To further characterize the experience-dependent properties of the CMM and NCM in female starlings, we also examined the FOS response bias in these females that had recent experience with long-bout or short-bout songs. Consistent with the ZENK response, FOS induction in response to long songs is greater than that in response to short songs (Sockman *et al.*, 2005). However, whereas this ZENK response to differences in song length is made sensitive by experience with the long-song category, the FOS response to differences in song length is made sensitive by experience with the short-song category. That is, FOS expression was greater in response to novel long than to novel short songs following a 1-week experience with short but not long songs. Thus, the ZENK- and FOS-signaling pathways are sensitized to variation in song-length categories by

experiences with songs at opposite ends of the starling song-variation continuum. This is likely because of the separate actions of songs in the long category on the ZENK-signaling pathway and songs in the short category on the FOS-signaling pathway. Experience sampling long songs apparently elevates subsequent ZENK expression in response to songs of a category that has common long-song features, whereas experience sampling short songs apparently suppresses subsequent FOS expression in response to songs of a category that has common short-song features. In other words, the ZENK pathway is made sensitive to long songs by experience sampling songs of the long category, and the FOS pathway is made less sensitive to short songs by experience sampling songs of the short category. These findings suggest the presence of complementary neural systems made sensitive in register with the natural axis of phenotypic variation, song length, that is fundamental to the female's mate choice.

Complementary ZENK- and FOS-signaling systems in the auditory forebrain could help establish the attractiveness of a given song within the context of the idiosyncratic distribution of song phenotypes sampled by the female. Potentially, the attractiveness of that song can vary with the song experienced locally, as suggested by studies on white-crowned sparrows, cowbirds, and canaries (see citations earlier). Whether starling mate choice varies with recent song-sampling experience awaits further research.

Of considerable interest would be to determine the functional significance of such complementary neural systems. The use in birds of conditional and localized ZENK and FOS knockouts, which have been developed in rodent systems (Bozon *et al.*, 2002; Fleischmann *et al.*, 2003; Jones *et al.*, 2001), might be helpful in this regard. However, at present there is no easy way to implement knockout technologies in birds so other methods, such as antisense oligonucleotide methods or small interfering RNA, is a more plausible approach to consider (Charlier *et al.*, 2005). But even in the absence of this technology, which would likely reveal the effects specific to the forebrain ZENK- and FOS-signaling systems, the data thus far strongly implicate an important role for the CMM and NCM in experience-dependent mate choice in the starling. How does this experience dependence arise, and what are the downstream neural and behavioral targets?

Experience-dependent representational plasticity of cortical neurons occurs in most sensory systems (Calford, 2002; Gilbert *et al.*, 2001). In the mammalian auditory system, it is tied to coincident activation of both cholinergic (Bakin and Weinberger, 1996; Kilgard and Merzenich, 1998) and catecholaminergic (Bao *et al.*, 2001) neurotransmitter systems. In rats, noradrenergic activity selectively modulates light-induced FOS but not

ZENK expression in the visual cortex (Yamada *et al.*, 1999). Thus, one might hypothesize that in the female starling and other songbirds, separate neurotransmitter or neuromodulator systems would mediate the IEGs ZENK and FOS and shape the real-time responses of single cells to stimuli as a function of recent experience. The noradrenergic system is one obvious candidate for shaping responses in the vertebrate forebrain. Among songbird species, noradrenergic inputs mediate state-dependent auditory responses in the song system forebrain nucleus interfacialis (Cardin and Schmidt, 2004), and evidence for catecholaminergic innervation of the auditory forebrain has been described in canaries (Appeltants *et al.*, 2001) and zebra finches (Mello *et al.*, 1998) and is probably a general phenomenon among songbirds. Lesions to noradrenergic projections through the administration of the noradrenergic-selective neurotoxin DSP-4 disrupts auditory processing in female canaries (Appeltants *et al.*, 2002), implicating an important role for this neurotransmitter system in modulating song-specific behavioral responses in female songbirds. Finally, social context effects on song-induced ZENK expression in area X of male zebra finches are also blocked by the administration of DSP-4, indicating that at least in some brain regions norepinephrine can modulate plasticity in the expression of IEGs (Castelino and Ball, 2005).

Some evidence exists for a role from other systems as well, such as those mediated by gamma-aminobutyric acid (GABA) and cannabinoids. GABA and ZENK colocalize in neurons of the CMM and NCM (Pinaud *et al.*, 2004), and activation of the CB1 cannabinoid receptor inhibits song-induced ZENK expression and habituation of ZENK expression in the NCM but has no effect in regions of the field L complex that show song-induced ZENK expression (Whitney *et al.*, 2003). Clearly a role of various neuromodulatory systems in song-induced IEG expression looks probable and merits further study.

How any neuromodulatory factor might influence IEG responses to conspecific song is not well understood, but some advances in this area have been made. In zebra finches, within the auditory forebrain exclusively, the phosphorylation of the extracellular signal-regulated kinase (ERK) shows remarkable similarity to ZENK in its response to conspecific song (Cheng and Clayton, 2004). Specifically, initial exposure to novel song but not tones or noise upregulates phosphorylation of this protein, which then habituates after repeated exposure to the same song. Presentation of a new novel song again elevates phosphorylation of ERK without affecting its habituation to the familiar song. Infusion into the auditory forebrain of the enzyme responsible for ERK activation blocks induction of ZENK. It seems conceivable therefore, that ERK might help to mediate any modulatory action of song-induced ZENK induction in the female

auditory forebrain by norepinephrine or other factors. Modulation of FOS may be similar, although two lines of evidence suggest that different IEGs may mediate the actions of different neuromodulatory systems: tuning properties of the forebrain FOS system in starlings that are opposite those of the forebrain ZENK system (Sockman *et al.*, 2005) and evidence from mammalian studies (Yamada *et al.*, 1999) suggesting that different IEGs may mediate the actions of different neuromodulatory systems.

In addition to the need for more research on mechanisms mediating the experience-based modulation of forebrain IEG tuning, there is also a need for studies on the behavioral outcome of this differential IEG expression in the songbird forebrain. The opposite tuning properties of the ZENK- and FOS-signaling systems raise intriguing questions. Do they both participate in systems that ultimately promote preference behavior? Does one mediate preference and the other aversion behavior? Evidence from behavioral and IEG studies on white-crowned sparrows suggests that ZENK induction is at least positively associated with strength of preference (Maney *et al.*, 2003), as indicated earlier. However, little is known about how FOS might participate in such a system. If the forebrain FOS-signaling system participates in mechanisms mediating aversion behavior, then the complementary properties demonstrated for ZENK and FOS might form a portion of a neural system for the mediation of mate preferences based on the prevalent song culture or the female's recent mate-sampling experience. Regardless of the outcome of these prospective studies, it is clear that female European starlings have evolved a highly plastic nervous system with properties that would seem capable, in part, of mediating mate-choice decisions based on the female's recent experience with a dynamic culture of male phenotypes.

