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Anonymous and gene-linked microsatellite markers reveal no correlation between heterozygosity and song complexity in a wild population of song sparrows

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Anonymous and gene-linked microsatellite markers reveal no correlation between heterozygosity and song complexity in a wild population of song sparrows (*Melospiza melodia*)

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Abstract

Correlations between heterozygosity and fitness are commonly reported in the literature, but there is disagreement about the frequency of such correlations and the mechanisms which underlie them. Using a multi-year data set acquired from a wild population of song sparrows I investigated the relationship between heterozygosity and song complexity, an important sexually selected trait thought to be an indicator of genetic quality. Heterozygosity was determined at 17 putatively neutral microsatellites and 5 microsatellites derived from the expressed sequence tag of zebra finch brain proteins. Song and syllable repertoire size were taken as measures of song complexity. Neither genetic marker type was predictive of song complexity. A further test failed to support the hypothesis that heterozygosity-fitness correlations at marker loci are due to closely linked coding genes. These results indicate that heterozygosity is unlikely to play a major role in individual song complexity in this outbred population of song sparrows.

Keywords: Heterozygosity-Fitness Correlations, Song Complexity, Song Sparrows, *Melospiza melodia*, EST-SSRs, Local Effects, Global Effects

Co-authorship

My supervisor, Dr. Elizabeth MacDougall-Shackleton will be a co-author on any publications arising from this work.

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Introduction

It has long been recognized that genetic diversity is important to the long term viability of populations as it provides the raw material required for evolution by natural selection (Rails et al. 1988; Frankham 1995). A population that lacks sufficient genetic diversity is, for example, unable to evolve to better suit its environment or to adapt to ecological changes. Given the widespread implications of population genetic diversity, from agriculture to conservation, it is important to understand the mechanisms that lead to changes in genetic diversity in natural populations. With low levels of mutation and sustained directional selection it might be expected that genetic diversity would steadily decrease over time (e.g. Makinen et al. 2008), but there are natural mechanisms which preserve population genetic diversity. Although neutral theory suggests that much of the genetic variation seen in nature may simply be due to drifting unselected variation (Kimura 1991) several evolutionary forces have been identified that contribute to the maintenance of population genetic diversity, including the influx of novel alleles via migration (e.g. Wooding 1998), spatial and temporal variation in selection pressure (e.g. Prout 1968, Ellner and Hairston 1994), and mate choice associated with inbreeding avoidance (Sherborne et al. 2007; Neff and Pitcher 2009).

Inbreeding depression, the often observed decrease in fitness of offspring produced by consanguineous mating (Charlesworth and Willis 2009), and heterosis, the increase in fitness observed in the offspring of matings between distantly related individuals (Darwin 1876; Tulu 2001) are thought to be caused by two major mechanisms, termed dominance and overdominance effects. First, when deleterious

recessive alleles are widespread, such effects may occur through dominance effects (Charlesworth and Willis 2009), such that mating between individuals of similar genotypes produces offspring with a higher level of expressed deleterious recessive alleles, whereas mating between dissimilar individuals is more likely to mask each parent's deleterious recessive alleles as they will express only the dominant non-deleterious alleles. Such dominance effects have been demonstrated by Xiao et al. (1995) in the *Oryza indica X Oryza japonica* rice hybrid. Second, inbreeding depression and heterosis can arise through heterozygote advantage or overdominance, due to advantages associated with heterozygosity per se. For example, heterozygosity at the major histocompatibility complex (MHC) is often associated with enhanced disease resistance, presumably because MHC heterozygotes can defend against a broader array of pathogens (e.g. Penn et al. 2002). Similarly, some enzymes may function more efficiently in heterozygotes (e.g. octopine dehydrogenase; Sarver et al. 1992), resulting in enhanced metabolic efficiency and fitness.

Inbreeding depression has been well demonstrated in laboratory studies. An extensive comparison of self-fertilized (selfed) versus non-self-fertilized (crossed) plants was carried out by Darwin (1876) and showed across several species that the offspring of crossed plants outperform the offspring of selfed plants in fitness-related traits such as plant size and seed production. Since then, there have been many demonstrations of inbreeding depression in laboratory populations. For example, a study by Van Oosterhout (2000) showed negative effects of inbreeding on fecundity, survival and longevity in a laboratory population of the butterfly *Bicyclus anynana*, suggesting that inbreeding presents a substantial risk to population viability. Importantly, theory predicts that

inbreeding depression should be even more pronounced in wild populations due to the harsher and less stable conditions typically encountered in the wild (Miller 1994). A meta-analysis by Crnokrak and Roff (1999) supported the prediction that inbreeding depression should be harsher in more stressful conditions, as have studies on both laboratory (*Drosophila melanogaster*; Miller 1994) and wild populations (*Geospiza scandens*; Keller et al. 2002). However, such studies generally rely on inbreeding coefficients derived from complete pedigrees, which are difficult to quantify in wild populations, especially those that are very large or experience frequent immigration such that pedigree analysis is not feasible. As a result of the difficulty of calculating inbreeding coefficients in large and open populations, most early demonstrations of inbreeding depression in the wild were restricted to a small number of isolated (often island) populations where the pedigree of every individual was known (e.g. Mandarte Island song sparrows *Melospiza melodia*, Keller et al. 1994, Keller 1998, Reid et al. 2003; Galapagos Island Finches *Geospiza sp.*, Keller et al. 2002; Soay sheep *Ovis aries*, Overall et al. 2005; Scandinavian wolves *Canis lupis*, Liberg et al. 2005). It is important to note that while inbreeding depression has been well demonstrated, outbreeding has been shown to be disadvantageous in certain situations as well (Fenster and Galloway, 1999) as it may break up locally adapted gene complexes or introduce alleles not suited to the local environment.

The advent of soluble allozyme markers, variable enzymes produced at a single locus, and later microsatellites, short repetitive sequences of DNA, gave researchers the opportunity to directly assess individual heterozygosity and thus extend studies of inbreeding effects into non-insular natural populations, even those with large population

sizes and frequent immigration. Early studies using allozyme markers in natural populations showed correlations between marker heterozygosity and a wide variety of traits such as growth rate in American oysters (*Crassostrea virginica*; Singh & Zouros 1978) and tiger salamander (*Ambystoma tigrinum*; Pierce and Mitton 1982) and mating success in brine shrimp (*Artemia franciscana*; Zapata et al. 1990). More recent studies using putatively neutral microsatellite markers, which are not thought to add to nor detract from individual fitness, also frequently show correlations between marker heterozygosity and many fitness components, including disease susceptibility in sea lions (*Zalophus californianus;* Acevedo-Whitehouse et al. 2003), parasite load in white-crowned sparrows (*Zonotrichia leucophrys*; MacDougall-Shackleton et al. 2005), song repertoire size in song sparrows (Reid et al. 2005), fecundity in Chinook salmon (*Oncorhynchus tshawytscha;* Heath et al. 2002) and juvenile survival in alpine marmots (*Marmota marmot;* Cohas et al. 2009). However, despite the widespread reporting of such heterozygosity-fitness correlations (HFCs) it is not clear whether heterozygosity at a set of markers is reflective of heterozygosity throughout the genome. Indeed, several studies have found little or no relationship between marker heterozygosity and inbreeding coefficient (e.g. Coopworth sheep *Ovis aries,* Slate et al. 2004; great tits *Parus major,* Chapman and Sheldon 2011). Moreover, recent theory has also shown that the number of markers generally used in HFC studies (often fewer than ten) may be inadequate for detecting genome-wide heterozygosity (Balloux et al. 2004; Dewoody and Dewoody 2005). Given the complicating factors, the degree to which such findings reflect inbreeding depression is by no means certain.

The debate over the underlying cause of HFCs centres around three major hypotheses: the direct, global and local effects hypotheses (Hansson and Westerberg 2002). The direct effects hypothesis, which posits that HFCs result from heterozygote advantage at the marker loci themselves (Thelen and Allendorf 2001; Pujolar et al. 2005), applies primarily to studies of allozyme heterozygosity. Microsatellites were thought to sidestep the question of direct effects, as these markers are putatively non-coding and neutral (Da Silva et al. 2009; but see Westgaard and Fevolden 2007). The global effects hypothesis (e.g. Coltman et al. 1998) posits that HFCs observed at microsatellite loci can be attributed to the effects of genome-wide heterozygosity on fitness (i.e. inbreeding depression); this assumes that microsatellite heterozygosity reflects genome-wide heterozygosity and inbreeding coefficient. Alternatively, however, the local effects hypothesis (Hansson et al. 2004) posits that HFCs result from fitness effects of genes closely linked to the marker loci, and that heterozygosity at a set of marker loci does not necessarily reflect genome-wide heterozygosity and inbreeding coefficient.

Distinguishing between local and global effects hypotheses in explaining microsatellite-related HFCs has historically involved several types of analyses. First, the locus by locus dropout approach asks whether the removal of any single locus from an analysis significantly affects the overall relationship of heterozygosity with fitness; if not, global effects have been inferred (e.g. Hoffman et al. 2004; Charpentier et al. 2005). Local effects have also been inferred when the model that best predicts fitness includes effects of all individual loci (Brouwer et al. 2007), or when significant correlations with fitness are found at one or more individual loci (Lieutenant-Gosselin and Bernatchez 2006). Another frequently used test involves determining whether heterozygosity is

significantly correlated among marker loci, which Balloux et al. (2004) have argued is necessary if the global effects hypothesis is to be supported. However, Szulkin et al. (2010) recently criticized many of these approaches as being insufficiently rigorous tests for local effects, and described what they consider more appropriate testing while noting that local and global effects may not be mutually exclusive alternatives.

