# GENETIC, SOCIO-ECOLOGICAL AND FITNESS CORRELATES OF EXTRA-GROUP PATERNITY IN THE EUROPEAN BADGER (*Meles meles*)


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*Board of the Faculty of Physical Sciences,*
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# Genetic, socio-ecological and fitness correlates of extra-group paternity in the European badger (*Meles meles*)

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## Abstract

The evolution of extra-group paternity (EGP) is a contentious issue in evolutionary biology. This thesis examines the factors and adaptive benefits driving EGP in a high-density, group-living population of European badgers (*Meles meles*).

To improve power to assign parentage, I isolated and characterised 21 new polymorphic microsatellite markers. I genotyped 83% of 1410 badger trapped 1987–2010 using 35 autosomal microsatellite markers. Maternity and paternity were assigned at 80% confidence ca. 82% of individuals. 48% of paternities were extra-group, where 85% were attributable to neighbouring-group males and EGP was detected in 47% of litters; thus badger social group do not correspond with a breeding unit.

I tested whether indirect genetic benefits explain these high EGP rates. *(1) 'Good-gene-as-heterozygosity Hypothesis':* Paternal heterozygosity, but not maternal or an individual's own heterozygosity, associated positively with first-year survival probability. Under benign environmental conditions, cubs fathered by more heterozygous males had a higher first year survival probability. Despite this correlation, the EGP rate per litter correlated with neither average nor maximum within-group heterozygosity of candidate fathers. *(2) Fitness benefit Hypothesis:* Extra-group offspring (EGO) had lower first-year survival probability and lived 1.3 years less than within-group offspring (WGO). Female WGO produced more litters and offspring over their lifetime than female EGO, whereas male EGO produced more offspring than male WGO. (*3) Inbreeding avoidance hypothesis:* The EGP rate within a litter increased with greater average pair-wise relatedness between mothers and within-group candidate fathers. No inbreeding depression on first-year survival probability was detected, but small sample sizes limited statistical power. Socio-ecologically, at the litter level, EGP correlated negatively with the number of within-group candidate fathers, and positively with neighbouring-group candidate fathers.

In conclusion, EGP in badgers may reduce inbreeding and be maintained in the population through a sex-specific antagonistic selection and indirect genetic benefits may occur when the total fitness benefits of producing extra-group sons outweigh the costs of producing extra-group daughters. These indirect genetic benefits only partially explain the evolution of promiscuity in European badgers, highlighting that evolutionary factors underlying promiscuity remain unclear.

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# Chapter 1:

# General Introduction

## 1.1 Fitness

The concept of fitness is central in evolutionary theory. Fitness is defined as the ability of an individual to survive in a particular environment long enough to pass on its genes to the next generation (King & Stansfield, 1990). Fitter individuals leave more descendants than their conspecifics and thus gain a selective advantage, under-scoring adaptive changes by natural selection (Barker, 2009; Orr, 2009). Individuals in a population vary in morphology, physiology and behaviour. These phenotypic variations contribute to differences in individual fitness if a certain trait affects survival or reproduction (Barker, 2009). Traits that optimise survival rate and reproductive success are thus strongly selected and evolutionarily stable (Maynard-Smith, 1978). Empirical studies on long-lived animals in natural populations have observed that considerable variation in fitness exists between individuals over their lifetime, often with a gender bias (e.g., Coulson et al., 1998a; Charpentier et al., 2006; Sardell et al., 2011). Understanding this variance, and the factors influencing the differential performance between individuals, is thus central for studies of population dynamics and in natural and sexual selections.

## 1.2 Group-living

The evolution of group-living is favoured when the net benefits of association with other conspecifics in the group exceed the costs (Krause & Ruxton, 2002). Living in groups provides advantages for many animals, such as protection against predators, enhanced foraging efficiency, reduced vulnerability to infanticide or reduced energetic costs of movements (Hass & Valenzuela, 2002; Silk, 2007; Kazahari & Agetsuma, 2008). Animals can, however, also form groups even without any direct benefits, when resources (food and mates) are distributed patchily in space and time such that the smallest economically

defensible territory that provides adequate resources for a single individual is predicted to have sufficient resources to share with additional individuals (the 'Resource dispersion Hypothesis', RDH, Macdonald, 1983; Carr & Macdonald, 1986; Woodroffe & Macdonald, 1993).

RDH has been used to explain group-living in many animals (see review Johnson et al., 2002). Social groups that are formed initially, in accordance with the RDH, are maintained through the retention and recruitment of offspring in their natal social groups (i.e., natal philopatry – see also the 'Territorial inheritance Hypothesis', Lindstrom, 1986) or through dispersal of individuals into new groups (Cheeseman et al., 1988; Woodroffe et al., 1995). Natal philopatry and restricted dispersal (Pope et al., 2006) of offspring leads to clustering of closely related individuals, which can increase the potential for inbreeding and inbreeding depression (Crnokrak & Roff, 1999; Keller & Waller, 2002b). Group-living also inevitably leads to competition between group members for access to resources (e.g., mates, food and breeding sites: where additional individuals to the primary pair are only viable when they can accept secondary food security, and have the capacity to buffer metabolic budgets over a limited time frame, Newman et al., 2011) and variation in the availability and distribution of these resources may therefore influence group membership and both male and female mating strategies within groups (Kokko & Rankin, 2006; Silk, 2007; Cameron et al., 2011; Huchard & Cowlishaw, 2011).

### 1.3 Mating system

Where species form social groups, mating systems span a diverse spectrum, from monogamy to polygamy (polygynous, polyandrous) and from fidelity to promiscuity (polygynandrous) (Orians, 1969; Wittenberger, 1979; Clutton-Brock, 1989). For instances,

females are socially monogamous, where offspring is attributed to a within-group male (e.g., dik-dik, *Madoqua kirkii*, Brotherton et al., 1997; California mouse, *Peromyscus californicus*, Ribble, 1991). Within such social groups however, females may still surreptitiously seek out promiscuous matings (Kleptogamy, see Palphramand et al., 2007), with paternities distributed between within-group males and sometimes to also include subordinate males (e.g., in mammals: Alpine marmots, *Marmota marmot*, Cohas et al., 2006; meerkat, *Suricata suricata*, Young et al., 2007; wild chimpanzees, *Pan troglodytes schweinfurthii*, Muller et al., 2007; see Wolff & Macdonald, 2004; in birds: e.g., Brouwer et al., 2011; see Cornwallis et al., 2010). One type of mating system that has attracted much interest in the 1980's is extra-group mating (or extra-pair mating in socially monogamous species), which results in a mixed mating strategy as paternity may be gained by extra-group males as well as within-group males.

### 1.3.1 Extra-group paternity

The use of molecular and analytical tools for accurate parentage assignment has led to the discovery that social parents are not necessarily the genetic parents of all their offspring (Burke & Bruford, 1987). Infidelity to the breeding pair among socially monogamous species is generally referred to as extra-pair paternity (EPP: i.e., offspring are not fathered by the female's social male, but instead are fathered by a within-group male such as a subordinate male in the social group, Burke & Bruford, 1987); in social group-living species infidelity results in extra-group paternity (EGP: i.e., offspring that are fathered by males from outside of the female's social group). EPP/EGP has been found to occur in more than two-third of mammals (Isvaran & Clutton-Brock, 2007; Soulsbury, 2010) and over 90% of birds (Griffith et al., 2002) so far investigated, but the reasons for these reproductive strategies are less well understood.

### 1.3.2 Genetic and non-genetic hypotheses for extra-group paternity

#### 1.3.2.1 Male benefits from EGP mating strategies

Males produce an abundance of cheap gametes (relative to females), and so they are capable of increasing their reproductive success by mating with multiple females to father multiple litters – constrained only by inter-male competition within the social system (Bateman, 1948; Trivers, 1972). When the availability of females is restricted within groups (e.g., a male-biased operational sex ratio, Emlen & Oring, 1977), male-male competition for access to mates may become more intense, favouring a mating strategy where males may seek extra-group mating opportunities, due to increased within-group costs relative to extra-group risks (often mating with proximate females from adjacent social group). Males are also more likely to engage in extra-group matings when within group reproduction is suppressed by dominant males (Creel & Creel, 1991) or females (Creel & Macdonald, 1995). Male reproductive success or promiscuity however, is necessarily a function of female willingness to participate in promiscuity (Wade & Shuster, 2005), modulated further by post-copulatory sexual selection, through sperm competition and cryptic choice by females (Birkhead & Pizzari, 2002).

#### 1.3.2.2 Female benefits from EGPs

While the adaptive benefits of extra-group mating are clear for males, it is less obvious why group-living, or monogamous-pair bonded females should need to, or be prepared to risk mating with extra-group males, particularly in mammals (and birds) where females produce a limited number of gametes and complete fertility can be provided by a single (within-group) male (Arnqvist & Rowe, 2005). Extra-group mating can be costly for females, increasing risks of sexually transmitted diseases, parasitic infection, predation

and/or injury inflicted by males (Daly, 1978; Keller & Reeve, 1995; Muller et al., 2007). Despite these costs, the high level of EGP recorded in many species has raised the question how females benefit, where for this strategy to provide a selective advantage implicitly benefits must outweigh costs. Several ecological, social and genetic hypotheses have thus been proposed to explain EGP in females and the advantages that they may gain by mating with extra group males (e.g., Westneat et al., 1990; Zeh & Zeh, 1997; Griffith et al., 2002; Westneat & Stewart, 2003; Akçay & Roughgarden, 2007; Isvaran & Clutton-Brock, 2007; Slatyer et al., 2012). Alternatively, EGP in female could evolve through indirect selection on the males' extra-group mating behaviour (Forstmeier et al., 2011).

***Ecological hypotheses***

Intra- and inter-specific variation in the frequency of EGP (or EPP) can be influenced by a variety of ecological factors (reviewed in Petrie & Kempenaers, 1998; Griffith et al., 2002). Food availability in space and time especially during cub rearing, may influence extra-group mating behaviour in female (Westneat et al., 1990; Cameron et al., 2011). When food resources are abundant, the need for male parental care to offspring survival become less important (Gowaty, 1996) and its benefits to the females should be overwhelmed by the benefits of EGP. Females might be expected to obtain EGP as insurance against the functional infertility of their social-group male(s) (Sheldon, 1994; Vedder et al., 2011). EGP could be also as the consequences of sexual harassment or infanticide risk from extra-group males (Woff & Macdonald, 2004).

The spatio-temporal distribution of extra-group mating opportunities is an important factor affecting the intensity of sexual selection (Shuster & Wade, 2003). In particular, the number of breeding males available at neighbouring-group is linked with the extra-group

mating opportunities, hence the frequency of EGP (e.g., Westneat et al., 1990; Griffith et al., 2002). The number of males within a females' social group may also influence EGP rate. As the number of defending males (or males providing required levels of copulation) within-group increases, EGP rate would be predicted to decrease (van Noordwijk & van Schaik, 2004). Ecological evidence of EGP (or EPP) is accumulating for birds' species (reviewed in Griffith et al., 2002). In mammals, however reliable studies of EGP frequency and its ecological correlations are still few (see Isvaran & Clutton-Brock, 2007).

***'Inbreeding avoidance Hypothesis'***

Inbreeding is defined as mating among related individuals, leading to an increase in genome-wide homozygosity (Keller & Waller, 2002b) with a consequent loss of genetic variation (Bensch et al., 2006). Inbreeding is posited to have a negative influence on offspring survival rate (Boakes et al., 2007), lifetime breeding success (Kruuk et al., 2002), growth rate (Slate et al., 2000) and other morphological traits that may affect the persistence of natural populations (Keller & Waller, 2002b). The potential for inbreeding is high in group-living animals; particularly those species forming social groups comprised by closely related individuals, and where both sexes are philopatric with limited dispersal opportunities (Carpenter et al., 2005; Dugdale et al., 2007). In such instances, EGP has been proposed as an alternative strategy to avoid inbreeding (e.g., Brouwer et al., 2011).