## B. ELECTROPHYSIOLOGICAL RESPONSES TO SONG

There is a relatively long tradition of studying electrophysiological responses to song in auditory areas and in the song control system to assess their potential significance for song perception (Katz and Gurney, 1981; Leppelsack and Voigt, 1976). This is a large area of research that has been reviewed in some detail elsewhere (Mooney, 2004; Theunissen *et al.*, 2004). We will only cover certain aspects relevant to our discussion of starling song. One hallmark of cells within many of the song system nuclei is their selective response to a "bird's own song" (BOS; Margoliash, 1987). That is, one readily finds neurons throughout the song system whose firing rates and/or temporal response properties are "tuned" to the acoustics of the

song that the bird sings. In the nucleus HVC, the stimulus specificity observed for BOS has both spectral and temporal components, with responses contingent on the presence (and absence) of acoustic energy in specific frequency bands or on specific temporal combinations of sounds (Margoliash, 1983; Margoliash and Fortune, 1992). In addition, these so-called “BOS responses” are strongly modulated by behavioral state (Dave *et al.*, 1998; Schmidt and Konishi, 1998) and are, in at least some cases, observed in cells that also show strongly coupled premotor activity during song production (Dave and Margoliash, 2000; Yu and Margoliash, 1996). The sensorimotor integration at both the cellular and system level, which gives rise to the BOS response, is an active area of research among birdsong neuroethologists. Understanding these physiological mechanisms will likely impact the broader study of sensorimotor learning in other systems, and may be of value in understanding the perceptual role of self-generated sounds in human speech processing (Margoliash, 2003).

Despite early suggestions that BOS selective responses in song system nuclei, specifically the vocal motor pathway (VMP), might reflect a “motor theory” of song perception (*sensu* Liberman *et al.*, 1967), recent data suggest a somewhat different interpretation. The state-dependent nature of these responses and the absence of auditory responses altogether in many VMP neurons in the awake animal (Dave *et al.*, 1998) argue against the notion that the pathways controlling vocal output also contribute to sensory representations of song. Instead, BOS selectivity more likely reflects the involvement of acoustic feedback in ongoing regulation of song production mechanisms. It now appears that only very specific sorts of auditory information, namely BOS, are admitted to the song system so that the bird can detect, and thus correct, any deviations between the intended song and that actually produced. Nonetheless, there are data that suggest a production-independent role for both HVC and the AFP in adult song perception. Lesions to lMAN in canaries affect auditory but not visual discrimination (Burt *et al.*, 2000); lesions to HVC in female canaries abolish female behavioral preferences for conspecific over heterospecific song (Brenowitz, 1991) and for sexually attractive song phrases over other phrases of conspecific song (Del Negro *et al.*, 1998). However, this is not the case for female zebra finches where HVC is quite small and not connected with nucleus RA (MacDougall-Shackleton *et al.*, 1998). In both male and female starlings, HVC lesions affect the ability to form new associations with familiar songs while leaving retention of learned conspecific song discriminations intact (Gentner *et al.*, 2000). Clearly the role of these structures, and by extension the general role of vocal-motor systems, in sensory perception and cognition requires further study—ideally through electrophysiology in awake animals.

As noted previously, auditory areas, such as NCM and CMM, have received attention as key sites for the processing of auditory information related to song. The extensive amount of data on IEG expression just reviewed is certainly consistent with this new direction. In European starlings, electrophysiological data demonstrate directly a role for CMM in learned recognition of song. After training starlings to recognize two sets of conspecific songs, Gentner and Margoliash (2003) observed that single neurons and populations of neurons in the medial CMM respond selectively to acoustic features contained in those songs that the birds had learned to recognize. In contrast, no neurons were selective for similar features in songs that were novel to the birds. This argues very strongly that the response functions of CMM, at both the single unit and the population level, are a direct product of each bird's unique sensory experience. Mechanisms of experience-dependent plasticity act to modify the responses of CMM neurons based on the functional demands of song recognition. Several additional results from this study are consistent with this notion. First, the spectrotemporal tuning properties of CMM cells correspond closely to song features correlated with individual motifs. That is, the same auditory objects that control recognition behavior also predict the responses of selective cells in CMM. Second, the variation in neuronal response strength among the familiar songs was dependent on the reinforcement contingencies used for recognition training. For animals trained with a go/no-go procedure to discriminate between two sets of songs, the S+ songs (i.e., the songs that were reinforced after a go response) elicited the significantly stronger responses than S- songs (i.e., the songs that were not reinforced after a go response), which in turn elicited significantly stronger responses than novel songs (Gentner and Margoliash, 2003). When positive reinforcement is available for both sets of songs, the response strengths associated with each set of familiar songs are similar, but still greater than those associated with novel songs. Thus, the response profiles of neurons in CMM are shaped not only by task relevant acoustic features of conspecific songs in a "bottom-up" fashion, but also by so-called "top-down" mechanisms presumably through reward systems (Gentner and Margoliash, 2003). Studies of ZENK expression in starlings have extended this view of the different roles played by these two areas (the CMM and NCM) in song recognition behavior (Gentner *et al.*, 2004). In NCM, ZENK expression is elevated in association with an operant song recognition task while the starlings are acquiring novel song discriminations (Gentner *et al.*, 2004). In CMM, there is elevation of ZENK expression during recognition of familiar songs during the acquisition of novel associations with familiar songs and when acquiring novel song discriminations (Gentner *et al.*, 2004).

## VI. FUNCTIONAL BASIS OF SONG PREFERENCES IN EUROPEAN STARLINGS

As discussed previously in this chapter, the primary adaptive function of male starling song is in attracting and obtaining mates (Cuthill and Hindmarsh, 1985; Eens, 1997). Males significantly increase their rate of singing in the presence of a female, much more so than in the presence of another male (Eens *et al.*, 1993). Females choose their mates on the basis of this song, demonstrating clear preferences for males that sing in longer bouts or possess large repertoires (Eens *et al.*, 1991b; Gentner and Hulse, 2000a; Mountjoy and Lemon, 1996). Earlier in this chapter, we discussed the physiological correlates of this behavioral response bias among females. Now we turn our attention to the evolutionary function of female song preferences. What reproductive advantage(s), if any, do choosy females gain by exhibiting song-based mating preferences? What information, if any, do males provide about themselves by singing long complex songs?