Beyond the debate over the underlying mechanisms of HFCs, the true extent of HFCs may be less than previously thought. Recent theory (Szulkin et al. 2010) predicts that HFCs should often be very weak and detectable only in populations with substantial variation in inbreeding coefficient. In contrast, many empirical studies have reported strong and positive HFCs, even in small populations where most individuals are highly inbred (e.g. Reid et al. 2005) and in large contiguous populations where most individuals are very genetically diverse (e.g. MacDougall-Shackleton et al. 2005). Recent studies have also identified widespread publication bias in the field (Coltman and Slate 2003), with a meta-analysis by Chapman et al. (2009) suggesting that studies with high effect size but low sample size are more likely to be published than studies with high sample sizes, because the latter often show little or no correlation between heterozygosity and fitness.

Several ways of improving the reliability and interpretability of HFC studies have been proposed. These include using appropriate statistical tests for local effects (Szulkin et al. 2010), increases in sample sizes and in the number of marker loci used (Balloux et al. 2004), publishing negative and null results in addition to positive results (Chapman et al. 2009), examining fitness components from multiple life stages (Szulkin et al. 2007)

and using a variety of marker types to compare patterns observed (Hansson et al. 2004; Szulkin et al. 2010). Although obtaining large sample sizes from wild populations is often easier for theoreticians to prescribe than for field biologists to achieve, newly identified genetic markers now allow us to increase the number of marker loci and to compare patterns among different marker types. Expressed sequence tag short sequence repeats (EST-SSRs) are microsatellites closely linked to coding genes, and have been found to be highly transferrable between relatively distantly related taxa (Karaiskou et al. 2008). Thus, after EST-SSRs have been identified from sequence analysis of a well studied model species they can be applied to other, non-model, species, thus avoiding the need to develop species-specific microsatellites (Karaiskou et al. 2008). Another advantage of using EST-SSRs in combination with traditional ('anonymous') microsatellites is that together they provide a test for local effects, because the local effects hypothesis predicts that microsatellites that are linked to genes may have much higher correlations with fitness than anonymous microsatellites.

Free-living song sparrows (*Melospiza melodia*) can provide a good study system for examining HFCs. Studying the fitness consequences of genetic variability in wild populations is important because the relatively harsh and variable environment encountered in the wild is reflective of natural conditions; the fitness consequences of inbreeding may not be apparent in captive laboratory studies with their relatively benign and predictable environments (Halverson et al. 2006). More specifically, song sparrows occupy a wide variety of environments and comprise of many subspecies that differ widely in migration behaviour, population size and history, and degree of genetic structuring (e.g. island vs. mainland populations). There has been extensive study of

HFCs in this species. In the sedentary Mandarte island population, for example, inbreeding coefficient is negatively related to survivorship (Keller et al. 2002), immunocompetence (Reid et al. 2007) and to song complexity, an important sexually selected trait (Reid et al. 2005). In the migratory mainland study population, positive HFCs have been observed in nestling growth rate (Potvin and MacDougall-Shackleton 2009), maintenance of paternity and nestling provisioning by males (Kewin 2010) and song repertoire size (Pfaff et al. 2007), although this last study was based on only a small number of loci. Interestingly, however, a strong negative relationship between heterozygosity and overwinter return rate has also been observed in this population (Kewin 2010), suggesting that the relationship between heterozygosity and fitness in the study population may be more complex than that observed in more inbred populations.

In this thesis, my first objective was to examine the relationship between multilocus heterozygosity and song complexity as measured by song repertoire size and syllable repertoire size in this large and open population of song sparrows. Song complexity is an important trait in this and other populations of song sparrow; associated with many aspects of fitness including female choice (Searcy 1984), reproductive success (Reid et al. 2004), longevity (Reid et al. 2005), immunocompetence (Reid et al. 2005), and stress response (MacDougall-Shackleton et al. 2009a). Because they are costly, and therefore thought to be an honest indicator of genetic quality, face strong sexual selection and show a great degree of variation (Gonzalez et al. 2010), sexually selected traits have been predicted to show strong HFCs (Von Hardenberg et al. 2007; Gonzalez et al. 2010) and thus represent a promising area of study. A finding of a positive relationship between microsatellite heterozygosity and song complexity would support the idea that HFCs can occur even in large, open populations. Conversely, finding no relationship between

heterozygosity and song complexity would suggest that the population lacks sufficient variation in heterozygosity and inbreeding to generate HFCs or that overdominance effects on song complexity are simply not present at the markers studied in this population. Finally, a nonlinear relationship between heterozygosity and one or both aspects of song complexity might be observed, suggesting some optimal level of genetic diversity (Neff 2004).

The second objective in this thesis was to investigate the genetic mechanisms underlying any observed HFCs, that is, to distinguish between the local and global effects hypotheses. By using a combination of approaches, I attempted to conduct a robust analysis of these mechanisms. First, I present tests for local effects suggested by Szulkin et al. (2010) to determine if models incorporating single-locus effects explain significantly more variation than those using multilocus heterozygosity (MLH). As a complementary test, I compare HFCs at different marker types (anonymous microsatellites and gene-linked EST-SSRs). The global effects hypothesis would be supported if models incorporating single-locus effects fail to explain significantly more variation than those using only MLH and if both marker types explain similar proportions of variance in fitness. Conversely, the local effects hypothesis would be supported if models with single locus effects account for significantly more variation than those with only MLH, and if EST-SSRs show stronger associations with song complexity than do anonymous microsatellites. Although a test only comparing marker types may not be conclusive due to the linkage of the anonymous markers being unknown, together these approaches provide a comprehensive examination of heterozygosity-fitness relationships

in a large and free-living population open to immigration, as well as the genetic architecture underlying such patterns.

Materials and Methods

Study Population and Site

I conducted field work from April to June 2010 on a free-living population of song sparrows (*Melospiza melodia melodia*) breeding on the Bracken tract owned by the Queen's University Biological Station, near Newboro, Ontario (44° 38'60 N/ 76° 19'0 W). The study site consists of forest, swamp and old fields, with sufficient edge habitat to support at least 30-40 breeding pairs of song sparrows. The population is migratory, returning to breed in March or April and first nesting in late April or early May. The study population shows reasonably high adult philopatry, with a yearly return rate of 30-50% for breeding adults. Individuals that have bred at the site generally return to or within 30 meters of their previous territories (MacDougall-Shackleton et al. 2009b). By contrast, only about 5% of the nestlings banded at the study site return to breed the following year, and only about 15% of breeders at the study site are first banded as nestlings on the site, suggesting that natal philopatry is low relative to adult philopatry and that some juveniles disperse away from their natal populations to breed.

Field Methods

Between April and June 2010, the research team captured adult song sparrows in mist nets in combination with song playback, and in seed-baited treadle traps. We

identified the sex of birds based on the presence of a cloacal protuberance (in males) then outfitted each with a uniquely numbered metal leg band (Canadian Wildlife Service), plus a unique combination of coloured plastic leg bands for field identification. We collected a blood sample from each bird via brachial venipuncture. About 25 μL of whole blood was blotted onto high wet strength filter paper, fixed with a drop of 0.5 M EDTA (pH 8.0) and allowed to dry awaiting genetic analysis (described below).

Song Recording and Analysis

I recorded song repertoires from territorial males, using the criterion of either 300 consecutive or 450 non-consecutive songs established for this population by Pfaff et al. (2007). Songs were recorded onto Marantz PMD 671 solid state recorders using Telinga Twin Science Pro parabolic microphones. In some cases males had had their repertoires recorded (and scored, by previous members of the lab) in previous years. These birds did not need to be rerecorded as song sparrows are closed-ended learners that do not alter their repertoires in adulthood (Nordby et al. 2002). In all, when including data available from other projects, complete repertoires were available for 57 males breeding in 2010. Repertoire data collected by previous members of the lab were also available for an additional 32 males that bred in 2008 but not 2010 (see below).

Song recordings were visualized as spectrograms using SYRINX V2.6 (John Burt; www.syrinxpc.com). I visually sorted all spectrograms into distinct song types, following techniques used by Pfaff et al. (2007), to determine the number of song types in each male's repertoire (hereafter "song repertoire size"). As a complementary measure of song complexity, I followed the criteria of Stewart and MacDougall-Shackleton (2008) to identify each song type's component syllables and counted the total number of distinct

syllables within each male's song repertoire (hereafter "syllable repertoire size"). Syllable repertoire size and song repertoire size are correlated ($n = 89$, Pearson's $r = 0.517$, $p < 0.001$) but predict different aspects of fitness (MacDougall-Shackleton et al. 2009a) and singing behaviour (MacDougall-Shackleton et al. 2009b). Because song and syllable repertoire size are related to distinct and somewhat independent elements of fitness I examined both of these elements of song complexity separately.