***'Good-gene-as-heterozygosity Hypothesis'***

Many studies have reported positive associations between individual heterozygosity and fitness-related traits such as survival rate, reproductive success or parasite resistance in wild populations (Acevedo-Whitehouse et al., 2005; Cohas et al., 2009; Thob et al., 2011).

Although heterozygosity is not directly heritable (i.e., non-adaptive genetic variation; Bensch et al., 2006), correlation between parents and offspring heterozygosity can occur in wild populations under certain conditions (Mitton et al., 1993; Hoffman et al., 2007; Kempenaers, 2007). The 'Good-gene-as-heterozygosity Hypothesis' predicts that females should choose mates with a high level of heterozygosity (Brown, 1997). As heterozygosity may enhance fitness, females would be predicted to value heterozygosity in their offspring. Indeed, the average heterozygosity in offspring is positively correlated with the average heterozygosity of the parents and a choice for a (more) heterozygous mate would result in a higher frequency of heterozygosity in offspring than under random mating (Mitton et al., 1993; Brown, 1999). Females might therefore seek EGP in order to have their offspring fathered by more heterozygous males.

***'Fitness benefit Hypothesis'***

One key force driving the evolution of EGP/EPP is hypothesised to be that, under indirect selection, extra-group offspring (EGO or extra-pair offspring, EPO) produced via extra-group mating may be fitter than within-group offspring (WGO or extra-pair offspring, WPO) (Jennions & Petrie, 2000; Arnqvist & Kirkpatrick, 2005). The majority of studies that have investigated the fitness benefit of EGP/EPP are again in birds (e.g., Akcay & Roughgarden, 2007; Magrath et al., 2009; Brouwer et al., 2010; Sardell et al., 2011 & 2012). For mammals, Cohas et al. (2007a) give an example of the alpine marmot (*Marmota marmot*), a socially monogamous species, where extra-pair offspring exhibited higher survival rates during the critical period between birth and sexual maturity and higher reproductive success compared to within-pair offspring.

Fitness benefits obtained by females from EGP/EPP may depend on their offspring's sex. While few studies that have explicitly tested for differential sex-specific effects on fitness-related traits between extra-pair offspring and within-pair offspring in birds, have found no different between male and female offspring (see review Table S1 in Sardell et al,. 2011; Gerlach et al., 2012; Sardell et al., 2012), some reported an interaction between offspring-paternity type (whether EGO/EPO or WGO/WPO) and sex (Sardell et al., 2011; Schmoll et al., 2011). For example, females engaging in extra-group (or -pair) mating may do so if this ultimately leads to a greater number of offspring and grand-offspring (i.e., a "promiscuous-son benefit," similar to the "sexy-son benefit" in Fisher's runaway sexual selection; Fisher, 1930; Weatherhead and Robertson, 1979).

***'Between- and within- sex genetic correlation Hypotheses'***

Female extra-group mating behaviour (i.e. resulting in EGPs) can evolve through indirect selection in males to obtain EGP even when the behaviour appears maladaptive from the female perspective (Kirkpatrick & Barton, 1997; Forstmeier et al., 2011). The 'between-sex genetic correlation Hypothesis' states alleles that enhance infidelity in males could also enhance infidelity in females carrying the same alleles (because of pleiotropic effects; Halliday & Arnold, 1987). The 'within-sex genetic correlation Hypothesis' states females' sexual responsiveness towards within-group and extra-group males may be affected by the same set of alleles. Resistance to copulate with extra-group males would also convey resistance to within-group males, thus lead to infertility (Forstmeier et al., 2011).

**1.4 The European badger as a model species**

European badgers (*Meles meles*) are widely distributed across Europe, from the Arctic regions of Scandinavia and Russia, Asia and Japan (Marmi et al., 2006). They are biologically intriguing insofar that they are facultatively social Carnivores, being primarily solitary or pair- living at low density on the continent compared to group-living at high-density in lowland UK (reviewed in Woodroffe & Macdonald, 1993; Johnson et al., 2000; Palphramand et al., 2007). In high-density populations in lowland UK (e.g., Rogers et al., 1997; Macdonald & Newman, 2002) European badger are typically group-living, with up to 29 individuals forming a social group and holding a spatial territory (da Silva et al., 1994). Amongst several theories on the formation of badger social groups (see Revilla & Palomares, 2002) the RDH provides the most parsimonious explanation of passive group formation (Macdonald, 1983; Carr & Macdonald 1986; Johnson et al., 2002; but see Revilla, 2003a & b for perspective) and are maintained primarily by the retention of offspring in their natal group (Cheeseman et al., 1988; Woodroffe & Macdonald, 1993; da Silva et al., 1994; Woodroffe et al., 1995; Macdonald et al., 2008) and restricted dispersal (Pope et al., 2006; Macdonald et al., 2008).

Group formation is unusual among the Mustelidae (Powell, 1979; Erlinge & Sandell, 1986; Yamaguchi & Macdonald, 2003; Newman et al., 2011), but occurs in the badger despite only limited evidence of co-operative behaviour (Woodroffe & Macdonald, 2000; Dugdale et al., 2010). Theory predicts that both the occurrence and amount of co-operative behaviour are correlated negatively with the level of promiscuity (Cornwallis et al., 2010). High level of promiscuity recorded in badger (Dugdale et al., 2011) would decrease relatedness within social groups, which can lead to the complete or partial loss of co-

operative behaviour. Badgers therefore provide an informative model with which to advance the understanding of the evolution of sociality.

### 1.4.1 Mating system and extra-group paternity (EGP)

While badgers have been observed to mate during all months of the year in Britain (Neal & Cheeseman, 1996) they have two main peaks of reproductive activity, one in spring immediately post partum and a second smaller peak in autumn (Cresswell et al., 1992). Badgers give birth once a year around February after a period of delayed implantation (Thom et al., 2004). Cubs remain underground for the first eight weeks (Kaneko et al., 2010). The annual population sex ratio of post-emergence offspring does vary significantly from 50:50 the average is at par (Dugdale, et al., 2003).

In high-density populations, badgers exhibit a polygyandrous mating system both behaviourally and genetically; males have been observed to mate with multiple females, and females with more than one male (Dugdale et al., 2007 & 2011a). The genetic output of this plural breeding reveals that up to seven females and seven males may produce offspring in each year successfully within a social group (Dugdale et al., 2007). The average litter size is 1.4 (range = 1–5, Dugdale et al., 2007) and multiple-paternity occurs, in which different cubs within a litter are fathered by different males (Carpenter et al., 2005; Dugdale et al., 2007). Both Carpenter et al. (2005) and Dugdale et al. (2007) report high levels of EGP in two different badger populations, with up to half of cubs in each population sired by extra-group males (i.e., a male that lived in a different social group than the female), mainly from neighbouring groups.

There are several socio-ecological factors that may facilitate EGP in badgers. Previous studies have shown that object marking (Buesching & Macdonald, 2004), sequential allomarking (Buesching et al., 2003) and the use of boundary latrine (Kilshaw et al., 2009) by males peaked during the postpartum mating season, suggesting that males may mark their range to attempt to deter intruding extra-group males from their social group females. In addition, males can be aggressive towards extra-group males (Christian, 1995; Macdonald et al., 2004a; Dugdale et al., 2011a). However, given that badgers are highly promiscuous (Dugdale et al., 2007), forage solitarily (Macdonald, 1983; Carr & Macdonald, 1986; da Silva et al., 1993) and make extra-territorial excursions, often in the vicinity of neighbouring-group territories (Macdonald et al., 2008; Huck et al., 2008) during the mating period, it is unlikely that within-group males could mate-guard females effectively. Additionally, as female badgers can produce multiple-paternity litters, in which the cubs are fathered by within-group as well as extra-group males (Dugdale et al., 2007), it may benefit males to gain extra-group paternities, rather than to mate-guard within-group females. A high level of temporary inter-group movements in both males and females, where males tend to make more temporary movements than females (Macdonald et al., 2008), may further facilitate EGP in badgers.

Although a high frequency of EGP has been recorded in badgers, only one study (by Dugdale et al., 2007) has so far investigated the adaptive benefits of EGP strategy in this species. Dugdale et al. (2007) found that male badgers which fathered offspring both within and outside of their resident group, have greater lifetime breeding success than males that only fathered within-group offspring. Dugdale, (2007) also documented that extra-group fathers are less related to the females than within-group fathers, and showed that by mating with extra-group males, females may avoid inbreeding, although incestuous

pairings did occur. While males can improve their reproductive output through EGP, the benefits that female badger may derive from EGP are unresolved and warrant further investigations.

## 1.5 Thesis structure and aims

The objective of this thesis is to develop paradigm concerning the evolutionary causes and consequences of extra-group paternity in a socially polygynandrous and promiscuous species; the European badger (*Meles meles*). I used a combination of field methods, molecular techniques and analytical tools to determine parentage and further investigate how badgers benefit from EGP. The detailed aims of the four data chapters are as follows:

### 1.5.1 Molecular markers and parentage assignment

Sufficient numbers of highly polymorphic microsatellite markers are required to resolve parentage with high confidence, because badger social groups contain close relatives (Dugdale et al., 2008). The capacity to discriminate close relatives and the success of assigning parentage correctly are a function of the number of variable loci employed, the properties of these loci, genotyping error rates and parentage software used. In Chapter 2, I describe the characterisation of 21 novel microsatellite markers that have not been tested in any other badger population previously. The 4 markers that are fit into multiplex PCR sets, supplement the existing 31 markers (Bijlsma et al., 2000; Domingo-Roura et al., 2003; Carpenter et al., 2003; Huck et al., 2008), and advance capacity to resolve genealogies. In Chapter 3, I identify EGP using a total of 35 microsatellite markers, and two parentage analysis software packages: a) MasterBayes, which applies a maximum likelihood method but within Bayesian framework (Hadfield et al., 2006) and has an additional feature to incorporate phenotypic information along with genetic data to assign parentage; (b) Colony

2.0, which adopts maximum likelihood techniques to assign parentage and sib-ship simultaneously (Wang & Santure, 2009).

### 1.5.2 EGP: Ecological, heterozygosity, inbreeding and genetic quality hypotheses

The occurrence of EGP is well documented in badgers (Evan et al., 1989; da Silva et al., 1994), with half of paternities assigned to extra-group males (Carpenter et al., 2005; Dugdale et al., 2007). Despite a high level of EGP, the question of why female badgers engage in extra-group mating is still unresolved. I test several hypotheses that may explain the evolution of extra-group mating in badgers. The 'Good-genes-as-heterozygosity Hypothesis' predicts that females should prefer more heterozygous males (potentially extra-group males) over less heterozygous males, if higher heterozygosity is associated with higher fitness (Brown, 1997) and heterozygosity is inherited to their offspring. In Chapter 3, I examine these heterozygosity-fitness correlations by investigating the relationship between an individual's own, maternal and paternal heterozygosity and first-year survival probability. Subsequently, in Chapter 4 I test whether the average heterozygosity of within-group males is correlated with the rate of EGP at individual (litter) level. I also test the correlations between EGP rate and two ecological hypotheses, through the number of: (a) within-group males and (b) neighbouring-group males. In Chapter 4, I also examine the prediction of a higher EGP rate when the pair-wise relatedness between mothers and within-group candidate fathers is high, which would indicate inbreeding avoidance (as females are less related to extra-group than within-group males). In Chapter 5, I test the 'Fitness benefit Hypothesis' that predicts extra-group offspring are fitter than within-group offspring in terms of their first-year survival probability, estimated longevity (i.e., number of years a badger survived) and reproductive

success (i.e., the number of years a badger is assigned parentage and the number of cubs a badger is assigned in its lifetime).