Much attention has been given to the hypothesis that male starling song functions as an indicator of some aspect of male quality. For instance, song might provide information about the quality of a male's territory, his propensity and ability to provide parental care, his age, or even some underlying genetic quality. The first two possibilities are examples of traits that provide direct benefits to females while the latter are examples of indirect benefits.

### A. DIRECT BENEFITS

Male starlings, unlike some other species of songbirds, do not defend large territories that include food resources (Feare, 1984). Rather, starlings defend only a few square meters immediately surrounding the nest (Kessel, 1957) and forage in pairs or flocks away from the nest (Feare, 1984; Kessel, 1957). Starlings are cavity nesters and, thus, the availability of suitable nest sites is limited. Therefore, the quality of the nest cavity location will likely vary among males, which may lead to differential reproductive consequences for the females that mate with them (Eens, 1997; Mountjoy and Lemon, 1996). While it is true for a number of species that song performance and territory quality are correlated (Catchpole and Slater, 1995), such is not the case with starlings. Repertoire size, which is highly and positively correlated with song bout length, did not correlate with nest box location preferences in a controlled field experiment (Mountjoy and Lemon, 1996). However, repertoire size is correlated with a measure of female preference (delay between the claiming of a nest box by a male and the laying of the first egg), with males possessing the



largest repertoires being most preferred by females (Mountjoy and Lemon, 1996). Thus, in starlings, territory quality (or the location of the nest cavity) does not appear to be a factor in mate-choice decisions nor is it signaled by male song.

Another possible way in which choosy females could receive direct fitness benefits is if a male's song performance indicates his ability and propensity to help care for offspring. In starlings, both sexes incubate the eggs and feed the nestlings, with the females performing most (70%) of the incubation (Feare, 1984). Reports regarding the division of labor between the sexes for nestling provisioning vary, with females making more feeding trips to the nest than males or both sexes provisioning at equal rates (Feare, 1984). Thus, males provide a substantial amount of parental care and females would have a selective advantage if they could predict which males would provide the best care for their young. However, the evidence to date indicates that male song does not provide such a cue for females. Repertoire size was not correlated with time spent incubating eggs or the rate of nestling provisioning among males in a Canadian population of starlings (Mountjoy and Lemon, 1997). Furthermore, in a Belgian population of starlings in which polygyny is common, males with the most complex song are more likely to obtain a second mate, and polygynous males provide less parental care than their monogamous counterparts (Eens, 1997).

Taken together, these data indicate that male starling song probably does not function as an indicator to females regarding which males can offer the most direct material benefits. Thus, starlings are good candidates for investigating other forms of indicator mechanisms, often referred to as "good genes" models, in which females benefit indirectly by mating with males of superior genetic quality.

## B. INDIRECT BENEFITS

Much attention has been given to the hypothesis that song performance indicates potential indirect benefits for females via transmission of good genes to offspring. Good genes models refer to any male phenotype that is heritable and provides some selective advantage to offspring that inherit the trait, thus providing the female with increased reproductive success (Johnstone, 1995; Pomiankowski, 1988; Williams, 1966; Zahavi, 1975). The phenotype in question can be body condition in general, the ability to resist disease, or any aspect of genetic quality that increases the probability of survival or mating success of the female's offspring (Fisher, 1930; Folstad and Karter, 1992; O'Donald, 1967, 1980).

Male starlings with larger repertoires are, on average, older (Eens, 1997). Song also has been correlated with body condition in starlings (Mountjoy

and Lemon, 1996), with males possessing larger repertoires scoring higher on an index of body condition (measured as the residuals of a regression of body weight on tarsus length). Furthermore, some evidence indicates that males with larger repertoires are also more dominant (Eens, 1997; Spencer *et al.*, 2004), suggesting that these males may have better access to food resources. Thus, male song could be an indicator of some underlying genetic quality that increases survival ability.

### *1. Immunocompetence Handicap Hypothesis*

One way in which survival ability could manifest itself is in an individual's capacity to resist disease (i.e., immunocompetence). The immunocompetence handicap hypothesis (ICHH) proposes a mechanism through which an elaborate trait (such as complex song) could be an honest and reliable signal of immunocompetence (Folstad and Karter, 1992). The ICHH proposes that some factor, such as T, which enhances the development of secondary sexual characteristics, also suppresses immunity. According to the ICHH, only those males that possess superior immunocompetence will be able to tolerate the immunosuppressive effects of T and thus be able to maintain the high concentrations of T associated with song production during the breeding season. Thus, females who choose to mate with males exhibiting the most elaborate or most complex song would then benefit, either directly or indirectly, by mating with the most immunocompetent males. A possible direct benefit for choosy females would be that forming pair bonds with males possessing superior disease resistance may reduce their exposure to pathogens. Indirectly, females could enhance their reproductive success if immunocompetence is a heritable trait that is passed on to offspring, increasing their probability of survival (Folstad and Karter, 1992). The literature on the ICHH is vast (see Garamszegi, 2005; Roberts *et al.*, 2004 for reviews) and much of it is beyond the scope of this chapter, thus we will limit our review of the ICHH to how it relates to starlings.

In support of one component of the ICHH, recent reports provide strong evidence that male song is an indicator of immunocompetence in starlings (Buchanan *et al.*, 2003b; Duffy and Ball, 2002; Spencer *et al.*, 2004). The first such study revealed that, among adult males, two measures of song performance were positively correlated with two components of the adaptive immune response (Fig. 5; Duffy and Ball, 2002). Song rate was strongly correlated with cell-mediated immunity, measured as the skin-swelling response to injection of phytohemagglutinin (PHA). PHA is a common protein that is known to elicit proliferation of cells involved in cell-mediated immunity (T cells). When injected under the skin, it induces the trafficking of T cells to the site of injection, resulting in swelling. Song bout