I used a total of 89 adult males with song and syllable repertoire sizes scored between 2008 and 2010 to determine the relationship between song complexity and genetic diversity (see below). Because some males bred at the site in multiple years I assigned them to the year in which their repertoire was originally recorded and made note of the recording year for each individual to account for potential differences in song scoring between years due to different researchers.

Genetic Analysis

DNA was extracted from field blots using a protocol adapted from Laitinen et al (1994). Initial DNA concenentrations were determined using a spectrophotometer and adjusted to approximately 25ng/uL for use in PCR reactions by dilution with sterilized distilled water. I genotyped 57 adult males from the 2010 field season at 17 anonymous microsatellite loci (Table 2.1). In addition, all males that bred in 2008, 2009 and/or 2010 for whom I had repertoire data ($n = 89$) were genotyped at five polymorphic EST-SSR loci characterized in zebra finches (*Taenopygia guttata*; Table 2.1), although birds sampled in 2009 were subsequently dropped from the analysis due to low sample size. An additional eight EST-SSR loci (Tgu58, Tgu35, Tgu13, Tgu52, Tgu85, Tgu4, Tgu8 and Tgu66; Karaiskou et al. 2008) were screened for amplification and variability across ten

Table 2.1: Overview of loci. Table shows expected (H_e) and observed (H_o) heterozygosity, population allele count, size range and repeat size in base pairs, marker type (anonymous vs. EST-SSR) and marker source for 89 adult male song sparrows (*Melospiza melodia*). Loci that deviated significantly from Hardy-Weinberg equilibrium are marked with asterisks. One asterisk denotes a heterozygote deficit, two a heterozygote excess.

Locus Name	He	Ho	Allele Count	Size Range (BP)	Repeat Unit (BP)	Marker Type	Reference
Sosp1*	0.897	0.792	37	216-394	4	Anonymous	1
Sosp2	0.502	0.621	7	157-185	4	Anonymous	1
Sosp3*	0.936	0.885	30	178-256	2	Anonymous	1
Sosp4*	0.858	0.777	14	172-228	4	Anonymous	1
Sosp5*	0.758	0.590	10	98-142	4	Anonymous	1
Sosp7	0.522	0.583	7	86-106	4	Anonymous	1
Sosp9	0.250	0.276	3	90-106	4	Anonymous	1
Sosp12	0.533	0.397	16	170-248	2	Anonymous	1
Sosp13	0.846	0.862	22	159-159	2	Anonymous	1
Sosp14*	0.903	0.850	20	211-315	4	Anonymous	1
Mme1	0.751	0.773	16	135-183	2	Anonymous	2
Mme7*	0.924	0.847	20	110-150	2	Anonymous	2
Mme12*	0.833	0.756	20	121-193	2	Anonymous	2
Escu1	0.883	0.870	23	128-180	2	Anonymous	5
Pdou5	0.933	0.938	31	217-289	2	Anonymous	3
ZoleB03*	0.907	0.432	21	236-344	4	Anonymous	4
ZoleC02	0.853	0.837	23	176-284	4	Anonymous	4
Tgu 1	0.862	0.887	14	271-307	2	EST-SSR	6
Tgu 16	0.783	0.770	9	292-310	2	EST-SSR	6
Tgu 40*	0.862	0.757	16	179-213	4	EST-SSR	6
Tgu 69**	0.515	0.554	8	204-220	2	EST-SSR	6
Tgu 74	0.325	0.285	5	243-261	2	EST-SSR	6

References refer to:

1. LF Keller, unpublished sequences, pers. comm. to EA MacDougall-Shackleton
2. Jeffery et al. 2001
3. Griffith et al. 1999
4. Poesel et al. 2009
5. Hanotte et al. 1994
6. Karaiskou et al. 2008

unrelated individuals but either failed to amplify or showed only a single allele, so were not pursued further.

Sets of primers with similar annealing temperatures and amplification conditions were combined into multiplexes for reactions: these included multiplex 1 (Sosp1, 5, 7), multiplex 2 (Mme1, Pdou5, ZoleH05), multiplex 3 (Sosp3, 13, 14), multiplex 4 (Mme2,7,Escu1), multiplex 5 (Sosp2,4,9, ZoleC02), multiplex 6 (Tgu40, Tgu74) and multiplex 7 (Tgu69, Tgu16). Mme12, ZoleB03 and Tgu1 could not be easily multiplexed and were amplified individually.

Polymerase chain reaction (PCR) amplifications were conducted in a final volume of 10 μL and included 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton x-100, 0.2 mg/mL BSA, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1-0.4 mM of each primer, 0.5 U Taq polymerase (Fisher Scientific) and approximately 25 ng of DNA. One primer at each locus was dye-labelled (Applied Biosystems, Foster City, CA). For most multiplexes, PCR cycling began with an initial denaturing step of 180 s at 94°C, and then went through 28 cycles of 30 s at 94°C, 90 s at the annealing temperature (see below) and 60 s at 72°C; followed by a final extension step of 270 s at 72°C. Multiplex 4 differed in that it had an initial step at 94°C for 270 s and annealing times of 40 seconds. Annealing temperatures were 54°C for ZoleB03, 55°C for multiplex 1, and 57°C for Mme12, Tgu1, and multiplexes 2 and 3. Multiplexes 6 and 7 were run with a touchdown profile with annealing temperatures dropping from 54°C to 49°C and 52°C to 48°C, respectively. Amplified PCR products were sized using an Applied Biosystems 3130 Genetic Analyzer and Genemapper software.

I used IR Macro N4 (Amos et al. 2001) to estimate null allele frequencies at each locus. I tested for deviations from Hardy-Weinberg equilibrium (HWE) using Genepop

4.0.10 (Rousset 2008) with Markov Chain parameters including a dememorization number of 10,000, 20 batches and 5000 iterations per batch. Ten of the loci analyzed (Sosp5, Sosp1, ZoleB03, Sosp3, Sosp14, Mme7, Mme12, Sosp4, Tgu40 and Tgu69) deviated significantly from HWE, in almost all cases due to heterozygote deficit, except Tgu69 which showed a slight heterozygote excess (Table 2.1). Because deviations from HWE are not necessarily due to the presence of null alleles, especially considering the population history of selection against heterozygotes (Kewin 2010) the main analyses were run with all loci. However, as a check I also repeated all analyses using just the subset of 13 loci that were in HWE, and determined that the results were qualitatively similar. Moreover, standardized multilocus heterozygosity (sMLH; see below) calculated across all loci was highly and significantly correlated with that calculated across the subset of 13 loci conforming to HWE (Figure 2.1, Pearson's $r = 0.820$, $df = 88$, $p < 0.001$)

Several measures of individual genetic diversity have been proposed and used, the most common being multilocus heterozygosity (MLH; Chapman et al. 2009), which is a calculation of the proportion of loci at which an individual is heterozygous; internal relatedness (IR; Amos et al. 2001) which weights homozygosity for rare alleles more heavily than homozygosity for common alleles, standardized heterozygosity (SH; Coltman et al. 1999), which standardizes loci heterozygosity by weighting them according to the population observed heterozygosity and mean d^2 (Coulson et al. 1998), which takes into account differences in allele sizes for heterozygous individuals. Because these measures of individual genetic diversity are all highly correlated with one another, to avoid pseudo-replication I chose in advance to use only a single measure, as recommended by Chapman et al. (2009). Because there was high variation in heterozygosity between markers (Figure 2.1) and the dataset contained missing data I

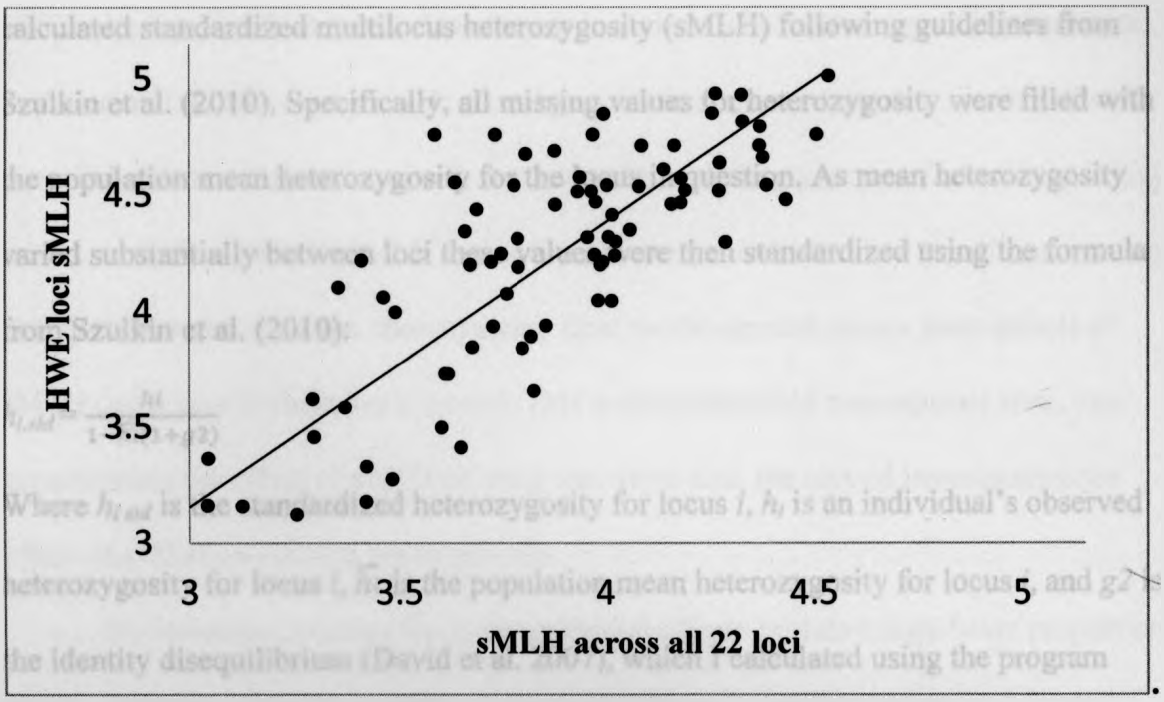

Figure 2.1: Standardized multi-locus heterozygosity at Hardy-Weinberg expectations loci vs. all loci in 89 male song sparrows (*Melospiza melodia*). sMLH (standardized multi-locus heterozygosity) calculated across the subset of loci conforming to Hardy-Weinberg expectations (HWE) was highly correlated with sMLH calculated across all loci in 89 male song sparrows (*Melospiza melodia*).