Finally, in Chapter 6, the major findings are synthesised and considered with respect to current understandings of promiscuous mating paradigm, noting future directions for studies on EGP.

# Chapter 2:

# Characterisation of twenty-one European badger (*Meles meles*) microsatellite loci facilitates the discrimination of second-order relatives

## 2.1 Abstract

The European badgers (*Meles meles*) in lowland England live in social groups that consist of close relatives and breed plurally. To understand the mating system and social structure, it is vital to elucidate the parentage patterns between individuals. To improve parentage assignment and discrimination of relatives, I isolated and characterised 21 polymorphic microsatellite loci in 24 individuals from Wytham Woods, Oxfordshire, UK. These 21 loci increased the discrimination power between full-siblings and half-siblings from 71% to 88%, when added to the existing 31 loci. Similarly, the combined non-exclusion probability increased from $3.0 \times 10^{-8}$ to $5.8 \times 10^{-13}$. Newly isolated *Mel-592* (FR745854) was X-linked, based on the genotypes of 48 known-sex individuals, which will facilitate genetic sex-typing of badgers.

## 2.2 Introduction

European badgers (*Meles meles*) are nocturnal, breed plurally in lowland England (Carpenter et al., 2005; Dugdale et al., 2007), and cubs are raised underground (Dugdale et al., 2010); thus, successful reproductive history and reproductive strategies are impossible to identify in this species from behavioural traits alone. The application of molecular genetics techniques for individual identification and parentage inference provides a powerful technique with which to resolve these questions and further elucidate the understanding of the evolution of badger life history strategies.

Fifty-seven microsatellites have previously been characterised for badgers (Bijlsma et al., 2000; Carpenter et al., 2003; Domingo-Roura et al., 2003; Huck et al., 2008) and 31 are polymorphic in the Wytham Woods population (Dugdale et al., 2007; Chapter 3). Blouin et al. (2003) proposed that around 50 polymorphic microsatellite loci are required to differentiate second-order relatives from first-order. As badger groups contain close relatives (Dugdale et al., 2008), adding extra polymorphic markers, can increase the resolution power in the parentage analysis (Chapter 3). The aim of this study is to characterise 21 new microsatellite loci from three genomic libraries.

## 2.3 Materials and methods

Libraries 1 and 2 were prepared as described in Carpenter et al. (2003). Library 3 was constructed from one female badger (BAP1556) sampled at Wilcot, England (Ordinance Survey reference: SU141608) using the method of Armour et al. (1994) and enriched separately for di- and tetranucleotide microsatellite motifs comprising $(GT)_n$, $(CT)_n$, $(GTAA)_n$, $(CTAA)_n$, $(TTTC)_n$, $(GATA)_n$ and their complements. These were denatured and

bound to magnetic beads following Glenn & Schable, (2005). Transformant colonies were directly sequenced.

Primer pairs were designed for 35 unique microsatellite loci using PRIMER3 0.4.0 (Rozen & Skaletsky, 2000) with annealing temperatures in the range of 55–61$^0$C. Twelve males and 12 females from Wytham Woods, UK (51˚46´ N 1˚20´ W) were genotyped at each locus. To ensure individuals were as unrelated as possible I selected individuals from different social groups. Genomic DNA was extracted from blood using a modified Chelex protocol (Walsh et al., 1991). Polymerase chain reactions (PCR) were performed in a 384-well Dyad Hybaid Touchdown™ thermal cycler (Thermo Hybaid, Ashford, UK). A negative control (ultrapure double-distilled water) was used to detect contamination and a positive (known genotype) control to ensure consistent allele-scoring. Each 2 ul Qiagen PCR reaction (Qiagen Inc., Valencia, USA), contained 1 ul of Qiagen master mix, 1 ul of fluorescently labelled primer mix (forward and reverse) at 0.2 uM and 50 ng of DNA. The touchdown PCR program was: 95°C for 15 min, then 30 cycles of 94°C for 30 s, 90 s at 61–55°C and 72°C for 1 min, followed by a final 30 min at 60°C. All 24 genotypes (35 loci) were derived from single-plex PCRs on an ABI 3730 DNA analyser. Alleles were scored using ROX500 size-marker and GENEMAPPER 3.7 (Applied Biosystems, California, USA). Loci were checked for sex linkage by comparing the genotypes of 24 females and 24 males.

Four hundred thirty-two new badger microsatellite sequences were isolated: *Mel-80–Mel-611* (FR745442–FR745873). Observed and expected heterozygosities and estimated null-allele frequencies were estimated using CERVUS 3.0 (Kalinowski et al., 2007). Means are reported ± standard errors (SE).

## 2.4 Results and conclusions

Of the 35 new loci tested 21 were polymorphic (Table 2.1), 6 were monomorphic and 8 failed to amplify or amplified non-specific products (Supplementary Table S2.1). There were 2–5 alleles per locus (mean = 3.05 ± 0.21), and expected heterozygosities ranged from 0.08–0.75 (mean = 0.44 ± 0.03). The combined non-exclusion probability of not excluding a single randomly-chosen unrelated individual from parentage increased from $3.0x10^{-8}$ (31 existing loci) to $5.8x10^{-13}$ (52 loci = 31 existing loci + 21 new loci from this study). KinInfor 1.0 (Wang, 2006) estimated that 31 existing loci distinguished full-siblings from half-siblings with 71% accuracy, or 88% with 52 loci. *Mel-592* (FR745854) was X-linked; all 24 males (XY) were homozygous, whereas 11 females (XX) were heterozygous and 13 females homozygous (Fisher's Exact Test $P < 0.001$; 95% confidence interval for the odds ratio = 0.00–0.19). This locus will assist the genetic sex-typing of badgers particularly when sampled non-invasively.

Deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were tested using GENEPOP 4.0.10 (Raymond & Rousset, 1995). Only *Mel-551* (FR745813) deviated significantly from HWE ($P = 0.0499$). Nine pairs of loci were in LD (*Mel-232–Mel-243*, *Mel-243–Mel-576*, *Mel-246–Mel-203*, *Mel-253–Mel-161*, *Mel-451–Mel-153*, *Mel-522–Mel-538*, *Mel-538–Mel-554*, *Mel-538–Mel-186*, and *Mel-153–Mel-203*) but not after adjusting for multiple testing by FDR control ($m = 210$, $\alpha = 0.05$, adjusted $P$ = 0.001–0.050; Benjamini & Hochberg, 1995).

These 21 polymorphic microsatellite loci will advance the capacity to resolve genealogical relationships between badgers.

**Table 2.1** Characterisation of 21 microsatellites for European badgers

| Locus | EMBL | Clone library | Clone | Repeat motif | Primer sequence (5'-3') | Fluoro label (FP) | Expected/ observed allele size (bp) | N | *A* | $H_0$ | $H_E$ | $P_{HWE}$ | *F (null)* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Mel-153* | AJ230722 | 2 | B18E04 | $(CA)_{12}$ | **F:**ATCTGGCATCTAACAGGAGATAC **R:**GATCTCTGCTGTCTTTGTGTG | 6FAM | 309/309–313 | 24 | 2 | 0.29 | 0.31 | 1.00 | 0.02 |
| *Mel-161* | AJ293385 | 1 | BAD03A | $(CA)_{15}$ | **F:**TTCTATGCTTGGACCTTCTACTCTTT **R:**ATTACCTAAGGAAGCGGAGGA | HEX | 361/355–359 | 24 | 3 | 0.50 | 0.58 | 0.40 | 0.06 |
| *Mel-186* | FR745448 | 3 | 09D01(S) | $(CA)_{20}$ | **F:**CTCAACATCCAAGGCTGACTG **R:**GATCATGACCTGAGCCGAAG | 6FAM | 439/431–437 | 23 | 3 | 0.43 | 0.52 | 0.10 | 0.06 |
| *Mel-191*[a] | FR745453 | 3 | 09E03(S) | $(CA)_{21}$ | **F:**GAATTAGAGAACCCAGAAATACC **R:**GTTGAAATTAGCTAACAGAGTGG | HEX | 273/273–275 | 24 | 2 | 0.08 | 0.08 | 1.00 | -0.01 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Mel-203* | FR745465 | 3 | 13F01(S) | $(GAAA)_8$ | **F:**TGCCTGCCTTAATGTTGATG **R:**TCACCAAGTTGGCACTGAAG | HEX | 453/441–453 | 24 | 4 | 0.54 | 0.60 | 0.30 | 0.04 |
| *Mel-211* | FR745473 | 3 | 10A04 | $(AC)_{19}$ | **F:**GGAGTCCTATGCCAAGAACAG **R:**AAATCTGTTACTCCTGCTCCATC | HEX | 267/265–267 | 23 | 2 | 0.44 | 0.43 | 1.00 | -0.01 |
| *Mel-213* | FR745475 | 3 | 10A06 | $(CA)_{15}$ | **F:**CCCATAAGGGTTGAGGACAG **R:**AATAAGGTGAAGACCCAAGGTG | HEX | 242/246–250 | 24 | 2 | 0.38 | 0.40 | 1.00 | 0.03 |
| *Mel-232* | FR745494 | 3 | 10C06 | $(CA)_{20}$ | **F:**TGGAACTGATGACTCACTAAACTC **R:**TACTTCCACTGGTCCAATCC | HEX | 272/268–274 | 22 | 3 | 0.27 | 0.25 | 1.00 | -0.07 |
| *Mel-243* | FR745505 | 3 | 10D08 | $(AC)_{21}$ | **F:**TGTTAGCTCCACCCTCAGATG **R:**ACCAATACAGAGTAAGCCTACAAACC | 6FAM | 275/269–273 | 24 | 3 | 0.50 | 0.67 | 0.17 | 0.14 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Mel-246* | FR745508 | 3 | 10D11 | $(GATA)_9$ | **F:**GGCGTTAAGGTTCTCCAAAG **R:**CTTGCCAGCCTTTGTAACTG | 6FAM | 157/151–159 | 24 | 3 | 0.33 | 0.40 | 0.66 | 0.07 |
| *Mel-253* | FR745515 | 3 | 10E07 | $(GT)_{15}$ | **F:**CACCCAATCCACCATGAG **R:**CCAGAATGCCTTATGGTCAC | HEX | 218/220–224 | 24 | 3 | 0.46 | 0.58 | 0.13 | 0.09 |
| *Mel-451* | FR745713 | 3 | 14A05 | $(AAAG)_{11}$ | **F:**CCGGTCTGAGGACAGCA **R:**CCCACCATAGGGCTCAAAC | 6FAM | 217/201–227 | 24 | 5 | 0.54 | 0.61 | 0.72 | 0.05 |
| *Mel-483* | FR745745 | 3 | 14F03 | $(AAAG)_{12}$ | **F:**CTGGGATTGAGCCCTGAG **R:**GGTTAATGGTCATCAAACCACT | 6FAM | 235/227–231 | 23 | 2 | 0.48 | 0.48 | 1.00 | -0.01 |
| *Mel-499* | FR745761 | 3 | 14H03 | $(TTTC)_{11}$ | **F:**TGACCTGAGCCGAAGTCAG **R:**AAAGCCAATACTTATTTACCATCCTG | 6FAM | 345/345–353 | 24 | 4 | 0.71 | 0.71 | 0.58 | 0.00 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Mel-522* | FR745784 | 3 | 15C09 | $(AAAG)_{11}$ | **F:**CTAATTGCTAGAATGAATGGGTTG **R:**TCAGTTTACCTCCATGGTTCC | HEX | 258/242–258 | 22 | 4 | 0.55 | 0.60 | 0.56 | 0.06 |
| *Mel-538* | FR745800 | 3 | 15F01 | $(ATCT)_{9}$ | **F:**CTAAGCCTTACGCCTACATTATACAC **R:**TATTGGCGAGTCTAGGTCCTG | HEX | 299/297–305 | 23 | 3 | 0.43 | 0.61 | 0.23 | 0.16 |
| *Mel-551* | FR745813 | 3 | 15G11 | $(GATA)_{11}$ | **F:**CCTGTAAGAGTTTGCATGGAG **R:**ACATGGTATCTACCTGGTCCTG | 6FAM | 227/217–237 | 24 | 5 | 0.58 | 0.77 | 0.05 | 0.13 |
| *Mel-554* | FR745816 | 3 | 15H03 | $(AAAG)_{15}$ | **F:**TGGTGCTTATCTGAACATGAGG **R:**GACTGAGCCAGCCAGGTG | HEX | 211/203–207 | 22 | 2 | 0.36 | 0.41 | 0.62 | 0.04 |
| *Mel-558* | FR745820 | 3 | 16A02 | $(AGAA)_{13}$ | **F:**GCAAGCACTGTGCATAATTG **R:**GATGCTCAACCCACTGAGTC | 6FAM | 255/233–245 | 24 | 4 | 0.75 | 0.72 | 0.52 | -0.03 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Mel-576* | FR745838 | 3 | 16D05 | $(AAGA)_9$ | **F:**ACTAGTGGCATGTTCATATAATGG **R:**CCTGCCTAGAGAGTTCCTACC | 6FAM | 258/246–258 | 23 | 3 | 0.30 | 0.27 | 1.00 | -0.08 |
| *Mel-592*[b] (X-linked) | FR745854 | 3 | 16F06 | $(CTTT)_{10}$ | **F:**AGCCAAATGACCAGCAATG **R:**AATTGGTATGTCTTATTAAGGAAGCAG | HEX | 233/233–237 (Female) | 24 | 2 | 0.54 | 0.50 | 1.00 | -0.05 |
| | | | | | | | 233/233–237 (Male) | 24 | 2 | 0.00 | | | |