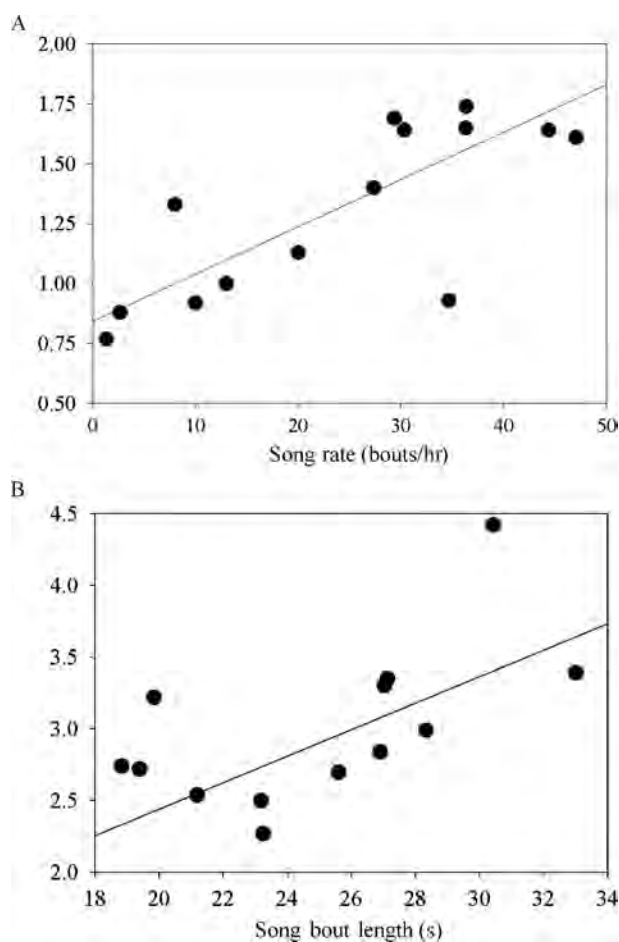


FIG. 5. (A) Wing web swelling (mm) 48 hr postinjection of PHA in relation to song rate. The linear regression line has the equation  $y = 0.77 + 0.02x$ ,  $r^2 = 0.719$ . (B) Anti-KLH antibody titers (mean absorbance values relative to negative controls), averaged across days 10 and 15, in relation to song bout length (s). The linear regression line has the equation  $y = 1.18 + 0.18x$ ,  $r^2 = 0.388$ . [Adapted from Duffy D. L., and Ball, G. F. (2002). Song predicts immunocompetence in male European starlings. *Proc. Roy. Soc. Lond. Ser. B-Biol. Sci.* **269**(1493), p. 849, Fig. 1.]

length was correlated with humoral immunity, measured as the antibody response to inoculation with another inert foreign protein (keyhole limpet hemocyanin or KLH; Fig. 5; Duffy and Ball, 2002). Similarly, a later report demonstrated a positive relationship between the PHA response

as fledglings and song bout length assessed 7 months later (Buchanan *et al.*, 2003b). In that same study the opposite relationship was found between PHA response and song bout length among birds that had received an unpredictable food supply as fledglings, suggesting that resource reallocation occurs during times of nutritional stress (Buchanan *et al.*, 2003b). Finally, the latest evidence indicates a positive relationship between PHA response and repertoire size among male starlings (Spencer *et al.*, 2004). Importantly, the birds in each of the aforementioned studies were the same age; thus age alone cannot account for the variation in song or immunocompetence.

Taken together, these experimental studies in starlings provide some of the strongest evidence in support of the hypothesis that song features can function as indicators of immune capability. The open question that remains to be established, however, is whether these relationships between male song performance and immune responsiveness translate into increased fitness among females. Mathematical modeling suggests that the advantage for females in choosing more immunocompetent males will vary depending on the dynamics of the pathogens prevalent in a specific population (Adamo and Spiteri, 2005). Furthermore, as researchers in the field have recently noted, the degree to which more robust responses to immune challenge reflect greater disease and/or parasite resistance remains an empirical question that is likely to be context and pathogen specific (Adamo, 2004; Viney *et al.*, 2005).

Finally, there is evidence suggesting that males that are more disease resistant are superior in other ways, some of which may be heritable (Garamszegi, 2005; Gleeson *et al.*, 2005). Thus, females may benefit not from males' immunocompetence per se, but from an overall superior genetic quality that manifests itself in increased resistance to disease among other things (Adamo and Spiteri, 2005). Song parameters are positively correlated with both age and body condition in starlings (Eens, 1997; Mountjoy and Lemon, 1996), lending support to this idea. Further study assessing various facets of immune function, resistance to disease and, most importantly, measures of fitness of choosy versus nonchoosy females is needed (Adamo, 2004; Adamo and Spiteri, 2005; Viney *et al.*, 2005). Despite these gaps in our knowledge, the evidence linking song to immune responsiveness in starlings is consistent and makes a compelling case in favor of one of the major tenets of the ICHH.

The primary feature that sets the ICHH apart from Hamilton and Zuk's original hypothesis (1982) linking secondary sexual traits and health is the double-edged-sword effect of some hormone (e.g., T) proposed by Folstad and Karter (1992) as the mechanism mediating the relationship. According to the ICHH, T should enhance song while suppressing immune function.

The presence of T is known to facilitate singing behavior in male birds of many species, including starlings (Arnold, 1975; Ball *et al.*, 2002; Catchpole and Slater, 1995; DeVoogd, 1991; Eens, 1997; Marler *et al.*, 1987). Similar to other songbirds, starlings increase their song output during the breeding season when T concentrations are elevated. However, starlings also sing during the fall when secretion of T from the testes is minimal at best (Eens, 1997). Thus, while song may be enhanced by high T concentrations, it does not appear to be dependent on high gonadal T secretion (Ball *et al.*, 2002). One report suggests that song bout length and the ability of certain social factors to induce singing by male starlings increase in the spring when T concentrations are high compared with autumn when T is undetectable (Riters *et al.*, 2000). Furthermore, singing in the absence of a female is not affected by castration and/or implantation with T; whereas castration results in a significant reduction of female-directed song by male starlings that is restored with subsequent T treatment (Pinxten *et al.*, 2002). However, at least three studies have failed to find any correlation between plasma T concentration and song bout length or song rate in starlings (Buchanan *et al.*, 2003b; Duffy and Ball, 2002; Sartor and Ball, 2005). Therefore, the relationship between T and singing in starlings appears to be context dependent and nonlinear.

The third main prediction of the ICHH that T should act as a handicap mechanism via immunosuppression remains its most controversial facet. Across various taxa, the evidence regarding whether T is immunosuppressive remains inconclusive. A meta-analysis that included birds, mammals, and reptiles found a suppressive effect of T on immunity; however, this effect disappeared when the analyses were corrected for multiple studies on the same species (Roberts *et al.*, 2004). While some evidence exists that T suppresses immune function in starlings (De Ridder *et al.*, 2002; Duffy and Ball, 2002; Duffy *et al.*, 2000), alternative explanations cannot yet be ruled out. As predicted by the ICHH, physiological doses of exogenous T in male starlings in nonbreeding condition (photorefractory) resulted in a reduction of both cell-mediated and humoral immune responses to PHA and KLH, respectively, while a similar dose in females significantly decreased humoral immune responses (Duffy *et al.*, 2000; Fig. 6). Furthermore, the effect of exogenous T on antibody responses to KLH was dose dependent (Duffy *et al.*, 2000). Similarly, a negative correlation was found between natural variation in endogenous T and antibody responses to KLH among male starlings (Fig. 7; Duffy and Ball, 2002). Furthermore, treatment of female starlings with male-like doses of exogenous T resulted in increased bacterial infection compared to controls (De Ridder *et al.*, 2002). It is notable that a consistent negative relationship between T and immunity has been observed in starlings, regardless of the source of