calculated standardized multilocus heterozygosity (sMLH) following guidelines from Szulkin et al. (2010). Specifically, all missing values for heterozygosity were filled with the population mean heterozygosity for the locus in question. As mean heterozygosity varied substantially between loci these values were then standardized using the formula from Szulkin et al. (2010):

$$h_{i,std} = \frac{hi}{1-\overline{hi}(1+g2)}$$

Where $h_{i\,std}$ is the standardized heterozygosity for locus *i*, h_i is an individual's observed heterozygosity for locus *i*, $\overline{hi}$ is the population mean heterozygosity for locus *i*, and *g2* is the identity disequilibrium (David et al. 2007), which I calculated using the program RMES (http://ftp.cefe.cnrs.fr). I calculated sMLH for each individual as the average of all single-locus values of $h_{i,std}$. For each individual, I also calculated two additional values of sMLH, one based solely on heterozygosity at the 17 genotypic (anonymous) loci and another based solely on heterozygosity at the five EST-SSR loci.

Statistical Analyses

I tested all continuous variables for normality using Kolmogorov-Smirnov tests. Both song repertoire size ($n = 89$, $KS = 0.03$, $p > 0.15$) and syllable repertoire size ($n = 89$, $KS = 0.05$, $p > 0.15$) were normally distributed, as was MLH ($n = 89$, $KS = 0.09$, $p = 0.06$) and sMLH ($n = 89$, $KS = 0.09$, $p = 0.10$). Because outbreeding depression, or heterozygote-disadvantage is a possibility all statistics were two-tailed. Tests were performed using the student version of Minitab14 (2003).

To test the hypothesis that individual genetic diversity predicts song complexity, I used linear regression with sMLH calculated over all 22 loci (i.e. pooling anonymous and

EST-linked markers). Because this analysis included males whose repertoires had been recorded and scored over multiple years by multiple student researchers, I controlled for potential effects of variation among researches by including year as a factor in the initial model, as well as year-by-sMLH interaction. However, I observed no significant year-by-sMLH interaction (data not shown) so my final models included only main effects of sMLH (with year included as a factor). This analysis involved two separate tests, one investigating the effect of sMLH on song repertoire size, the second investigating the effect of sMLH on syllable repertoire size.

To determine whether local chromosomal effects explain a significant proportion of heterozygosity-fitness correlations, for each of the relationships examined above I performed an F-ratio test to compare a model which included sMLH but no single-locus effects (model 1) to a more complex model including separate effects of $h_{i\ std}$ at each locus (model 2). F statistics were calculated following Szulkin et al. (2010), using the formula

$$F = \frac{(resSS1 - resSS2)/(df1 - df2)}{resSS2/df2}$$

Where resSS1 is the residual sum of squares for model 1, resSS2 is the residual sum of squares for model 2, df1 is the sample size (n) and df2 is (n – number of loci (L) - 1). I then assessed the statistical significance of the above F statistic using (df1-df2, df2) degrees of freedom to determine whether model 2 had significantly more predictive power than model 1 (Szulkin et al. 2010).

As a complementary test for local chromosomal effects, I compared the relationship between song complexity and sMLH calculated across the 17 anonymous microsatellites versus sMLH across the five EST-SSRs. Using a general regression model

(GRM) I tested for homogeneity of slopes between sMLH calculated at each marker type and song complexity by testing whether the interaction between marker type and sMLH was a significant component of a model predicting song complexity. If heterozygosity-fitness correlations are primarily due to linkage disequilibrium between the marker loci and loci associated with fitness (local chromosomal effects) then the gene-linked EST-SSRs should have stronger predictive power in relation to fitness traits than do an assortment of putatively unlinked loci. Conversely, if heterozygosity-fitness correlations arise primarily from variation in genome-wide heterozygosity, both marker types may have similar power to predict fitness traits.

Results

Variation in Song Complexity Between Years

Unexpectedly, the two years examined varied significantly in the average observed syllable repertoire size (Figure 3.1; ANOVA, $F_{1,85} = 9.58$, $p = 0.003$), although not song repertoire size (Figure 3.2; ANOVA, $F_{1,85} = 0.61$, $p = 0.438$). A follow-up analysis suggests that this among-year variation was due to differences among researchers in categorizing syllables, not to actual cohort differences in song complexity. Ten males whose vocal repertoires were scored in both 2008 and 2010 generally received lower syllable repertoire scores in 2010 than 2008 (Figure 3.3) despite the fact that song sparrows are closed-ended learners that do not change their repertoire content after maturity (Nordby et al. 2002).

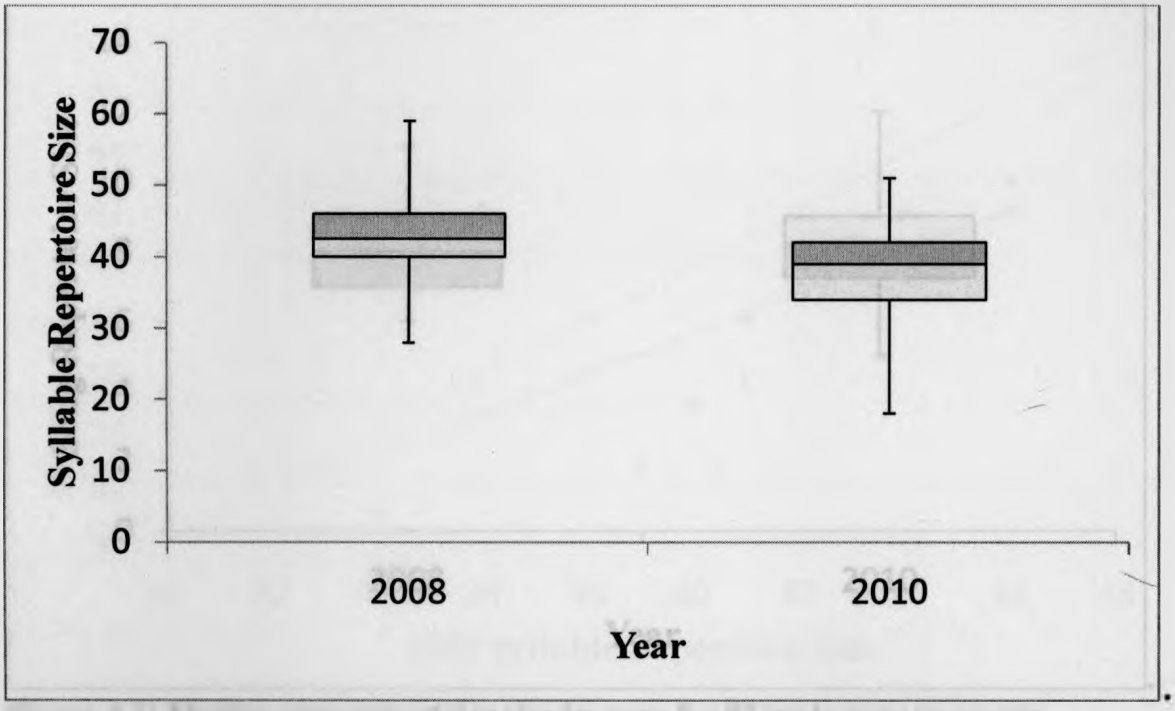

Figure 3.1: Median syllable repertoire size by year for 87 male song sparrows (*Melospiza melodia*). Mean measured syllable repertoire size was significantly lower for male song sparrows (*Melospiza melodia*) scored in 2010 than for those scored in 2008 (2008 n = 30, 2010 n = 57). Boxplots show medians, 3rd quartiles and 1st quartiles. Whiskers show minimum and maximum values.