(FP) = forward primer, (bp) = base pairs, $N$ = number of badgers genotyped, $A$ = number of alleles observed, $H_O$ = observed heterozygosity, $H_E$ = expected heterozygosity, $P_{HWE}$ = $P$-value from Hardy-Weinberg equilibrium, *F(null)* = estimated null allele frequency, (S) = clone sequenced at NBAF–Sheffield, all other clones isolated from library 3 were sequenced at NBAF–Edinburgh

[a]*Mel-191* (FR745453) displayed high sequence similarity to many mammalian X-chromosomes; however when tested in a larger number of individuals (569 females and 543 males) at later stage of this study, this locus was found to be autosomal by the present of male heterozygotes (n=125, Annavi et al., unpublished data) [b]*Mel-592* (FR745854) is X-linked based on the genotyping of 48 known-sex individuals

## 2.5 Supplementary materials

**Supplementary Table S2.1** European badger (*Meles meles*) microsatellite loci that were uninformative when amplified in badgers from Wytham Woods, Oxfordshire, England.

| Locus | EMBL | Clone library | Clone | Repeat motif | Primer sequence (5'-3') | Fluorescent label (FP) | $T_m$ | N | Expected allele size (bp) | Comments |
|---|---|---|---|---|---|---|---|---|---|---|
| *Mel-141* | AJ293362 | 1 | CGBA5 | $(CA)_{20}$ | **F:**AGCATGGAGCAGGCACTC **R:**ACCTGAGCCGAAGCCAG | 6FAM | 60 60 | 14 | 214 | Non-specific product(s) |
| *Mel-147* | AJ230716 | 2 | B02G06 | $(GA)_7$ | **F:**CCCTGGAATGCTTCTGAAC **R:**CCTTTATCATAGGAGAGGGAGTC | 6FAM | 58 57 | 15 | 403 | Monomorphic |
| *Mel-150* | AJ230719 | 2 | B10B06 | $(GA)_{10}$ | **F:**TTACCAGGAAGGTATGACTATTTAC **R:**TATTTCATTCTATTGCACTGTCA | 6FAM | 55. 55 | 15 | 234 | Monomorphic |
| *Mel-152* | AJ230721 | 2 | B17GO9 | $(CA)_{10}$ | **F:**TTGAATTGCAGTGTGTGGATG **R:**TTCGGTAATCCCAAATGACG | HEX | 61 61 | 15 | 391 | Monomorphic |
| *Mel-157* | AJ293380 | 1 | CGBA43 | $(GT)_{22}$ | **F:**CCTGAGATCACGCCAGTCAG **R:**TAAGTAGACCTGAAGCCTCTGC | 6FAM | 62 58 | 14 | 553 | Failed to amplify |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Mel-174X* | AJ230710 | 2 | B26D10 | $(GA)_8$ | **F:**TCAGGGAATTTTCCAAAGATG<br>**R:**TCCCAATTACCATGTGAGAAAG | HEX | 59<br>59 | 7 | 198 | Non-specific product(s) |
| *Mel-181* | FR745443 | 3 | 09B01(S) | $(GT)_{14}$ | **F:**AATTGAGGTTGCTTTCTCTCAC<br>**R:**TAATGATTGCCATGGGATG | 6FAM | 58<br>58 | 15 | 337 | Monomorphic |
| *Mel-183* | FR745445 | 3 | 09C01(S) | $(CA)_{21}$ | **F:**CCACAAGTGGATTTGTAAGATTATATG<br>**R:**ATGTGGTAGAGAGTAAATGCTACTGC | HEX | 59<br>59 | 15 | 130 | Monomorphic |
| *Mel-190* | FR745452 | 3 | 09E02(S) | $(CA)_{16}$ | **F:**AGATGTCCATCAACAGATGAAT<br>**R:**GATGGCTGCATAGTATTCCA | 6FAM | 56<br>56 | 15 | 102 | Monomorphic |
| *Mel-197* | FR745459 | 3 | 09H02(S) | $(GT)_{10}$ | **F:**CATGCCATTCTTAGGGGTTC<br>**R:**TCACTCTCTGACCCTCTGTCC | HEX | 59<br>59 | 15 | 472 | Failed to amplify |
| *Mel-209* | FR745471 | 3 | 10A02 | $(GT)_{17}$ | **F:**TCTGTCACCTATCATACTGACCTGAG<br>**R:**CAGGCAAGGAAGCGACATAG | 6FAM | 61<br>61 | 24 | 247 | Non-specific product(s) |
| *Mel-270* | FR745532 | 3 | 10G01 | $(AC)_{23}$ | **F:**AACAGCTGCCTCCGACTC<br>**R:**GCTCCAAAGCAGAACTGTTG | HEX | 59<br>59 | 24 | 249 | Non-specific product(s) |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Mel-570* | FR745832 | 3 | 16C06 | $(AAGA)_{13}$ | **F:**ACATTGGGCTCCATGCTG **R:**CACCAGTTGGTATTGTGATTGC | 6FAM | 61<br>60 | 24 | 271 | Non-specific product(s) |
| *Mel-586* | FR745848 | 3 | 16E08 | $(AAAG)_{12}$ | **F:**GGATGATGAGCTTCCAGAGG **R:**AAGAGTCATGAGTGAATCGAAGTG | HEX | 60<br>60 | 24 | 393 | Non-specific product(s) |

(FP) = forward primer, $T_m$ = melting temperature, $N$ = number of badgers tested from Wytham Woods, Oxfordshire, UK, (bp) = base pairs, (S) = clone sequenced at NABF-Sheffield, all other clones isolated from library 3 were sequenced at NBAF-Edinburgh

# Chapter 3:

# Heterozygosity-fitness correlations in a wild mammal population: single locus, paternal and environmental effects

## 3.1 Abstract

Heterozygosity-fitness correlations (HFCs) have been used to investigate the direct relationship between an individual's genetic diversity and fitness. The indirect effects of parental heterozygosity and the environmental-variability of HFCs are under-researched currently. I used a multi-model capture-mark-recapture framework to estimate the probability of European badger (*Meles meles*) survival, in a high-density UK population, in response to offspring and parental heterozygosity measured at 35 microsatellites. I detected inter-annual variation in first-year, but not adult, survival probability. Adult males had a lower annual survival probability than adult females. Paternal heterozygosity, but not maternal or an individual's own heterozygosity, was associated positively with first-year survival probability, but only in wet years. Moist soil conditions enhance badger food supply improving survival; therefore, higher indiscriminate mortality rates in dry years mask differential survival effects. Female badgers may thus increase their fitness by mating with heterozygous males, but these benefits only manifest during wet years. First-year survival probabilities were not correlated with the inbreeding coefficient (*f*); however, small sample sizes limited the power to detect inbreeding depression. Despite identity disequilibrium showing that the 35 loci had power to detect inbreeding, the local effect model (single-locus paternal HFCs) received 99.8% more support than the general effect model, with some effects only apparent in wet years. I posit that environmentally-interactive paternal effects may play an important role in the evolution of mate choice.

## 3.2 Introduction

Without individual genetic diversity within populations there would be no potential for natural selection. Understanding how genetic diversity is associated with fitness is thus fundamental for comprehending and predicting evolutionary processes (Stearns & Hoekstra, 2005; Ellegren & Sheldon, 2008).

This diversity manifests as heterozygosity, where genetic markers reveal effects on fitness-related traits, e.g., survival probability (Charpentier et al., 2008), reproductive success (Slate et al., 2000) and disease resistance (Acevedo-Whitehouse et al., 2005). Inbreeding reduces heterozygosity on a genome-wide scale, which increases the probability that deleterious mutations are expressed (Keller & Waller, 2002b). This can lead to inbreeding depression, when the offspring of related parents have lower fitness than offspring of unrelated individuals. The 'General effect Hypothesis' (Hansson & Westerberg, 2002) proposes that these 'heterozygosity-fitness correlations' (HFCs, David, 1998) arise due to inbreeding or outbreeding depression.

Two other hypotheses have also been applied to explain HFCs (Hansson & Westerberg, 2002). The 'Direct effect Hypothesis' (David, 1998) states that HFCs may arise due to functional over-dominance at scored loci that are under direct selection. Functional over-dominance occurs when a heterozygote has an intrinsically higher fitness than that of either homozygote. This hypothesis is generally rejected when using microsatellites, as they are usually assumed to be neutral markers, located in non-coding regions of the genome (Jarne & Lagoda, 1996). Nevertheless, some microsatellites have a functional role in structural and metabolic DNA processes, such as the regulation of gene activity i.e., DNA replication and recombination (Li et al., 2002).

The 'Local effect Hypothesis' (David, 1998) proposes that associative over-dominance explains HFCs, where some loci are in linkage disequilibrium with functional loci. Local effects may be weak however, and many studies may have overestimated these by using inappropriate statistical tests (Szulkin et al., 2010).