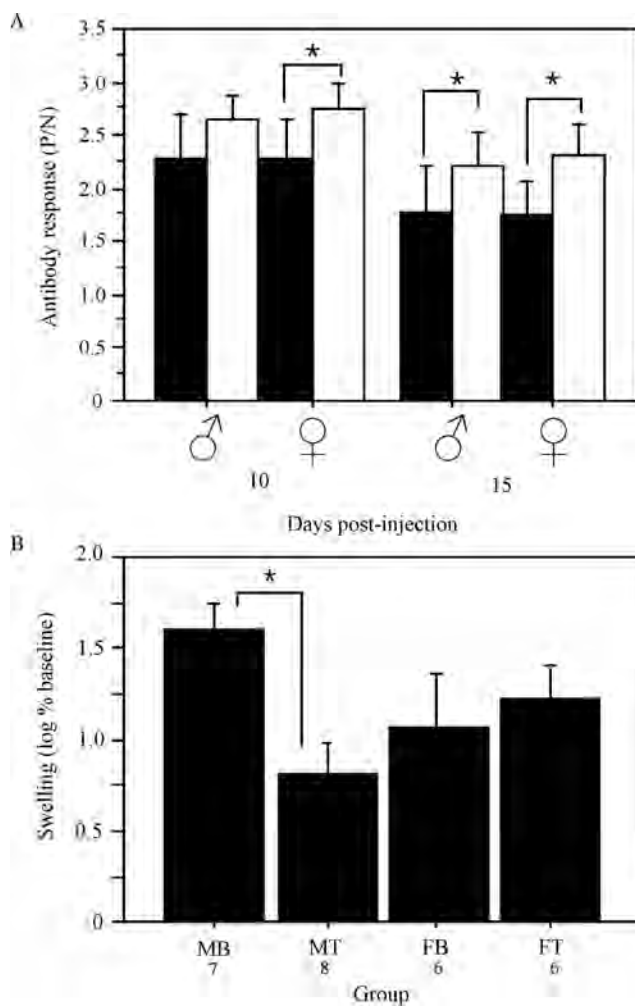


FIG. 6. (A) Anti-KLH antibody titers relative to negative controls at 10 and 15 days postinjection. Error bars represent 95% confidence intervals. Asterisk =  $p < 0.05$  relative to controls. Open bars = blank implants and filled bars = T implants. (B) Log transformed values of the mean ( $\pm$  SE) change in web swelling (% baseline) 24 hr after injection with PHA. Asterisk =  $p < 0.05$  relative to controls (MB = males with blank implants, MT = males with T implants, FB = females with blank implants, and FT = females with T implants). Numbers below the x-axis indicate the sample size for each group. [Adapted from Duffy, D. L., Bentley, G. E., Drazen, D. L., and Ball, G. F. (2000). Effects of testosterone on cell-mediated and humoral immunity in non-breeding adult European starlings. *Behav. Ecol.* **11**(6), p. 658, Fig. 4, by permission of Oxford University Press.]

T (exogenous or endogenous) and using different measures of immunity (response to antigenic challenge or bacterial infection). However, given that two of the three aforementioned studies with starlings involved administration of T to birds that normally would not experience high T concentrations

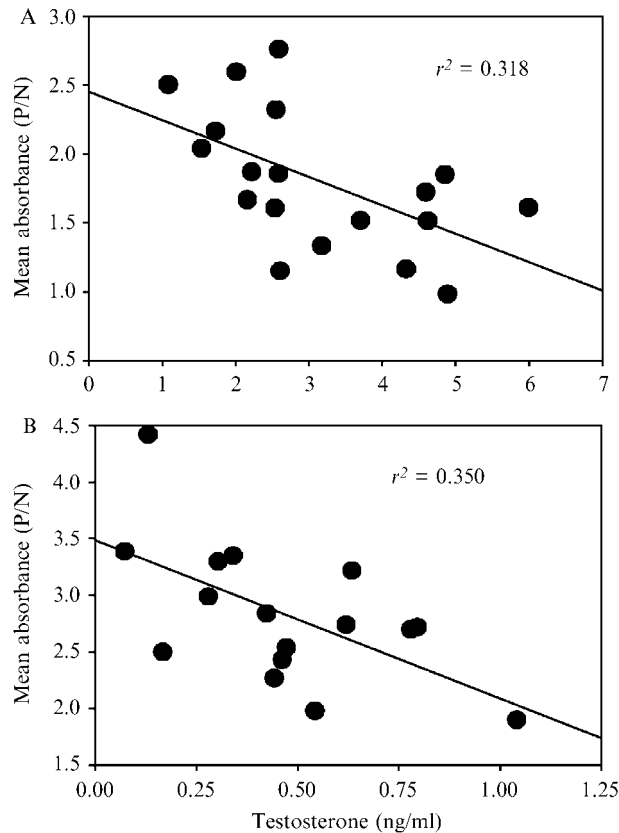


FIG. 7. (A) Anti-KLH antibody titers relative to negative controls (day 15 postinjection) of T-treated birds as a function of exogenous T concentrations (ng/ml). The linear regression line has the equation  $y = 2.450 - .206x$ ;  $r^2 = .318$ ,  $p < .01$ ; (B) Anti-KLH antibody titers relative to negative controls (averaged across days 10 and 15 postinjection) in relation to endogenous plasma T concentrations (ng/ml). The linear regression line has the equation  $y = 3.92 - 1.40x$ ;  $r^2 = 0.350$ . [Part A adapted from Duffy, D. L., Bentley, G. E., Drazen, D. L., and Ball, G. F. (2000). Effects of testosterone on cell-mediated and humoral immunity in non-breeding adult European starlings. *Behav. Ecol.* **11**(6), p. 657, Fig. 3, by permission of Oxford University Press; part B: adapted from Duffy, D. L., and Ball, G. F. (2002). Song predicts immunocompetence in male European starlings, *Proc. Roy. Soc. Lond. Ser. B-Biol. Sci.* **269**(1493), p. 850, Fig. 2.]

(nonbreeding males and females), further study is required to corroborate the immunosuppressive effect of T under natural conditions.