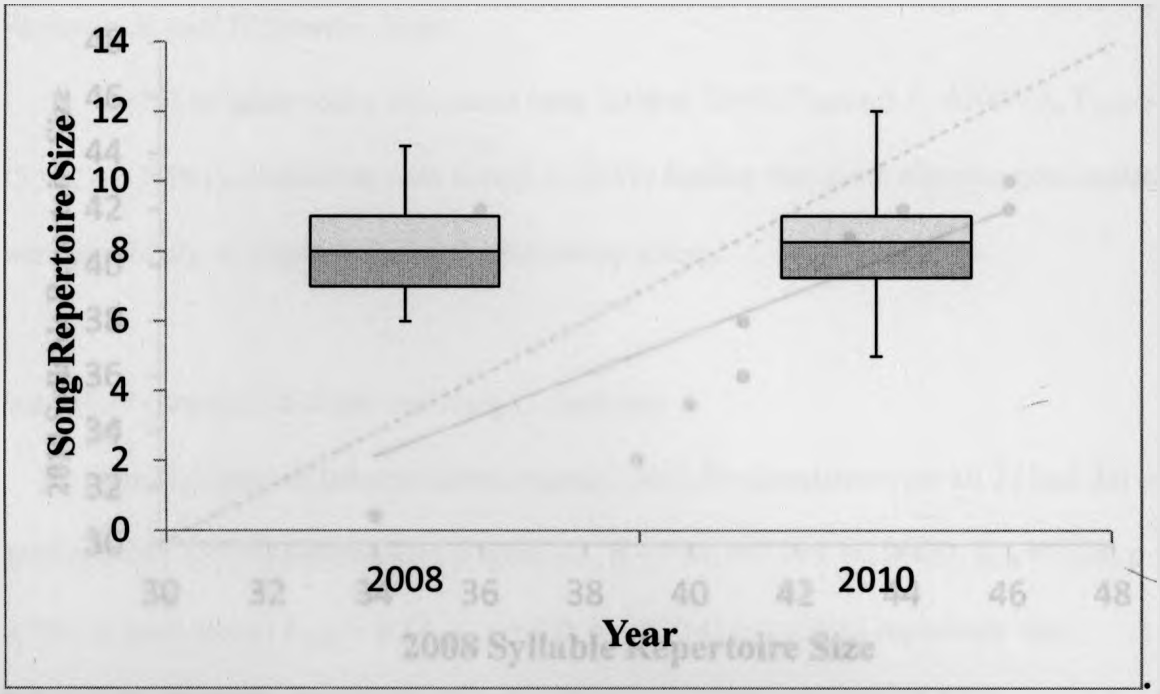

Figure 3.2: Median song repertoire size by year for 87 male song sparrows (*Melospiza melodia*). Mean song repertoire size did not differ significantly depending on in which year the bird's (*Melospiza melodia*) repertoire was scored (2008 n = 30, 2010 n = 57). Boxplots show medians, 3rd quartiles and 1st quartiles. Whiskers show minimum and maximum values.

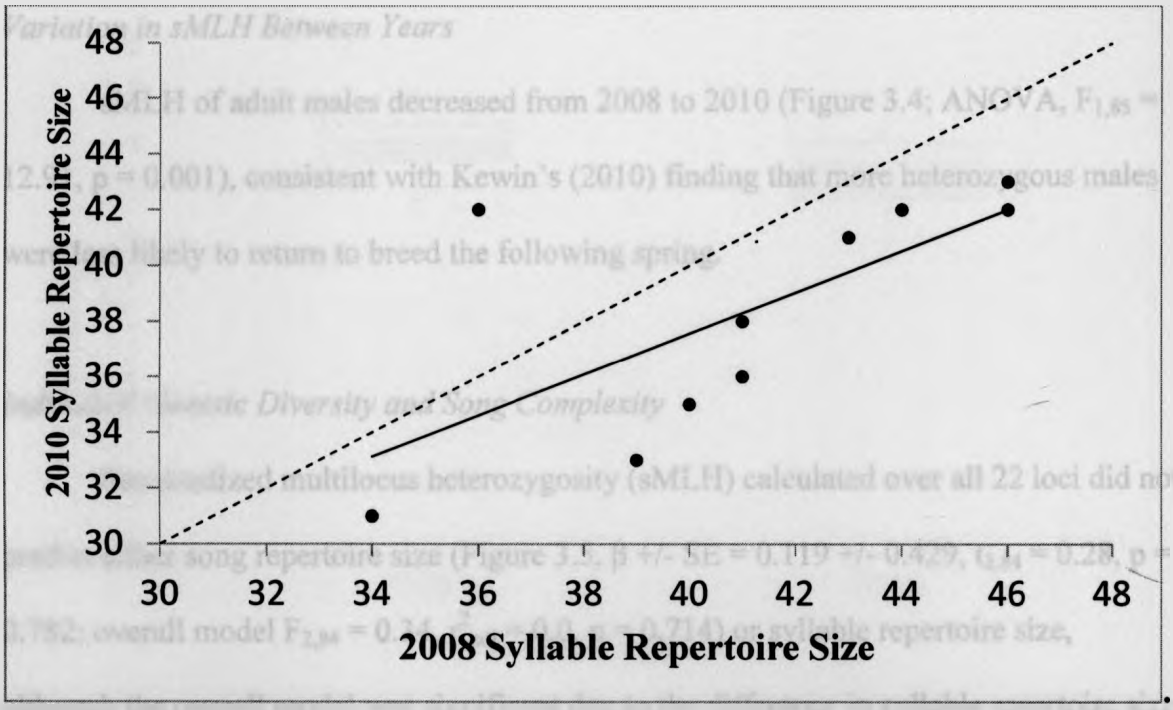

Figure 3.3: Syllable repertoire sizes for ten male song sparrows (*Melospiza melodia*) whose vocal repertoires were recorded and scored in both 2008 and 2010. The dashed line is the expected 1:1 line and the solid line is a line of best fit for the actual data. There is a clear tendency to lump rather than split similar syllables in 2010 compared to 2008, demonstrated by the fact that most syllable repertoire sizes were scored higher in 2008 than 2010.

Variation in sMLH Between Years

sMLH of adult males decreased from 2008 to 2010 (Figure 3.4; ANOVA, $F_{1,85}$ = 12.91, p = 0.001), consistent with Kewin's (2010) finding that more heterozygous males were less likely to return to breed the following spring.

Individual Genetic Diversity and Song Complexity

Standardized multilocus heterozygosity (sMLH) calculated over all 22 loci did not predict either song repertoire size (Figure 3.5, β +/- SE = 0.119 +/- 0.429, $t_{2,84}$ = 0.28, p = 0.782; overall model $F_{2,84}$ = 0.34, r^2_{adj} = 0.0, p = 0.714) or syllable repertoire size, although the overall model was significant due to the difference in syllable repertoire size between years (Figure 3.6, β +/- SE = 1.433 +/- 1.702, $t_{2,84}$ = 0.84, p = 0.402, overall model $F_{2,84}$ = 5.13, r^2_{adj} = 8.8, p < 0.01).

Local Vs. Global Effects On Song Complexity

I found no convincing evidence for local chromosomal effects on song complexity. F-ratio tests indicated that models containing sMLH did not explain significantly less of the variation in song complexity than did more complex models that incorporated single-locus values of $h_{i\,std}$, for either song repertoire size (Table 3.1; $F_{63,84}$ = 0.825 , p= 0.788) or syllable repertoire size (Table 3.2; $F_{63,84}$ = 0.877, p= 0.706). Heterozygosity at one locus (Mme1) was significantly positively related to syllable repertoire size in the model incorporating multiple single-locus effects (Table 3.2), but this is likely due to chance given the large number of loci tested.

A general regression model (GRM) with sMLH split by marker type (anonymous vs EST) did not reveal a significant difference between marker types in the slope of the