When testing these hypotheses it is important to choose a heterozygosity measure that reflects inbreeding reliably (Coltman & Slate, 2003). HFCs have become increasingly popular tools with which to quantify inbreeding depression in populations where pedigrees have not been derived (Grueber et al., 2008; Chapman et al., 2009). Empirical evidence however currently indicates that, even measured at relatively large numbers (16–23) of microsatellite loci, the correlations between molecular heterozygosity (e.g., standardised multilocus heterozygosity: Coltman et al., 1999) and inbreeding coefficient (*f*) are too weak to be of biological significance (e.g., Grueber et al., 2011).

It is therefore important that HFC studies quantify their power to detect inbreeding or outbreeding. HFCs are more likely to be detected in: (1) populations with a higher variance in *f,* due to incestuous matings yielding identity disequilibrium of loci across the genome (Slate et al., 2004, although see Chapman et al., 2009); (2) larger sample size, in which many markers are used to estimate heterozygosity (Balloux et al., 2004); and (3) under specific environmental conditions – inbreeding depression tends to increase linearly with the magnitude of environmental stress (Fox & Reed, 2010), such that HFCs manifest more strongly across the population under stressful conditions (e.g., Lesbarreres et al., 2005; Da Silva et al., 2006). Alternatively, HFCs may only be detected during favourable conditions, if unfavourable conditions exert stronger selection than the heterozygosity effect, and then the HFC will be masked (Harrison et al., 2011). Studies should therefore quantify the

variance in *f* in the focal population, use a large number of markers, and assess HFCs over a range of environmental conditions.

I investigated HFCs in a high-density population of European badgers (*Meles meles*) in southern England; a study system that fulfils the three critical points above. This population has a genetically-derived pedigree that includes inbreeding events (5% rate of incestuous matings: Dugdale, 2007), enabling variance in *f* to be quantified. Additionally, I have genotyped individuals with 35 microsatellite loci; more than most other HFC studies (Chapman et al., 2009). Furthermore, high-quality environmental data are available for the study site, to investigate any environmental-dependency of HFCs. This is important as summer temperature, summer rainfall and equinoctial conditions impact on the fitness of badgers and thus affect cohort survival rates (Macdonald & Newman, 2002; Macdonald et al., 2010).

To test for a relationship between genetic diversity and fitness, I examined whether first-year survival probability (i.e., survival from birth to 1 year), as a fitness metric, was predicted by heterozygosity and/or *f*. Offspring fitness is determined not only by their own genetic composition, but also by parental effects (Kirkpatrick & Lande, 1989), as can be suggested by correlations between offspring fitness and parental heterozygosity (Brouwer et al., 2007). I therefore investigated the effects of individual, maternal and paternal heterozygosity on first-year survival probability. As low May rainfall is known to interact with juvenile parasitic infection and higher cub morbidity and mortality rates (Macdonald et al., 2010), I also examined climatic effects on first-year survival probability, while controlling for parasitic infection statistically.

This approach allowed to test whether the Local or General Effect Hypotheses provided the greatest explanatory power for HFCs in the study population; in the absence of a badger genome map I could not test the 'Direct effect Hypothesis'. If estimated heterozygosity reflects genome-wide heterozygosity – thus predicting $f$ – this would indicate a general effect. Alternatively, should heterozygosity at any single locus exhibit a greater capacity to account for variation in fitness-related traits than multilocus heterozygosity, this would indicate a local effect.

## 3.3 Materials and methods

### 3.3.1 Study site, species and data collection

This study was conducted at Wytham Woods, 6 km northwest of Oxford in southern England (51˚46′ N 1˚20′ W), which comprises 415 ha of mixed deciduous/coniferous secondary and ancient woodland, surrounded by agricultural land (Savill et al., 2010). Over the study period (1987–2010) mean (with their 95% confidence interval [CI]) annual temperature and precipitation were 10.5 [10.1, 10.9] $^{o}$C and 665 [622, 708] mm, respectively (climate data were obtained from Oxford Radcliffe Metrological Station, School of Geography). The resident badger population inhabits a partly discrete geographical area, which limits, but does not eliminate, the potential for migration into or out of the study area (Macdonald et al., 2008). Dispersal is restricted, although temporary movements occur, most often to neighbouring groups (Macdonald et al., 2008). Based on modified 'minimum number alive' and capture-mark-recapture (CMR) techniques (Macdonald et al., 2009), the badger population size has increased substantially from the late 1980s, from ca. 100, and then stabilised after 2000 at ca. 250 individuals (Macdonald

et al., 2009). Social group territory boundaries within this population have been mapped using bait marking, approximately every two years (Kilshaw et al., 2009).

Badgers in this study site live in large groups (up to 29 individuals, da Silva et al., 1994) consisting of close kin (Dugdale et al., 2008), with up to seven breeding members of both sexes per year within a social group (Dugdale et al., 2007). Natal philopatry and high levels of relatedness between group-members may increase the likelihood of incestuous matings; however, the high rate of extra-group matings (ca. 50%: Carpenter et al., 2005; Dugdale et al., 2007) may reduce inbreeding, noting that dispersed kin may also be present in surrounding groups.

Badgers were captured 3–4 times per year over two weeks in late May, August and October–November, with one week of trapping in January in some years. Badger cubs are normally born in mid-February, in a highly altricial state and remain predominantly below ground for their first 8 weeks from birth (Roper, 2010). Because cubs are highly dependent during this period, trapping was suspended on welfare grounds until they were fully weaned at around fifteen weeks of age, in June (Macdonald et al., 2009). All trapping and handling procedures were in accord with the UK Animals (Scientific Procedures) Act, 1986, approved by an institutional ethical review committee, and carried out under government licenses (currently Natural England Licence 20104655 and Home Office PPL30/2835). Badgers were trapped using steel-mesh cages placed near the entrances of active setts (subterranean dens), sedated with ketamine hydrochloride (0.2 ml/kg body weight, Thornton et al., 2005) and given a unique tattoo number, in their inguinal region, for permanent individual identification. Individuals were sexed and classified as cub (animals in their first year) or adult based on their trapping history from birth. Of 1410

individuals trapped 1987–2010, 975 (69%) were of known age (trapped as cubs). Intact follicles from plucked hairs, along with jugular blood samples (c. 3 ml) were collected for genetic analyses.

### 3.3.2 Microsatellite genotyping

One thousand one hundred and seventy (83%) badgers, trapped 1987–2010, were genotyped. Of the 975 badgers that were first caught as cubs, 838 (86%) were genotyped (136 were not sampled, and one badger had only one sample that did not amplify). A minimum of 20 hair follicles or 25 µl of whole blood from each individual were used for DNA extraction, using a slightly modified Chelex protocol (Walsh et al., 1991). Individual genotypes were determined using 35 fluorescently labelled autosomal microsatellite markers (Supplementary Table S3.1), which were grouped into seven multiplexes (4–9 markers per set) using Multiplex Manager 1.0 (Holleley & Geerts, 2009). Primer pairs were analysed in a single computational run in AutoDimer 1.0 for potential cross-reactivity within and between primers (Vallone & Butler, 2004). A 2 µl Qiagen Multiplex PCR reaction was used, as described in Chapter 2, and these samples were then sequenced and analysed using GENEMAPPER 3.5.

Genotyping was 97% complete, with each individual genotyped for a minimum of 18 loci (mean [CI] = 34.0 [33.8, 34.1]). A GENEPOP 4.0.10 (Raymond & Rousset, 1995) analysis of 30 adults from three different years, selected randomly, showed that none of the markers violated Hardy Weinberg Equilibrium ($m = 35$, $\alpha = 0.05$, adjusted $p = 0.050–0.001$) and no pair of loci was linked consistently, after false discovery rate control (Benjamini & Hochberg, 1995). Mean allelic dropouts ($\varepsilon_1$) and false alleles ($\varepsilon_2$) were estimated at 0.005 using PEDANT 1.0 (Johnson & Haydon, 2007), by re-genotyping 5% of individuals,

chosen at random. CERVUS 3.0.3 (Kalinowski et al., 2007) and MICRO-CHECKER 2.2.3 (van Oosterhout et al., 2004) were used to estimate allelic diversity, observed heterozygosity and null alleles for each marker (Supplementary Table S3.1). Mean observed and expected heterozygosity [CI] were 0.45 [0.39, 0.51] and 0.49 [0.43, 0.55], respectively. The mean number of alleles [CI] was 4.46 [3.79, 5.13].

### 3.3.3 Parentage assignment

A step-wise procedure was used to attempt to assign parentage to the 813 genotyped cubs (trapped 1988–2010; 25 cubs trapped in the first year of capture-mark-recapture study (1987) were excluded due to incomplete candidate parents; Figure 3.1). Bayesian parentage analysis was applied to each cub cohort, in a restricted analysis, using MasterBayes 2.47 (Hadfield et al., 2006) in R 2.12.2 (R Development Core Team, 2008), where females aged ≥ 2 years, which were present in the cubs' natal group, were included as candidate mothers; all males of breeding age (> 1 year old), across the entire population, were included as candidate fathers (Dugdale et al., 2007). Where mothers were unassigned, I re-tested parentage in an open analysis, considering all extant females aged ≥ 2 years as potential candidate mothers. I included the geographical locations (GPS coordinates of each group's main sett) of all offspring and candidate parents in my models to estimate the probability with which parentage assignment decreases with distance. Un-sampled males were placed at the mean distance between all phenotyped individuals. A starting pedigree, for the final model was estimated by running 10,000 iterations and extracting the mode of the posterior distribution of the parents. The starting model used default tuning parameters but specified the number of unsampled candidate mothers and fathers (Supplementary Table S3.2) estimated from CMR (Dugdale et al., 2007), the two genotyping error rates ($\varepsilon_1$ and $\varepsilon_2$) as 0.005, and the allele frequencies were extracted from all genotypes. Tuning

parameters were specified in the final model (ß=5 restricted analysis, ß=1 open analysis) to ensure that the Metropolis-Hastings acceptance rates were between 0.2 and 0.5. Allele frequencies in the final model were extracted from all genotypes and the maximum number of mismatches tolerated was set to three. Markov chains were run separately for each year, for 1.5 million iterations, with a thinning rate of 500 and burn-in of 500 000. Successive samples from the posterior distribution had low autocorrelation ($r < 0.02$). Sib-ships were then reconstructed in COLONY 2.0 by partitioning each cub cohort (including cubs that were and were not assigned parent(s) in MasterBayes) into full- and half-sibship families using a maximum likelihood method (Wang & Santure, 2009). Parentage was accepted with $\geq 0.8$ probability both in MasterBayes and Colony. Maternity and paternity were assigned to 683 (84% of genotyped cubs) and 655 (81%) cubs, respectively. Both parents were assigned to 561 (69%) cubs (see Supplementary Table S3.3 for summary statistics of pedigree and Supplementary Table S3.4 for summary of mismatches between parents and their offspring).