While the data from starlings thus far appear to support the predictions of ICHH, it remains possible that a more complex mechanism may be involved. For instance, artificial elevations of T have been demonstrated to induce concomitant increases in circulating corticosterone (CORT) in a number of bird species, including dark-eyed juncos (*Junco hyemalis*, Casto *et al.*, 2001; Klukowski *et al.*, 1997), house sparrows (*Passer domesticus*, Evans *et al.*, 2000; Poiani *et al.*, 2000), song sparrows (Owen-Ashley *et al.*, 2004), and starlings (Duffy *et al.*, 2000). Chronic elevation of CORT (e.g., days or weeks) generally has been demonstrated to be immunosuppressive (Buchanan, 2000). Thus, the immunosuppressive effects of T treatment observed in the aforementioned studies could in fact be due to the concomitant T-induced rise in CORT. One study in house sparrows indicates that when the immunosuppressive effects of CORT are statistically controlled for, T has an enhancing effect on immune responses (Evans *et al.*, 2000). However, the relationship between naturally occurring fluctuations in T and CORT remains poorly understood.

Most studies have found an inverse relationship between T and CORT, primarily via a stress-induced rise in CORT simultaneous with a decrease in T (Knol, 1991; Silverin, 1998). For example, following mate-choice trials in which males competed for females, chosen males were found to have elevated CORT and decreased T concentrations (Sorenson *et al.*, 1997). In contrast, a positive correlation between naturally occurring seasonal fluctuations in T and baseline CORT has been reported in dark-eyed juncos (Deviche *et al.*, 2000). And yet, evidence in house sparrows indicates that, while exogenous T increased CORT concentrations in the postbreeding season, a negative correlation was seen with endogenous T and baseline CORT concentrations in intact males during the breeding season (Buchanan *et al.*, 2003a). Thus, various factors must be considered when assessing physiological and behavioral correlates of one or both of these steroids, such as the timing of measurement (e.g., time of year, baseline or stress induced), and whether the observed relationship involves naturally occurring endocrine responses to environmental stimuli or is the result of manipulation of either T or CORT.

The issue is further complicated by the observation that T also can cause an increase in corticosteroid-binding globulin (CGB), which binds to both T and CORT (although CORT has a greater affinity; Deviche *et al.*, 2001; Schoech *et al.*, 1999). The complexity of the interactions between T and CORT remains a challenge to understanding the relationship between the endocrine and immune systems. Additional studies are needed in which factors, such as dose and duration of hormone treatment, social milieu, and



the limitations of various immune measures, are considered (Braude *et al.*, 1999; Poiani *et al.*, 2000). Furthermore, new hypotheses that have been proposed to explain the relationships among hormones, immunocompetence, and sexually selected signals cannot be ruled out by the existing data and deserve closer attention (Braude *et al.*, 1999; Buchanan, 2000; Poiani *et al.*, 2000).

In sum, these studies provide strong evidence in support of the hypothesis that features of male starling song that are important to females during mate selection are indicators of immune function. At this point, however, the mechanism underlying this relationship remains unclear. It has become increasingly evident that the mechanism as originally proposed by the ICHH is too simplistic to explain the relationship between song and immune responsiveness. Though T is likely involved to some degree, more recent efforts have indicated that CORT probably plays a pivotal role. Accordingly, new hypotheses have been proposed in which the stress response is a key factor underlying the development or expression of condition-dependent signals (Braude *et al.*, 1999; Buchanan, 2000; Nowicki *et al.*, 1998, 2002a; Poiani *et al.*, 2000). One such hypothesis is the “developmental stress hypothesis” (Nowicki *et al.*, 1998, 2002a).

## 2. Developmental Stress Hypothesis

Brain structures important for song learning and production, and their interconnections, develop early in life within weeks after hatching (Catchpole and Slater, 1995). It is during this time that young birds are most likely to experience physiological stress, for example, undernourishment (Nowicki *et al.*, 1998). According to the developmental stress hypothesis (originally termed the “nutritional stress hypothesis”), stressors, such as inadequate nutrition during the period of development of the song control system, result in a delayed or decreased ability to learn song, for which birds may not be able to fully compensate (Nowicki *et al.*, 1998, 2002a). Thus, in adulthood, learned song features may reliably reflect the extent of or the response to stressors experienced early in life. By choosing males based on learned song features, such as repertoire size, females are able to select those males who were better able to cope with early developmental stress and, thus, are presumably of superior genotypic quality (Nowicki *et al.*, 1998, 2002a).

Recent reports in starlings add to a growing body of literature in support of the developmental stress hypothesis (Buchanan *et al.*, 2003b; Spencer *et al.*, 2004). Starlings that experienced unpredictable short-term food deprivation on a daily basis for 3 months after fledging showed a decrease in the quality and quantity of song produced the following spring compared to controls that received an *ad libitum* food supply (Buchanan *et al.*, 2003b;

Spencer *et al.*, 2004). Birds that received the unpredictable food supply spent less time singing, showed a longer latency to begin singing, sang fewer and shorter song bouts, and had smaller repertoires compared to controls (Buchanan *et al.*, 2003b; Spencer *et al.*, 2004; Fig. 8).

Furthermore, birds in the unpredictable food supply group tended to have higher peak CORT concentrations than controls (Buchanan *et al.*, 2003b). This again suggests that this stress hormone could be acting as a physiological constraint on the development or expression of sexually selected song features. Conversely, peak CORT during development was positively correlated with repertoire size as adults (Spencer *et al.*, 2004). While the role of CORT in song development in starlings remains unclear, a study of zebra finches demonstrated that experimental manipulation of

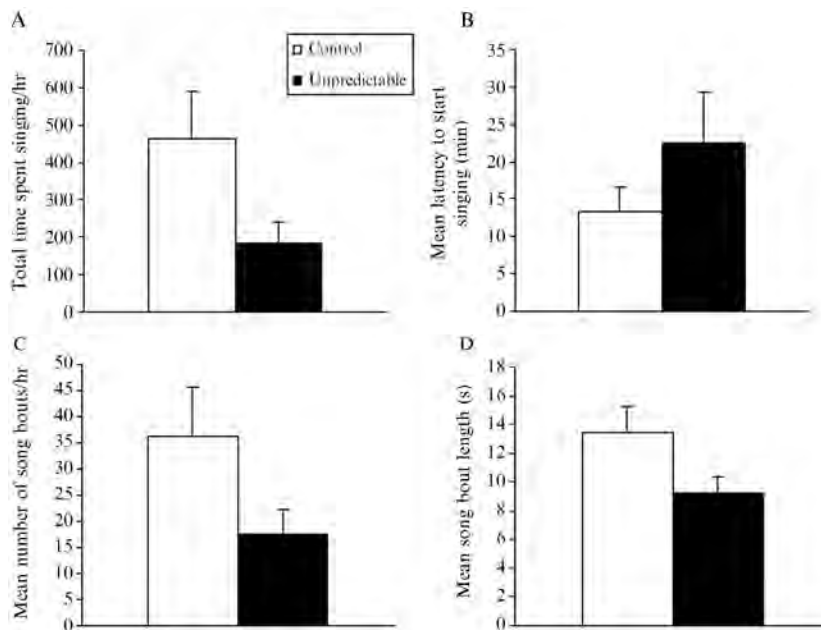


FIG. 8. Song production of males in two treatment groups: unpredictable food supply and *ad libitum*-fed controls. (A) Mean total time spent singing (s/hr); (B) mean latency to start singing; (C) mean number of songs bouts/hr; and (D) mean song bout duration. [Adapted from Buchanan, K. L., Spencer, K. A., Goldsmith, A. R., and Catchpole, C. K. (2003b). Song as an honest signal of past developmental stress in the European starling (*Sturnus vulgaris*). *Proc. Roy. Soc. Lond. Ser. B-Biol. Sci.* **270**, p. 1154, Fig. 4, by permission of The Royal Society.]