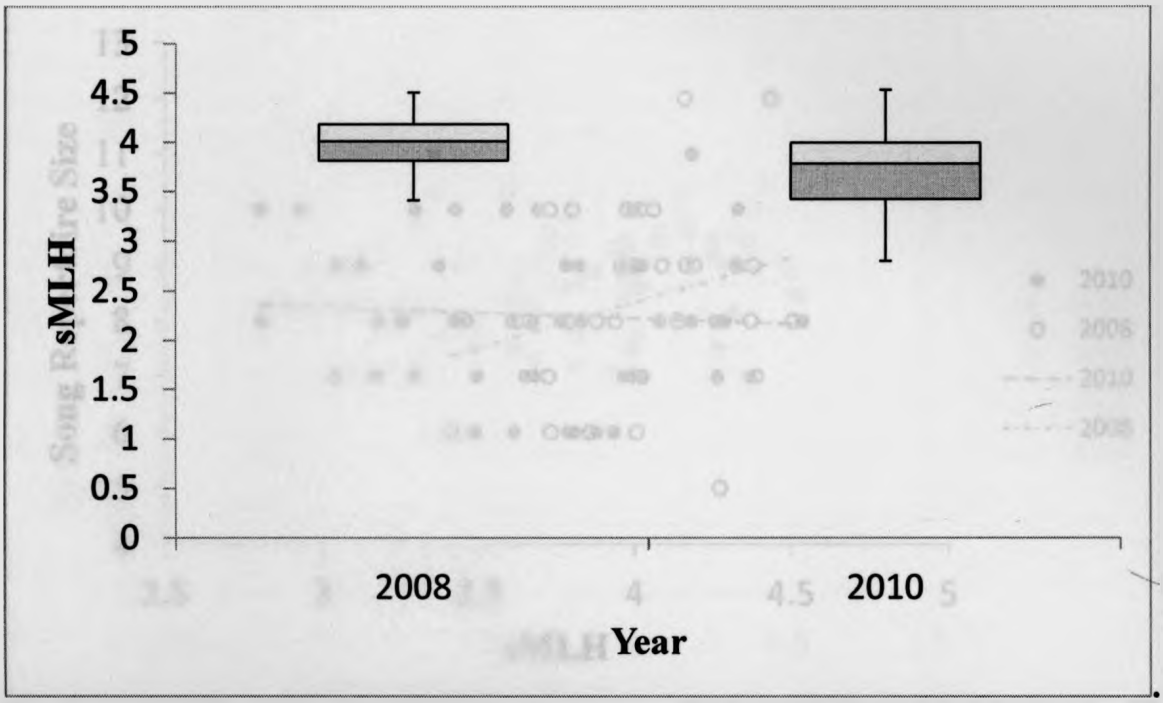

Figure 3.4: Median standardized multi-locus heterozygosity by year for 87 male song sparrows (*Melospiza melodia*). Mean sMLH (standardized multi-locus heterozygosity) calculated across all 22 loci was significantly lower among adult male song sparrows (*Melospiza melodia*) breeding in 2010 (N = 57) than in 2008 (n = 30). Boxplots show medians, 3rd quartiles and 1st quartiles. Whiskers show minimum and maximum values. Males breeding in both 2008 and 2010 are included only in the year that their song complexity was scored.

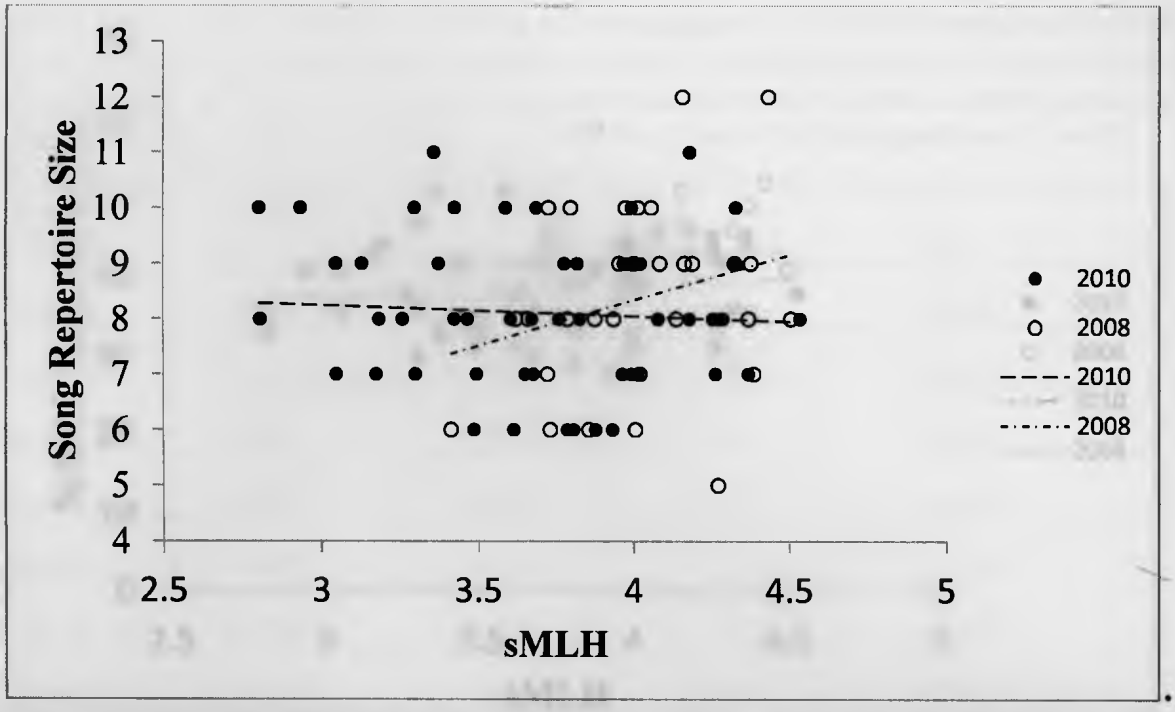

Figure 3.5: Standardized multi-locus heterozygosity and song repertoire size for 87 male song sparrows (*Melospiza melodia*). sMLH (standardized multi-locus heterozygosity) calculated across all 22 loci does not predict song repertoire size in 87 male song sparrows (*Melospiza melodia*; 2008 n = 30, 2010 n = 57).

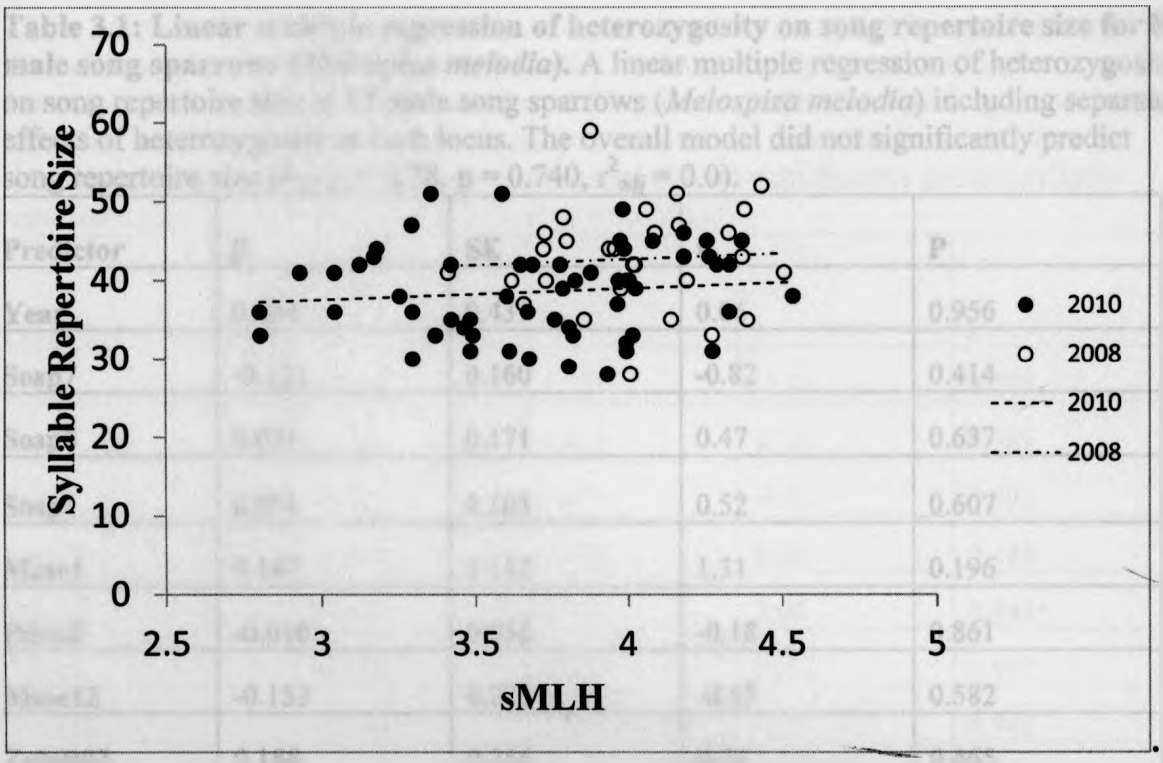

Figure 3.6: Standardized multi-locus heterozygosity and syllable repertoire size for 87 male song sparrows (*Melospiza melodia*). sMLH (standardized multi-locus heterozygosity) calculated across all 22 loci does not predict syllable repertoire size in 87 male song sparrows (*Melospiza melodia*; 2008 n = 30, 2010 n = 57).

Table 3.1: Linear multiple regression of heterozygosity on song repertoire size for 87 male song sparrows (*Melospiza melodia*). A linear multiple regression of heterozygosity on song repertoire size of 87 male song sparrows (*Melospiza melodia*) including separate effects of heterozygosity at each locus. The overall model did not significantly predict song repertoire size ($F_{23,63} = 0.78$, $p = 0.740$, $r^2_{adj} = 0.0$).

Predictor	β	SE	t	P
Year	0.024	0.439	0.06	0.956
Sosp7	-0.131	0.160	-0.82	0.414
Sosp5	0.081	0.171	0.47	0.637
Sosp1	0.054	0.103	0.52	0.607
Mme1	0.147	0.112	1.31	0.196
Pdou5	-0.010	0.056	-0.18	0.861
Mme12	-0.153	0.277	-0.55	0.582
ZoleB03	0.188	0.256	0.74	0.465
Sosp13	0.000	0.082	0.00	0.998
Sosp3	-0.013	0.081	-0.16	0.870
Sosp14	0.030	0.084	0.36	0.719
Mme7	0.024	0.084	0.29	0.773
Mme12	-0.067	0.122	-0.55	0.584
Escu1	0.008	0.082	0.10	0.924
Sosp9	-0.128	0.346	-0.37	0.713
Sosp2	-0.020	0.154	-0.13	0.896
Sosp4	-0.041	0.111	-0.37	0.712
ZoleC02	0.123	0.098	1.25	0.215
Tgu 40	-0.210	0.134	-1.57	0.121
Tgu 69	0.226	0.186	1.22	0.228
Tgu 74	-0.504	0.445	-1.13	0.262
Tgu 16	0.038	0.114	0.33	0.744
Tgu 1	-0.088	0.078	-1.13	0.264

Table 3.2: Linear multiple regression of heterozygosity on syllable repertoire size for 87 male song sparrows (*Melospiza melodia*). A linear multiple regression of heterozygosity on syllable repertoire size of 87 male song sparrows (*Melospiza melodia*) including separate effects of heterozygosity at each locus. Asterisks indicate loci with significant effects at $p < 0.05$. The overall model did not significantly predict syllable repertoire size ($F_{23,63} = 1.23$, $p = 0.253$, $r^2_{adj} = 0.059$).