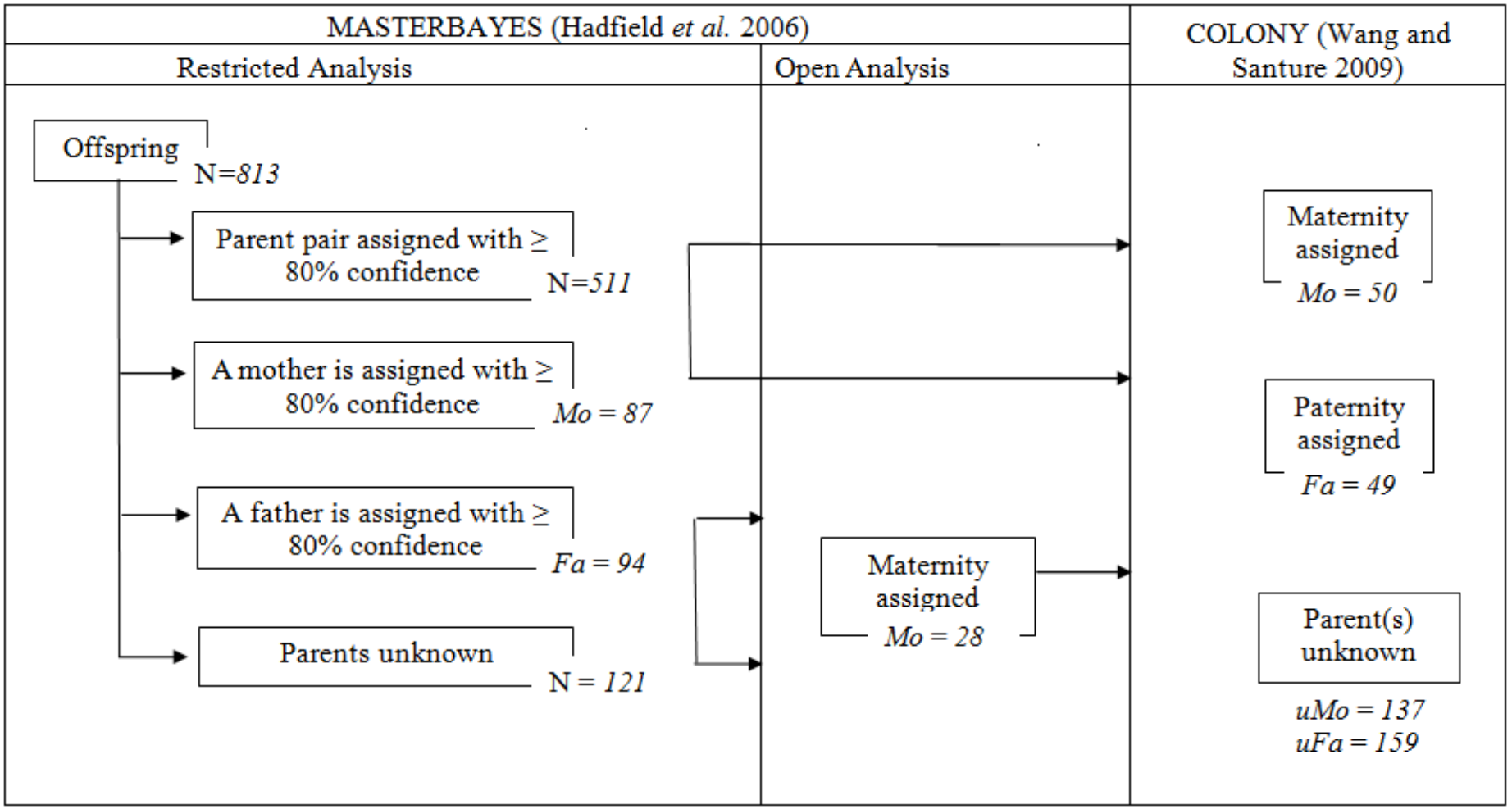


**Figure 3.1** Flowchart of the parentage assignment rules used in MasterBayes 2.47 and Colony 2.0. Both parents were assigned to 511 cubs; 165 were assigned mothers only; 143 were assigned fathers only. N = total number of cubs; *Mo* = number of cubs with an assigned mother; *Fa* = number of cubs with an assigned father; *uMo* = number of cubs with an unassigned mother; *uFa* = number of cubs with an unassigned father. Parentage was assigned with ≥ 80% confidence.

### 3.3.4 Estimating genetic diversity and inbreeding

Three microsatellite-derived measures of multilocus heterozygosity (standardised heterozygosity [SH], Coltman et al., 1999; homozygosity by locus [HL], Aparicio et al., 2006; internal relatedness [*Ir*], Amos et al., 2001) were estimated for 1007 badgers using GENHET in R 2.12.2 (Coulon, 2010). I excluded the mean $d^2$ estimator (Coulson et al., 1998b) because of difficulties with its interpretation (Hansson, 2010). SH values were highly correlated with HL and *Ir* (Spearman's rank correlation, $r = -0.96$, $P < 0.001$). I therefore present analyses based on SH, which gave similar outputs to the other two indices (Supplementary Table S3.5 and S3.6). Pedigree Viewer 6.3 (Kinghorn, 1994) was used to calculate *f* for 561 of the 813 genotyped cubs that were assigned both parents with ≥ 0.8 probability. I also followed the approach of Szulkin et al. (2007), by restricting this dataset to 420 (52%) or 88 (11%) cubs that had at least one or all four grandparents assigned, respectively (Supplementary Table S3.7).

### 3.3.5 Data analyses

A recapture history file was compiled, consisting of 24 annual trapping records for 975 individuals of known age between 1987 and 2010. If an individual was trapped at least once in a particular year it was denoted as '1' (years commenced with the birth of cubs in February) or '0' if it was not caught in that year.

#### 3.3.5.1 Age, sex, cohort and population size

A multi-step strategy in MARK 6.1 (White & Burnham, 1999) was used within a Cormack-Jolly-Seber framework, to obtain a 'reduced model' (B1, Table 3.1, that was used later to investigate the effects of SH and *f* on first-year survival probability). Cubs

typically exhibit lower mean inter-annual survival rates than do adult badgers (Macdonald et al., 2009). Also, adults and cubs exhibit year-dependent survival rates (Macdonald & Newman, 2002). My starting model was therefore based on year-dependent (t) and age-dependent (two-age classes, a2 = [cub < 1 year old] / [adult ≥ 1 year old]) survival (Φ) and recapture probabilities (*p*) [starting model = Φ (a2-t/t) *p* (a2-t/t)]. Sex, cohort size and population size were then included to test their effects on cub and adult survival probabilities.

#### 3.3.5.2 Goodness-of-fit and model selection

The goodness-of-fit of my starting model was assessed using a bootstrap method (Pradel, 1996), implemented in MARK 6.1 (White & Burnham, 1999). The variance inflation factor (ĉ), estimated by dividing the model deviance by the bootstrapped deviance, indicated that the starting model was slightly over-dispersed (ĉ = 1.03; N = 100 replicates); I adjusted the AICc (Akaike information criterion, corrected for sample size; Akaike, 1973) value accordingly, through quasi-likelihood (QAICc).

I employed information-theoretic (IT) approaches to select plausible models and to estimate the overall importance of each effect (Burnham et al., 2011). The QAICc, was used to identify the 'top model' (i.e., model with the lowest QAICc value, Burnham et al., 2011). Following Burnham & Anderson, (2002), if the difference in QAICc (ΔQAICc) between the top model and another model was ≥ 7, I considered the top model to be more plausible, given the data. If ΔQAICc was < 7, both models were considered plausible, given the data. A model's relative weight (ω) was calculated as the model's relative likelihood given the data (exp[-0.5 * ΔQAICc]) divided by the sum of the likelihoods for

all considered models (whether plausible or not). The 'evidence ratio' between two models, was calculated as the ratio of ω for those two models.

#### 3.3.5.3 Effects of SH and f on first-year survival probability

The reduced model (B1, Table 3.1; Φ [a2-t/sex] *p* [t]) was used to investigate the effects of an individual's own heterozygosity ($SH_{Ind}$; N = 839), as well as the heterozygosity of their assigned mother ($SH_{Mat;}$ N = 683) and father ($SH_{Pat}$; N = 655), on first-year survival probability. Models tested for climatic effects (standardised mean summer [May–October] and winter [November–February] temperature, and standardised total summer rainfall [May–October]). I included all first-order interactions between each SH measure and these climatic variables, to test for climate dependency of heterozygosity effects on first-year survival probability. Quadratic SH effects were included to test for nonlinearity (Neff, 2004). To control for the effect of endoparasitc infection on first-year survival, a sub-set of models was run, retaining $\log_e(x+1)$ transformed coccidial (gut parasite) load (even if not significant), using a restricted dataset. The restricted dataset consisted of coccidial loads derived from faecal counts of *Eimeria melis* oocysts, from 143 genotyped cubs (N = 47 [1993]; 23 [1994]; 34 [1995]; 28 [1996] and 11 [1997]) caught between May and November (standardised across months; Newman et al., 2001).

The detected paternal heterozygosity effect may be due to immigrants into the study population that produced heterozygous offspring, which then bred. These fathers may not only have high heterozygosity but also rare alleles that may influence survival. I therefore included a measure of rare alleles (the number of rare alleles [frequency of < 5%] that an offspring's father had, divided by the number of alleles that the father was typed for) when modelling the effect of paternal heterozygosity on first-year survival probability.

Similar models, incorporating climatic variables, were used to test for effects of *f* on first-year survival probability. Badgers with $f \geq 0.125$ were defined as inbred ('1') and those with $f < 0.125$ as outbred ('0'). I conducted three separate analyses, with different datasets, to test how the effect differed depending on my increased ability to estimate *f* which at the same time reduced the statistical power (Szulkin et al., 2007; Marshall et al., 2002): (a) $f_{561}$ included 561 cubs that had both parents assigned with ≥ 0.8 probability, (b) $f_{420}$ was restricted to 420 of these cubs that had at least one grandparent assigned, and (c) $f_{88}$ was restricted to 88 individuals that had all four grandparents assigned. Models were analysed with the logit link function in MARK 6.1 (White & Burnham, 1999). Recapture probabilities were fixed at a predetermined value from the reduced model (B1, Table 3.1).

#### 3.3.5.4 'General effect Hypothesis'

The extent to which heterozygosity reflects genome-wide heterozygosity, and ultimately the level of *f*, was tested (using two datasets: $f_{561}$ and $f_{88}$). The observed correlation between an individual's $SH_{Ind}$ and *f* was computed using a Spearman's rank correlation, the expected correlation (*r*) between $SH_{Ind}$ and *f* was then calculated as:

$$r(SH_{Ind}, f) = \frac{-\sigma(f)}{(1 - E(f))\sigma(SH_{Ind})}$$

[Equation 4, Slate et al., 2004]

where E(*f*) and σ(*f*) represent the mean and standard deviation of *f*. $\sigma(SH_{Ind})$ represents the standard deviation of SH, calculated using Equation 1 in Slate et al. (2004).

Following Balloux et al. (2004), I subdivided 35 loci at random into two sets (consisting of 17 and 18 loci) and tested whether the mean heterozygosity of the first set of loci was correlated with the second set using the stats package in R. This procedure was repeated 100 times with different combinations of two sets of loci (heterozygosity-heterozygosity

correlation or HHC). I then calculated the mean and standard deviation of the Spearman rank HHC correlation coefficient. To detect identity disequilibrium (the correlation in homozygosity and/or heterozygosity among loci within an individual) due to variance in inbreeding, I also calculated the parameter, $g_2$ (and it's SE) using 1000 iterations in the software RMES (David et al., 2007), as this provides a more powerful statistic (Szulkin et al., 2010).

**3.3.5.5 'Local effect Hypothesis'**

To test whether the HFCs resulted from local effects, I ran two fixed effect linear models following Szulkin et al. (2010). I used MARK 6.1 and included either: 1) all 35 single-locus individual or paternal heterozygosity ($SLH_{Ind}$ or $SLH_{Pat}$) measures (homozygous = 0; heterozygous = 1), or 2) either the multilocus $SH_{Ind}$ or $SH_{Pat}$ measure, respectively. Climate variables (standardised mean winter [November–February] temperature, and standardised total summer rainfall [May–October]) were included in both models. As MARK does not allow for missing individual covariates, in the $SH_{Pat}$ analysis, I restricted the analysis to the 375 cubs assigned to 116 males genotyped at all 35 loci; *Mel-114* was later excluded, because only two males were heterozygous at this marker. QAICc was used to establish whether the model with all single-loci had greater explanatory power than the model with either the $SH_{Pat}$ or $SH_{Ind}$ estimator. I then tested the paternal heterozygosity effect and its interaction with total summer rainfall at each locus separately.