CORT in nestlings affected adult song output in a similar manner as food restriction (Spencer *et al.*, 2003).

The core of the developmental stress hypothesis is that variation in exposure or response to stressors early in life results in variation in neural development, specifically within the song control system, which is reflected in variation of learned song features as an adult (Nowicki and Searcy, 2004; Nowicki *et al.*, 2002). One assumption of this hypothesis is that the morphology of the song control system correlates with production of learned song features. In agreement with this assumption is the observation that the volumes of both RA and HVC are correlated with song bout length and HVC is correlated with song rate in male starlings (Bernard *et al.*, 1996; Sartor and Ball, 2005), as mentioned earlier in this chapter. However, because this evidence is correlational, the direction of causality cannot be determined. Although Buchanan *et al.* (2003b) did not examine the song control system in their study, it would be interesting to learn whether manipulation of developmental stressors early in life result in subsequent differential development of the song control system. In swamp sparrows (*Melospiza georgiana*), Nowicki *et al.* (2002) demonstrated that a restricted diet early in development led to smaller volumes of HVC and RA in adulthood; however, when differences in telencephalon volume were controlled only the difference in RA volume remained significant. At the time of this writing, only a handful of studies have tested the developmental stress hypothesis but thus far the evidence is promising.

Importantly, the ICHH and the developmental stress hypothesis are not mutually exclusive. In addition to neural development, stress experienced early in development also can affect immune function. In fact, a study of starling nestlings indicates that the effect of early developmental stress on immune responses is sex specific and resource dependent (Chin *et al.*, 2005). Studying natural variation in a wild population of starlings, Chin *et al.* (2005) found that when resource availability was low, thus lowering parental provisioning rate and chick growth rate, larger brood size negatively affected cell-mediated immune responsiveness to PHA in male nestlings, whereas no effect was observed in female nestlings. In a resource-rich environment, however, brood size had no effect on immune responses of nestlings of either sex, suggesting that the trade-off responsible for higher sensitivity to developmental stress among males compared to females may be compensated for under resource-abundant conditions (Chin *et al.*, 2005). This resource-dependent effect of developmental stress echoes that found by Buchanan *et al.* (2003b) in which unpredictable food resources resulted in a negative relationship between immune responsiveness as nestlings and later song performance, while abundant food resources revealed a positive relationship. Furthermore, the sex-specific nature of these relationships would be

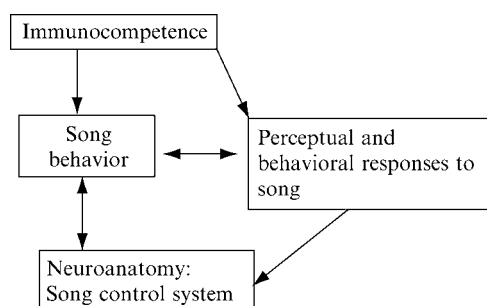


FIG. 9 Schematic diagram illustrating interrelationships among song production in male starlings, the associated neuroanatomy of the song system that is positively correlated with song bout length, song preferences in female starlings that are related to biases in *zenk* expression in their auditory forebrain, and immunocompetence. Plasmas T is one physiological variable that is involved in coordinating these different traits. See text for more details.

expected if there is a trade-off between the development of sexually-selected characteristics and other energetically expensive processes (e.g., somatic growth, immune function).

A hypothesis has recently been put forward that links immunity to neural development (Moller *et al.*, 2005). Specifically, it has been hypothesized that parasitic infection can have negative consequences for neural development and learning. In turn, higher susceptibility to infection in males may result in greater investment in immune defense compared to females. The prediction that follows is that males that have evolved strong immune systems will be least likely to suffer from infection and therefore should be capable of developing larger brains (Moller *et al.*, 2005). When Moller *et al.* (2005) compared the relative size of various body organs to brain size across different bird species, they found that relative size of organs involved in immune function (bursa of Fabricius and spleen) covaried positively with relative brain size. Furthermore, there was a significant positive correlation between the relative mass of immune defense organs and the degree of sexual dimorphism in brain size, indicating stronger selection pressure for males compared to females. In contrast, scant evidence indicated such covariation among the sizes of immune defense organs or brain size, and with the sizes of the heart or liver (Moller *et al.*, 2005).

### 3. *Sexy Son Hypothesis*

Given what we know about the relationships between multiple aspects of male quality (e.g., age, condition, immunocompetence, developmental stress, brain morphology) and song features that are important during mate

choice, it seems reasonable to conclude that song functions as a condition-dependent indicator in starlings. However, it is notable that one recent study offers support for a hypothesis that does not require a male's attractiveness to be dependent on his condition. The "sexy son hypothesis" states that females benefit by mating with highly attractive (polygynous) males at the cost of reduced paternal care when their sons inherit those attractive qualities and thus have better mating success, providing their mothers with more grandchildren (Weatherhead, 1994; Weatherhead and Robertson, 1979, 1981).

Gwinner and Schwabl (2005) reported that sons of polygynous starlings possessed superior competitive ability in contests for nest boxes and performed more female-directed song compared to sons of monogamous males (Gwinner and Schwabl, 2005). However, there was only a nonsignificant tendency for sons of polygynous males to attract more females than sons of monogamous males in an aviary setting. The authors suggest that their hand-rearing and housing conditions may have hindered adequate testing of female choice, leading to the lack of a significant effect. Importantly, the competitive advantages gained by sons of polygynous males could not be attributed to differences in maternal deposition of hormones into the eggs, though other early environmental effects during the nestling stage could not be ruled out.

It is worth noting that although the sexy son hypothesis does not *require* attractiveness to be condition dependent, it does not preclude it either. In fact, it has been recently proposed that the Fisherian sexy son hypothesis and the quality-indicator good genes' models are complementary to one another and their relative weights depend on the costs associated with being choosy (Kokko, 2001; Kokko *et al.*, 2002; Radwan, 2002). Taken together, the studies discussed herein strongly suggest that, by selecting males based on singing performance, choosy females are likely to obtain a variety of indirect fitness benefits.