Predictor	β	SE	t	P
Year	-2.259	1.731	-1.31	0.197
Sosp7	0.187	0.629	0.30	0.767
Sosp5	-0.007	0.674	-0.01	0.992
Sosp1	0.284	0.408	0.70	0.488
Mme1	0.914	0.443	2.06	**0.043***
Pdou5	-0.116	0.221	-0.52	0.602
Mme12	-0.080	1.091	-0.07	0.942
ZoleB03	0.344	1.009	0.34	0.734
Sosp13	-0.174	0.325	-0.53	0.595
Sosp3	0.522	0.318	1.65	0.105
Sosp14	0.176	0.330	0.53	0.596
Mme7	-0.479	0.332	-1.44	0.155
Mme12	-0.193	0.483	-0.40	0.690
Escu1	-0.023	0.322	-0.07	0.945
Sosp9	0.652	1.362	0.48	0.634
Sosp2	-0.056	0.605	-0.09	0.926
Sosp4	-0.565	0.437	-1.29	0.201
ZoleC02	0.385	0.386	1.00	0.323
Tgu 40	-0.091	0.527	-0.17	0.864
Tgu 69	0.585	0.733	0.80	0.427
Tgu 74	-0.659	1.752	-0.38	0.708
Tgu 16	-0.185	0.450	-0.41	0.683
Tgu 1	0.380	0.308	1.23	0.222

relationship between heterozygosity and song repertoire size as shown by the non-significant effect of the interaction between marker type and sMLH on song repertoire (Figure 3.7; Table 3.3; β +/- SE = -0.419 +/- 0.410, $t_{4,169}$ = -0.102, p = 0.309, overall model: $F_{4,169}$ = 0.590, p = 0.668, r^2_{adj} = 0.0%). A second GRM with sMLH split by marker type (anonymous vs EST) did not reveal a significant difference between marker types in the slope of the relationship between heterozygosity and syllable repertoire, as shown by the non-significant effect of the interaction between marker type and sMLH on syllable repertoire, although the overall model was significant due to between-year differences in measured syllable repertoire sizes (Figure 3.8; Table 3.4; β +/- SE = -0.129 +/- 1.635, $t_{4,169}$ = -0.08, p = 0.937, overall model: $F_{4,169}$ = 4.99, p = 0.001, r^2_{adj} = 8.4%).

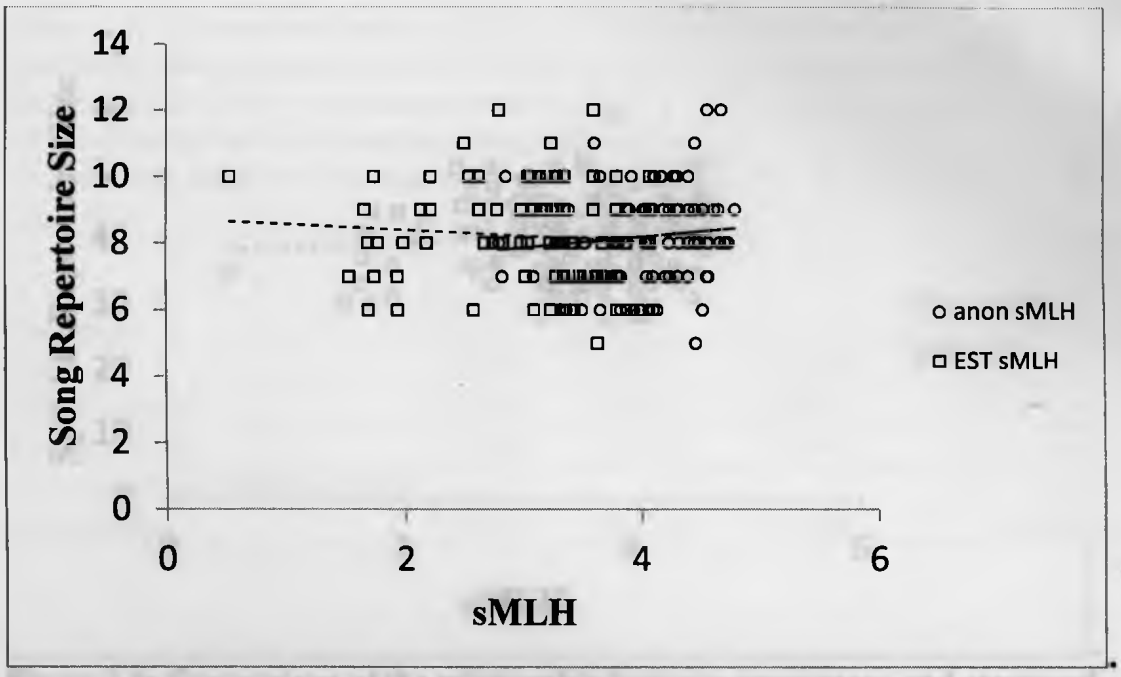

Figure 3.7: Comparison of the relationship between anonymous and expressed sequence tag microsatellite heterozygosity on song repertoire size for 87 male song sparrows (*Melospiza melodia*). The slope of the relationship between song repertoire size and sMLH (standardized multi-locus heterozygosity) calculated at 17 anonymous microsatellite loci does not differ significantly from that calculated at 5 EST-SSR loci in 87 male song sparrows (*Melospiza melodia*; 2008 n = 30, 2010 n = 57).

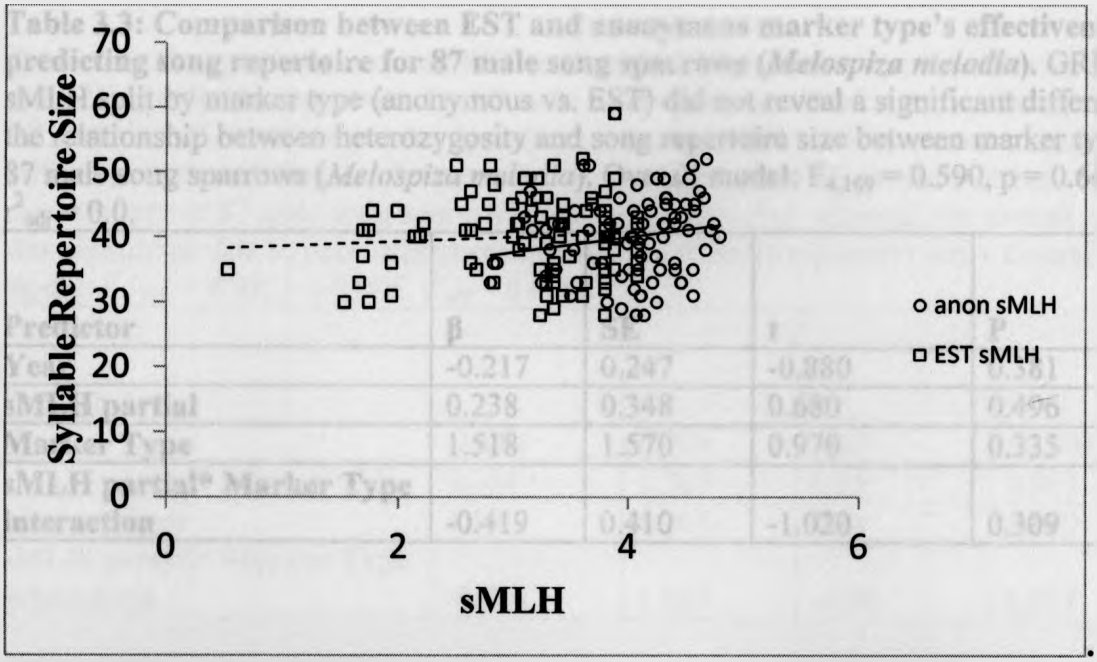

Figure 3.8: Comparison of the relationship between anonymous and expressed sequence tag microsatellite heterozygosity on syllable repertoire size for 87 male song sparrows (*Melospiza melodia*). The slope of the relationship between syllable repertoire size and sMLH (standardized multi-locus heterozygosity) calculated at 17 anonymous microsatellite loci does not differ significantly from that calculated at 5 EST-SSR loci in 87 male song sparrows (*Melospiza melodia*; 2008 n = 30, 2010 n = 57).

Table 3.3: Comparison between EST and anonymous marker type's effectiveness in predicting song repertoire for 87 male song sparrows (*Melospiza melodia*). GRM with sMLH split by marker type (anonymous vs. EST) did not reveal a significant difference in the relationship between heterozygosity and song repertoire size between marker types in 87 male song sparrows (*Melospiza melodia*). Overall model: $F_{4,169} = 0.590$, $p = 0.668$, $r^2_{adj} = 0.0$.