Unless otherwise stated, all statistical analyses were carried out in R 2.13.1 (R Development Core Team, 2008) and all means are reported with their 95% confidence interval, CI.

## 3.4 Results

### 3.4.1 Age, sex, cohort and population size effects

Two plausible age-specific models of annual survival (Φ) and recapture probabilities were identified, given my data (Table 3.1: A1 probability = 88%, A2 probability = 12%). Recapture probabilities were year-dependent, and cubs had a lower annual survival probability (mean Φ = 0.69 [0.65, 0.72]) than adults (0.81 [0.80, 0.83]), with year-dependence in the cub age-class (Table 3.1, Figure 3.2). Constant adult survival in the adult age-class was 7.3 times more likely than year-dependent adult survival (evidence ratio A1/A2 = 0.88/0.12, Table 3.1). Including sex, only one model was supported by the data, where the mean survival probability of adult females (0.84 [0.82, 0.86]) exceeded that of adult males (0.79 [0.76, 0.81]), but there was no apparent sex effect on first-year survival probability (B1, Table 3.1). The model incorporating a cohort-size effect on survival dynamics was not supported (C2 probability = 0%, Table 3.1). Although the model that included population size was listed as the top model (D1, Table 3.1), the parameter estimate overlapped zero (β = -0.002 [-0.004, 0.0004]), and the data provided only 1.3 times more support for including (D1) than excluding population size (D2, Table 3.1). Model B1 (Table 3.1) was therefore used as my 'reduced model' to investigate the effects of SH and *f* on first-year survival probability.

**Table 3.1** Model selection statistics for age, sex and cohort size effects on cub and adult annual survival (Φ) and recapture (*p*) probabilities (N = 975) in multi-step strategy to obtain a reduced model. No = model number; k = number of parameters; Δ = QAICc (Akaike's information criterion, corrected for sample size and adjusted through quasi-likelihood) difference from the top model (i.e., model with lowest QAICc); ω = relative QAICc weight (exp[-0.5 * ΔQAICc], divided by the sum of this quantity for all considered models); t = year; a2- = two-age classes (cub/adult); * = interaction term; (.) = constant; C = cohort size. These models include ω values ≥ 0.01 except for A3-4 and C2, which are presented to illustrate the age and cohort size effects, respectively.

| No | Model | k | QDeviance | Δ | *ω* |
|---|---|---|---|---|---|
| ***Age-specific models*** | | | | | |
| A1 | Φ (a2-t/.) *p*(t) | 47 | 1655.8 | 0.00 | 0.88 |
| A2 | Φ (a2-t/t) *p*(t) | 67 | 1618.2 | 3.98 | 0.12 |
| A3 | Φ (a2-./.) *p*(t) | 25 | 1744.7 | 43.81 | 0.00 |
| A4 | Φ (.) *p*(t) | 24 | 1785.2 | 82.26 | 0.00 |
| ***Sex-specific models*** | | | | | |
| B1 | Φ (a2-t/sex) *p*(t) | 48 | 2099.5 | 0.00 | 0.98 |
| B2 | Φ (a2-t/.) *p*(t) | 47 | 2110.3 | 8.72 | 0.01 |
| B3 | Φ (a2-t/sex) *p*(sex*t) | 71 | 2060.9 | 9.36 | 0.01 |
| ***Cohort size effect models*** | | | | | |
| C1 | Φ (a2-t/sex) *p*(t) | 48 | 2099.5 | 0.00 | 1.00 |
| C2 | Φ (a2-t/sex*C) *p*(t) | 90 | 2065.8 | 54.44 | 0.00 |
| ***Population size effect models*** | | | | | |
| D1 | Φ (a2-t/sex + population size) *p*(t) | 27 | 5500.5 | 0.00 | 0.57 |
| D2 | Φ (a2-t/sex) *p*(t) | 26 | 5503.1 | 0.53 | 0.43 |

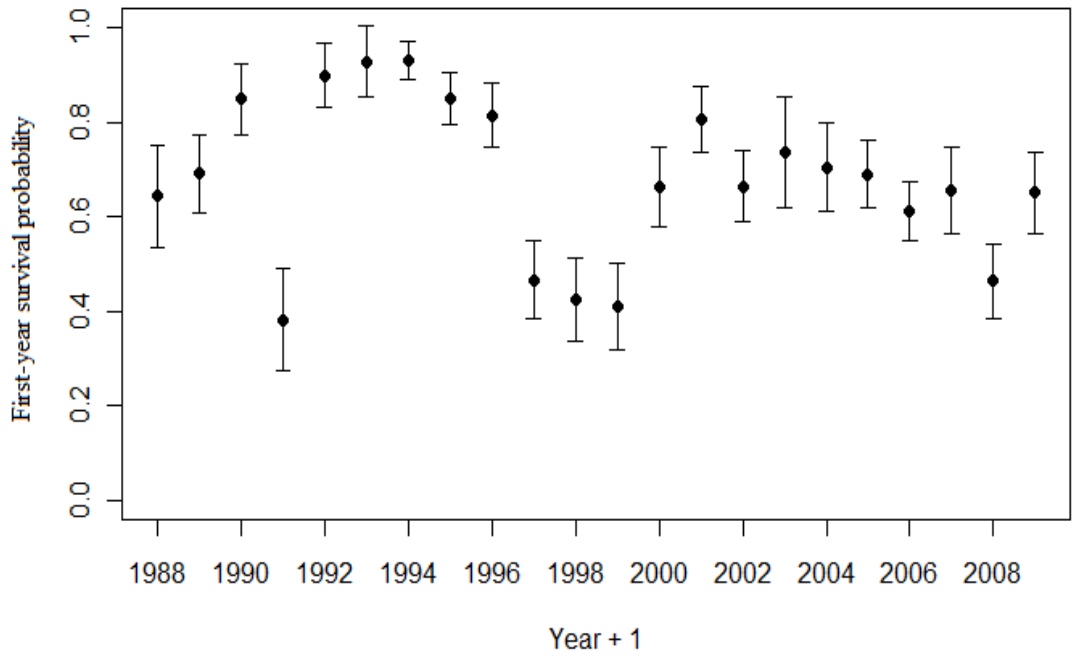


**Figure 3.2** Estimated annual survival probabilities (SE) for cub cohorts from 1987 to 2009 (N = 975). Survival estimates were derived by model averaging

### 3.4.2 Effects of SH and f on first-year survival probability

Total summer rainfall (= 0.23 [0.03, 0.44]) correlated positively as did winter temperature (= 0.35 [0.14, 0.56]). The positive interactions between summer rainfall and an individual's own ($SH_{Ind}$), maternal ($SH_{Mat}$) or paternal ($SH_{Pat}$) SH, were included among the plausible models of first-year survival probability (Table 3.2). The positive interaction between total summer rainfall and $SH_{Pat}$ was included in the top model (H1). This model was 2.2 times better supported by the data than was the model without SHPat*SR (= 1.38 [0.05, 2.71]; ratio of ω: 0.11/0.05, H1 vs. H4, Table 3.2). When summer rainfall was high, offspring sired by males with higher levels of heterozygosity had higher survival probabilities than offspring sired by males with lower levels of heterozygosity (Figure 3.3A). The interaction remained when I included rare alleles in the model; the estimate of the rare allele effect did not differ from zero (Supplementary Table S3.8). Although the

interactions between summer rainfall and both $SH_{Ind}$ and $SH_{Mat}$ showed similar positive trends as $SH_{Pat}$, the confidence intervals overlapped zero (Figure 3.3B and 3.3C). In a restricted dataset of 143 cubs for which coccidial load data were available, the effect of $SH_{Ind}$ on first-year survival probability was included in the plausible models, while accounting for climate variables and coccidial load (F3, Table 3.2), but the top model (F1) did not contain $SH_{Ind}$ and received 2.2 times more support.

*f* was not associated with first-year survival probability, when winter temperature and summer rainfall were included, using datasets of: 561 (both parents assigned; β = -1.01 [-1.83, -0.20]). 420 (at least one grandparent assigned; β = -0.44 [-1.50, 0.62]) or 88 (all four grandparents assigned; β = -0.69 [-3.97, 2.60]) cubs. Never-the-less *f* was included among the plausible models, receiving 0.47 ($f_{561}$), 0.49 ($f_{420}$) times and 0.37 ($f_{88}$) times the support as the models without *f* (Table 3.3).

**Table 3.2** Plausible models, and their model selection statistics, of the effect of an individual's own ($SH_{Ind}$) and their maternal ($SH_{Mat}$) and paternal ($SH_{Pat}$) standardised heterozygosity, and $SH_{Ind}$ after including coccidial infection (*Eimeria melis*) on first-year survival probability. No = model number; k = number of parameters; Δ = difference in QAICc from the top model (i.e., model with lowest QAICc); ω = relative QAICc weight (exp[-0.5 * ΔQAICc], divided by the sum of this quantity for all considered models); Φ = survival probability; $T_{wt}$ = mean winter temperature (November–February); SR = total rainfall (May–October); $T_{sm}$ = mean summer temperature (May–October); $SH_{Ind}^2$, $SH_{Mat}^2$ and $SH_{Pat}^2$ = quadratic effects. Models with Δ < 2 are presented.

| No | Model | k | QDeviance | Δ | ω |
|---|---|---|---|---|---|
| ***$SH_{Ind}$ models*** | | | | | |
| E1 | $\Phi(T_{wt})$ | 4 | 5075.3 | 0.00 | 0.11 |
| E2 | $\Phi(T_{wt}, SR)$ | 5 | 5073.5 | 0.28 | 0.09 |
| E3 | $\Phi(T_{wt}, SH_{Ind})$ | 5 | 5074.0 | 0.70 | 0.07 |
| E4 | $\Phi(T_{wt}, SR, SH_{Ind})$ | 6 | 5072.2 | 0.97 | 0.07 |
| E5 | $\Phi(T_{wt}, SR, SH_{Ind}, SH_{Ind*}SR)$ | 7 | 5070.5 | 1.26 | 0.06 |
| E6 | $\Phi(T_{wt}, SH_{Ind}, SH_{Ind}^2)$ | 6 | 5072.8 | 1.52 | 0.05 |
| E7 | $\Phi(T_{wt}, SR, SH_{Ind}, SH_{Ind}^2)$ | 7 | 5071.0 | 1.75 | 0.04 |
| E8 | $\Phi(T_{sm}, T_{wt})$ | 5 | 5075.1 | 1.79 | 0.04 |
| ***$SH_{Ind}$ models (restricted coccidial dataset)*** | | | | | |
| F1 | $\Phi(T_{wt}$ , SR, *Eimeria melis*) | 6 | 1079.7 | 0.00 | 0.43 |
| F2 | Φ(SR, *Eimeria melis*) | 5 | 1082.8 | 1.15 | 0.24 |
| F3 | Φ(SR, *Eimeria melis,* $SH_{Ind}$) | 6 | 1081.2 | 1.56 | 0.20 |
| ***$SH_{Mat}$ models*** | | | | | |
| G1 | $\Phi(T_{wt}, SR)$ | 5 | 4234.7 | 0.00 | 0.12 |
| G2 | $\Phi(T_{wt}, SR, SH_{Mat}, SH_{Mat*}SR)$ | 7 | 4231.7 | 1.01 | 0.07 |
| G3 | $\Phi(T_{sm}, T_{wt}, SR)$ | 6 | 4234.2 | 1.53 | 0.06 |
| G4 | $\Phi(T_{wt})$ | 4 | 4238.5 | 1.82 | 0.05 |
| G5 | $\Phi(T_{wt}, SR, SH_{Mat})$ | 6 | 4234.5 | 1.86 | 0.05 |
| G6 | $\Phi(T_{wt}, SR, SH_{Mat}, SH_{Mat}^2, SH_{Mat*}SR)$ | 8 | 4230.5 | 1.86 | 0.05 |
| ***$SH_{Pat}$ models*** | | | | | |
| H1 | $\Phi(T_{wt}, SR, SH_{Pat}, SH_{Pat*}SR)$ | 7 | 3891.7 | 0.00 | 0.11 |
| H2 | $\Phi(T_{sm}, T_{wt}, SR, SH_{Pat}, SH_{Pat*}SR)$ | 8 | 3890.1 | 0.44 | 0.09 |

| | | | | | |
|---|---|---|---|---|---|
| H3 | $\Phi(T_{sm}, T_{wt}, SR)$ | 6 | 3895.1 | 1.47 | 0.05 |
| H4 | $\Phi(T_{wt}, SR)$ | 5 | 3897.3 | 1.66 | 0.05 |
| H5 | $\Phi(T_{wt}, SR, SH_{Pat}, SH_{Pat*}SR, SH_{Pat*}T_{wt})$ | 8 | 3891.3 | 1.67 | 0.05 |
| H6 | $\Phi(T_{wt}, SR, SH_{Pat}, SH_{Pat}^2, SH_{Pat*}SR)$ | 8 | 3891.5 | 1.88 | 0.04 |