#### 4. Future Directions

The next logical step in exploring the functional basis of starling song is to empirically establish the fitness benefits gained by choosy females that mate with males that display superior song performance. To accomplish this, it is necessary to turn more focus toward the offspring. For instance, do females paired with robust singers fledge more young (Forstmeier and Leisler, 2004; Gil and Slater, 2000; Reid *et al.*, 2005; Sheldon *et al.*, 1997)? Do their offspring experience mating or reproductive advantages compared to the offspring of nonchoosy females (Head *et al.*, 2005)? Do the offspring of highly immunocompetent males exhibit superior disease resistance (Johnsen *et al.*, 2000; Kleven and Lifjeld, 2004)?

In addition, more focus needs to be directed toward the mechanisms and evolution of female mate preferences. For example, how much variation exists among (and within) females in their preferences and what maintains that variation (Forstmeier and Birkhead, 2004)? Earlier in this chapter, we discussed how recent exposure to short song bouts versus long song bouts can alter neural responses to novel songs of different bout lengths (Sockman *et al.*, 2002). How does this neural plasticity translate into behavioral responses to song? What factors influence plasticity in mate choice and what are the fitness advantages for various alternative strategies? Furthermore, why have particular mechanisms evolved as opposed to some other alternative processes? For example, a few different hypotheses have been put forward to explain the function of steroid-induced immunosuppression (Besedovsky and DelRey, 1996; Derting and Virk, 2005; McEwen *et al.*, 1997; Muehlenbein and Bribiescas, 2005; Raberg *et al.*, 1998; Wedekind and Folstad, 1994). Could evolution has co-opted these processes and applied them to sexually selected traits? The answers to these questions would broaden what we know about the functional significance of song in starlings.

#### VII. PUTTING IT ALL TOGETHER: SONG PRODUCTION/PERCEPTION AND HORMONES

In this chapter, we have tried to tie together a number of different types of findings about the mechanism and function of song in European starlings. In this concluding section we make a few general points. First, one theme that emerges from this work is how a basic behavioral observation, namely that male starling song of a particular type (i.e., long-bout song) is attractive to female starlings and is used for mate choice, can guide an entire research program in valuable ways. This fact along with the related observation that male song sung in the breeding season is responsive to the presence of a female, while song produced outside the breeding season is not has guided an entire series of mechanistic studies in valuable ways. For example, the regulation of male starling song by the steroid hormone T can only be explained if one realizes that female-directed song is enhanced by T but not song produced for other reasons outside the breeding context (Pinxten *et al.*, 2002). The importance of long-bout song for female choice has also guided studies on song production and perception. Variation in the volume of key song nuclei HVC and RA in starlings positively correlates with song bout length in males older than 1 year (Bernard *et al.*, 1996).

The expression of the IEG *zenk* in the auditory telencephalon of female starlings is best explained if one considers variation in song bout length (Gentner *et al.*, 2001; Sockman *et al.*, 2002, 2005). Thus, aspects of both male neuroanatomy and female neural responses in starlings can be explained by taking account of the functional significance of behavior. Finally, the potential functional significance of song as a predictor of male immunocompetence was again revealed when simple immune measures were correlated with the appropriate measures of song performance (Duffy and Ball, 2002). The challenge now is to understand the web of causality that explains these intriguing correlations between song behavior and various aspects of brain and physiology.

The gonadal hormone T is involved in the interplay between female choice and various aspects of male behavior, neuroanatomy, and physiology in that it regulates to some degree all these aspects of the male phenotype in starlings. As just noted, T stimulates male song behavior during the breeding season (Pinxten *et al.*, 2002) and it promotes the growth of song nuclei such as HVC and RA (Bernard and Ball, 1995, 1997). It also inhibits cell-mediated and humoral immune measures in male starlings, although this may be through increased CORT secretion (Duffy *et al.*, 2000). What is needed now is to take neuroendocrine studies to the next level of sophistication. The arrow of causation needs to be elucidated to explain these correlations. Is variation in the volume of song nuclei, such as HVC and RA, permissive for long-bout starling song or a consequence of engaging in such song (Sartor and Ball, 2005). How and where does T act to enhance only male-directed song? Is that an effect of its action in the preoptic area and other areas related to song motivation (Riters and Ball, 1999; Riters *et al.*, 2000)? Is the fact that female-directed song only occurs during the breeding season related merely to seasonal variation in T concentrations or does the central control of song as it relates to stimulus context also change in a seasonal fashion? For example, this seasonal change in the stimulus that elicits male song could be related to seasonal changes in the volume of the preoptic nucleus or perhaps to dramatic seasonal changes in hormone receptor types that have been described in the song system of starlings (Bentley and Ball, 2000). We know in zebra finches that song directed at females exhibits a very different pattern of IEG expression than song that is undirected (Jarvis *et al.*, 1998). This context effect is regulated at least in part by ascending noradrenergic projections to song nuclei such as area X (Castelino and Ball, 2005). Are these modulatory inputs to the song system changing seasonally and resetting the way in which song is used? It is clear that an entirely new generation of mechanistic studies is now in order.

If these continue to be guided by a sensitivity to behavior and its functional context, the future for the neuroethological approach is promising indeed.

### VIII. SUMMARY

Male European starlings produce a long complex song that is used by females to choose mates in a breeding context. Females tend to prefer males that produce songs organized into long bouts. The production of song is controlled by a specialized neural circuit called the song control system that is present in all oscine songbirds. Using long-bout song as a tool allowed us to explicate several aspects of the neural control of song perception and production in starlings. Variation in the volume of key song control nuclei, such as HVC and RA, correlates positively with variation in song bout length in adult starlings. IEG expression in the auditory forebrain of females is enhanced in response to long-bout song as compared to conspecific song organized into short bouts. Long-bout song as well as high rates of singing also predicts variation in measures of cellular and humoral immunity in male starlings. The cause and effect relationships among these variables still need to be elucidated. But T enhances the size of song control nuclei in starlings that correlate with the production of long-bout song preferred by females. Female choice therefore drives aspects of male physiology that results in long-bout song. In the female auditory forebrain, there is evidence for physiological responses to song as measured by IEG expression and electrophysiology that are tuned to aspects of male song that they prefer. However, these neural biases exhibit a plasticity that allows the females to modify their neural responses and memorize male songs as a function of the types of songs they encounter in their local social environment. These studies illustrate how behavioral investigations of functional significance can provide tools to implement a neuroethological investigation of behavioral mechanisms (see Fig. 9).

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