Predictor	β	SE	t	P
Year	-0.217	0.247	-0.880	0.381
sMLH partial	0.238	0.348	0.680	0.496
Marker Type	1.518	1.570	0.970	0.335
sMLH partial* Marker Type interaction	-0.419	0.410	-1.020	0.309

Table 3.4: Comparison between EST and anonymous marker type's effectiveness in predicting syllable repertoire for 87 male song sparrows (*Melospiza melodia*). A GRM with sMLH split by marker type (anonymous vs. EST) did not reveal a significant difference in the relationship between heterozygosity and syllable repertoire between marker types in 87 male song sparrows (*Melospiza melodia*), although the overall model was significant due to year differences in observed syllable repertoire sizes. Overall model: $F_{4,169} = 4.99$, $p < 0.005$, $r^2_{adj} = 0.084$.

Predictor	β	SE	t	P
Year	-3.983	0.984	-4.05	<0.005
sMLH partial	0.764	1.387	0.55	0.583
Marker Type	1.106	6.261	0.18	0.860
sMLH partial* Marker Type interaction	-0.129	1.635	-0.08	0.937

Discussion

Individual genetic diversity was not related to song complexity as measured by song or syllable repertoire size, in this large, free-living population of song sparrows. Because song complexity has previously been linked to inbreeding coefficient in song sparrows (Reid et al. 2005) this result may appear inconsistent with previous work. However, as described below, there are several reasons why the apparent discrepancy is not unexpected. I also observed differences in one measure of song complexity (syllable repertoire size) between years; this finding was unexpected, but appears to reflect an artefact of different years being scored by different researchers. Consistent with previous observations by Kewin (2010) that less heterozygous males were more likely to return the following year, I found a significant decrease in individual genetic diversity between 2008 and 2010. In addition to the lack of support for a relationship between MLH and song complexity, I found no evidence for local chromosomal effects, either when comparing models with effects for individual loci to models based on MLH, or when comparing models using anonymous vs. gene-linked markers. Overall this work highlights the importance of population structure to HFC as well as the context-dependent nature of such correlations.

Year Differences in Syllable Repertoire Size and Heterozygosity

Syllable repertoire size varied significantly between years (Figure 3.1), but this is likely an artefact. This is supported by the analysis of birds scored in multiple years, which showed that individuals which should have had the same syllable repertoire size in both years, due to the closed ended learning program of song sparrows (Nordby et al.

2002), had repertoires consistently scored lower when scored by the 2010 group of researchers compared to the 2008 group (Figure 3.3).

sMLH also decreased between 2008 and 2010 (Figure 3.4) and I believe this represents an actual drop in mean population heterozygosity rather than an artefact of different years being scored by different researchers. Unlike syllable repertoire size, heterozygosity was scored to a greater extent by the same researchers; for example, I scored all EST-SSR loci for both 2008 and 2010. Moreover, there is a much lower degree of subjectivity in the scoring of heterozygosity than in sorting syllables. Additionally, there is a previously established trend in the population of decreased heterozygosity between years, possibly due to low return rates of highly heterozygous males (Kewin 2010). However, the effect was much weaker in relation to female return rates and Potvin and MacDougall-Shackleton (2009) showed apparent advantages of heterozygosity to nestlings so it remains to be determined whether the entire population is becoming less genetically diverse or whether variation in strength and direction of HFCs across sexes and age classes tends to balance out over time. If neutral locus diversity reflects adaptive genetic diversity in this population then such a balancing effect may preserve the population's ability to adapt to changing environmental conditions by preserving some level of population genetic diversity.

Heterozygosity-Fitness Correlations with Song Complexity

Previous work with song sparrows has reported a correlation between song complexity and inbreeding coefficient (Reid et al. 2005) so it seemed reasonable to expect a correlation between sMLH and song complexity in this case; however, the lack of correlation was not completely unexpected. The null result found is consistent with the

idea that HFCs will be rare and often weak, especially in populations with low variance in inbreeding (Szulkin et al. 2010). Because of the female-biased dispersal and low return rates for adult females in this population there is little opportunity for daughters to mate with fathers or brothers, or for mothers to mate with sons, and so it seems likely that variance in inbreeding is low. This result is consistent with numerous studies that have reported a lack of correlation between marker heterozygosity and fitness traits in a wide variety of taxa. Examples include a range of life history, morphological and fitness traits in a similarly large and non-inbred population of great tits (*Parus major*, Chapman and Sheldon 2011) and morphological traits in Coopworth sheep (*Ovis aries*, Slate et al. 2004).

One explanation for the lack of correlation between heterozygosity and song complexity in this population given the previously reported correlation between inbreeding coefficient and song complexity in another population of the same species, (Reid et al. 2005) is that, due to the context-dependent nature of HFCs, the two populations with distinct life history (e.g. migratory vs. sedentary), predators and local climactic conditions may not be expected to exhibit the same HFCs. In particular, the Mandarte Island population is highly philopatric, with only about one immigrant per generation (Keller et al. 2001), whereas natal philopatry is much lower for the Bracken population with most new recruits apparently immigrating from off the study area. Such a context-dependent nature of inbreeding effects has been well-demonstrated (Miller 1994; Neff 2004). Indeed, these results are consistent with the idea that the Mandarte population, with its philopatry and repeated population crashes, could be closer to the lower end of the heterozygosity spectrum where positive HFCs are expected (Neff 2004), while the Bracken population should be more outbred, closer to the middle of the

spectrum where HFCs are weaker, and sometimes negative (as in the case of adult male return rate.

A second explanation for the apparent discrepancy between this result and the one reported by Reid et al. (2005) is that multilocus heterozygosity is often a poor predictor of inbreeding coefficient. Theoretical work showing that MLH will often be a poor predictor of inbreeding (Balloux et al. 2004; Dewoody and Dewoody 2005) has been corroborated by experiments that showed little or no correlation between pedigree-calculated inbreeding coefficients and MLH in a diverse set of taxa with various population structures (e.g. great tits *Parus major,* Chapman and Sheldon 2011; Coopworth sheep *Ovis aries,* Slate et al. 2004). If MLH is a poor predictor of f or of genome-wide diversity then even if a relationship did exist between inbreeding and song complexity in our population, which is itself uncertain, then it may be difficult to detect using marker heterozygosity.

Finally, it is consistent with HFC theory that, given the population structure of the two populations, the reported result would be found. While the link between song complexity and inbreeding coefficient was reported in an isolated and inbred population, the current study was carried out in a large, migratory population. HFC theory predicts that correlations are likely to be found in populations with a high degree of variance in inbreeding level, while in large, open populations such correlations are much less likely to be uncovered (Szulkin et al. 2010). Although this explanation seems quite satisfying it should be noted that correlations between genetic diversity and measures of fitness including nestling growth (Potvin and MacDougall-Shackleton 2009) and adult return rate (Kewin 2010) have previously been uncovered in our population, indicating that marker

heterozygosity may indeed be an effective tool for assessing the relationship between genetic diversity and some, if not all, measures of fitness in this population.

Local Vs. Global Effects

Although no HFCs were found using MLH, it is still important to consider the possibility of local effects as the antagonistic effects of heterozygosity on fitness at several individual markers could mask each individual effect leading to no overall correlation. By comparing models with effects for individual loci with models with MLH as predictors as in Szulkin et al. (2010) I was able to rule out significant relationships between individual locus heterozygosity and either measure of song complexity.

In addition to the individual loci vs. MLH F-ratio test, I also investigated whether the correlation between MLH and song complexity differed depending on whether MLH was calculated at gene-linked or anonymous markers. The results showed that there was no observable difference in the predictive power of anonymous vs. EST SSRs. This is in contrast to a recent study by Olano-Marin et al. (2011; neutral microsatellites vs. gene-linked microsatellites), which uses evidence from Blue Tits to argue that neutral microsatellites may be a better predictor of the effects of inbreeding than those linked to functional genes and a study by Pujolar et al. (2005; neutral microsatellites vs. allozymes) that uses evidence from European eels (*Anguilla anguilla*) to argue that gene-linked markers should show stronger HFCs. At this point it is difficult to draw a final conclusion on whether gene-linked or neutral markers are generally better predictors of HFC given the variation in results found in such studies.

Conclusions

I observed no relationship between song complexity, an important sexually selected trait in this species, and heterozygosity assessed at either anonymous or EST-linked microsatellite loci. While contrasting with many previous studies reporting positive relationships between heterozygosity and various components of fitness (Hansson et al. 2004; Lieutenant-Gosselin and Bernatchez 2006; Da Silva et al. 2009), these findings do support the recent prediction that HFCs in nature, and especially in non-inbred populations, should be rare and generally weak (Chapman et al. 2009). Further work comparing gene-linked and putatively neutral markers is still advisable as such studies have the potential to shed substantial light on the local vs. global effects debate. If enough studies were produced using these methods it is probable that a meta-analysis could show conclusively whether gene-linked loci show significantly stronger correlations with fitness traits than neutral marker loci. Additionally, more studies using appropriate tests for local effects will lead to a much more robust understanding of the genetic mechanisms which lead to HFCs when they are observed.

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