**Table 3.3** Plausible models, and their model selection statistics, of the effect of inbreeding coefficient (datasets with at least: both parents ($f_{561}$), one grandparent ($f_{420}$) and all four grand-parents ($f_{88}$) assigned) on first-year survival probability ($\Phi$). k = number of parameters; $\Delta$ = difference in QAICc from the top model; $\omega$ = relative QAICc weight (exp[-0.5 * $\Delta$QAICc], divided by the sum of this quantity for all considered models). $T_{wt}$ = mean winter temperature (November–February); SR = total summer rainfall (May–October).

| **No** | **Model** | **k** | **QDeviance** | **Δ** | ***ω*** |
|---|---|---|---|---|---|
| ***$f_{561}$ models*** | | | | | |
| I1 | $\Phi(T_{wt}, SR)$ | 5 | 3418.2 | 0.00 | 0.68 |
| I2 | $\Phi(T_{wt}, SR, f_{561})$ | 6 | 3419.7 | 1.48 | 0.32 |
| ***$f_{420}$ models*** | | | | | |
| J1 | $\Phi(T_{wt}, SR)$ | 5 | 2286.3 | 0.00 | 0.67 |
| J2 | $\Phi(T_{wt}, SR, f_{420})$ | 6 | 2285.7 | 1.42 | 0.33 |
| ***$f_{88}$ models*** | | | | | |
| K1 | $\Phi(T_{wt}, SR)$ | 5 | 359.7 | 0.00 | 0.73 |
| K2 | $\Phi(T_{wt}, SR, f_{88})$ | 6 | 359.6 | 1.94 | 0.27 |

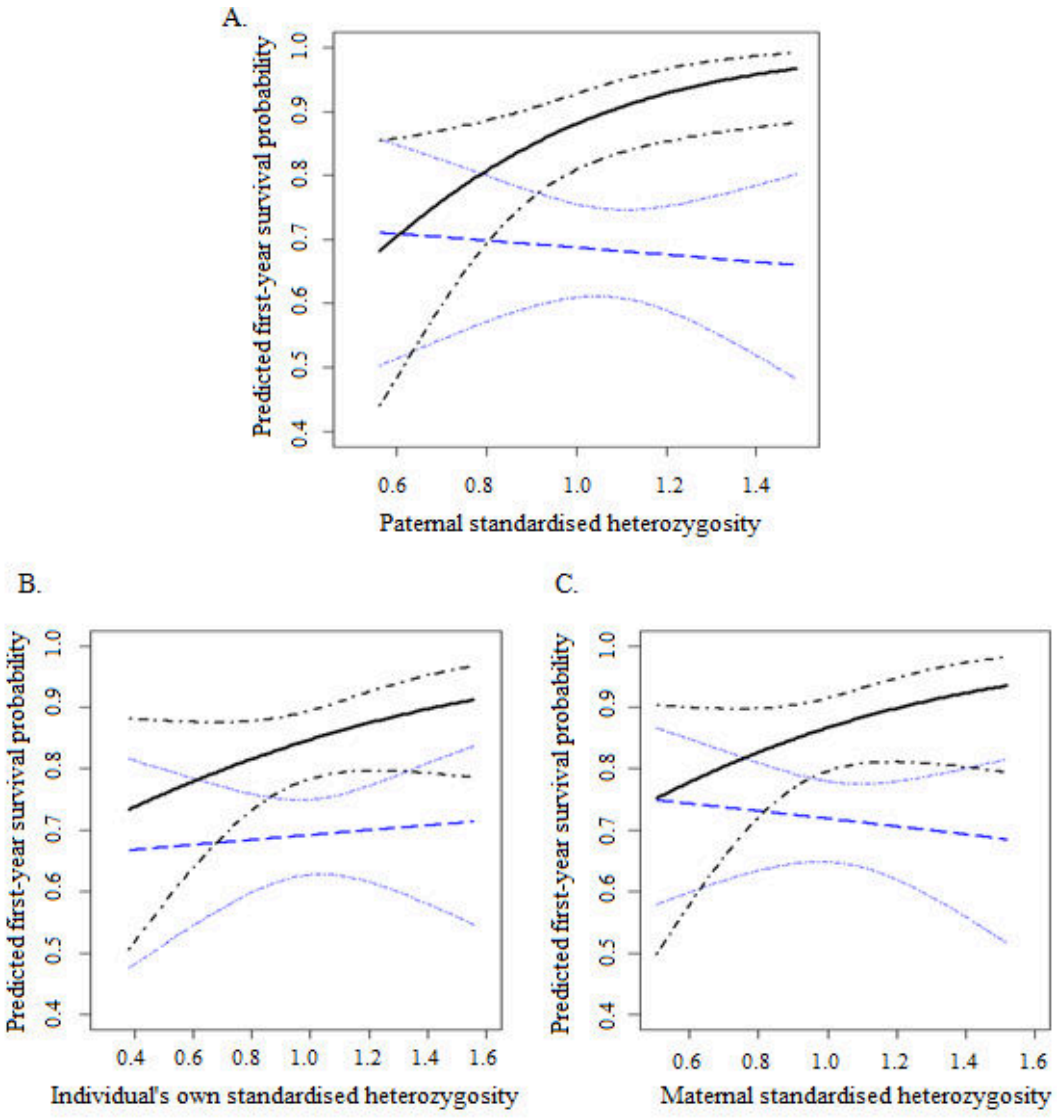


**Figure 3.3** The relationship between first-year survival probability and: (A) paternal standardised multilocus heterozygosity; (B) individual's own heterozygosity; (C) maternal heterozygosity. Probabilities are plotted under conditions of high (solid line) and low (dashed line) total summer rainfall (May–October). Standardised total summer rainfall (SR) was categorised for ease of visualization; SR > 0 and SR ≤ 0 were years with above and below mean rainfall respectively. The dot-dash lines represent 95% confidence intervals.

### 3.4.3 'General effect Hypothesis'

In the dataset $f_{561}$ (mean $f$ = 0.010 [0.006, 0.014]), 42 cubs had $f > 0$. Mean $SH_{Ind}$ was 0.99 [0.97, 1.01]; inbred badgers ($f \geq 0.125$, N = 25) had a lower mean $SH_{Ind}$ (0.77 [0.68, 0.85]) than outbred badgers ($f < 0.125$, N = 536; 1.01 [0.99, 1.02]; Mann-Whitney U = 2590, z = -5.19, $P < 0.001$). In the restricted dataset $f_{420}$ (mean $f$ = 0.014 [0.009, 0.019]), 42 cubs had $f > 0$. Mean $SH_{Ind}$ was 1.00 [0.98, 1.02]; inbred badgers ($f \geq 0.125$, N = 25) had a lower mean $SH_{Ind}$ (0.77 [0.69, 0.85]) than outbred badgers ($f < 0.125$, N = 395; 1.01 [0.99, 1.03]; Mann-Whitney U = 8026, $P < 0.001$). In the further restricted dataset $f_{88}$ (mean = 0.010 [0.002, 0.018]), 11 cubs had $f > 0$. In $f_{88,}$ mean $SH_{Ind}$ was 1.04 [1.00, 1.08]; the mean $SH_{Ind}$ of inbred ($f \geq 0.125$; 0.93 [0.62, 1.79], N = 3) and outbred (1.05 [1.00, 1.09], N = 85) badgers did not differ (Mann-Whitney U = 157.5, $P < 0.490$). Although the predicted correlation coefficient between $SH_{Ind}$ and $f$ ($r(SH_{Ind}, f)_e$) was -0.25 ($f_{561}$; versus -0.18 for $f_{88}$), the observed correlation was relatively weak ($f_{561}$: $r(SH_{Ind}, f)_o$ = -0.16, $P < 0.001$; $f_{88}$: $r(SH_{Ind}, f)_o$ = -0.02, $P$ = 0.878). This is consistent with the significant but weak heterozygosity-heterozygosity correlation (HHC) detected between two random subsets of the loci (mean HHC $r$ [standard deviation] = 0.146 [0.025], range = 0.085–0.200, $P <$ 0.001). The molecular markers, however, had the power to detect inbreeding ($g_2$ = 0.01, $SD$ = 0.003, $P < 0.001$). The observed correlation between $SH_{Pat}$ and $f_{561}$ was also weak 0.03, $P$ = 0.471 ($f_{88}$: $r$ = 0.01, $P$ = 0.317).

### 3.4.4 'Local effect Hypothesis'

The multilocus $SH_{Ind}$ model had 99.9% support than the model with an individual's single-locus heterozygosity ($SLH_{Ind}$: QAICc [$SH_{Ind}$] = 3110.5 versus QAICc [$SLH_{Ind}$] = 3130.4, ΔQAICc [$SLH_{Ind}$] = 19.83, ω [$SH_{Ind}$] vs. ω [$SLH_{Ind}$] = 0.9995 vs. 0.0005). The model with

34-marker paternal single-locus heterozygosity ($SLH_{Pat}$) explained the variation among first-year survival probability 99.8% better, given the data, than the model including $SH_{Pat}$ (QAICc [$SLH_{Pat}$] = 2209.1 versus QAICc [$SH_{Pat}$] = 2221.6, ω[$SLH_{Pat}$] vs. ω[$SH_{Pat}$] = 0.998 vs. 0.000). I found that 9/34 (26%) paternal loci were associated with first-year survival probability; six exhibited positive (*Mel-102, Mel-103, Mel-113, Mel-117, Mel-135* and *Mel-137*; Figure 3.4A and 3.4B) and three negative (*Mel-106, Mel-108* and *Mel-186*; Figure 3.4A) associations. Furthermore, positive interactions between $SLH_{Pat}$ and total summer rainfall on first-year survival probability were observed at *Mel-104* (β = 1.00 [0.27, 1.72])*, Mel-113* (7.23 [0.81, 13.66]) and *Mel-161* (0.74 [0.10, 1.39]), whereas *Mel-10* (-0.89 [-1.64, -0.14]) exhibited a negative interaction.

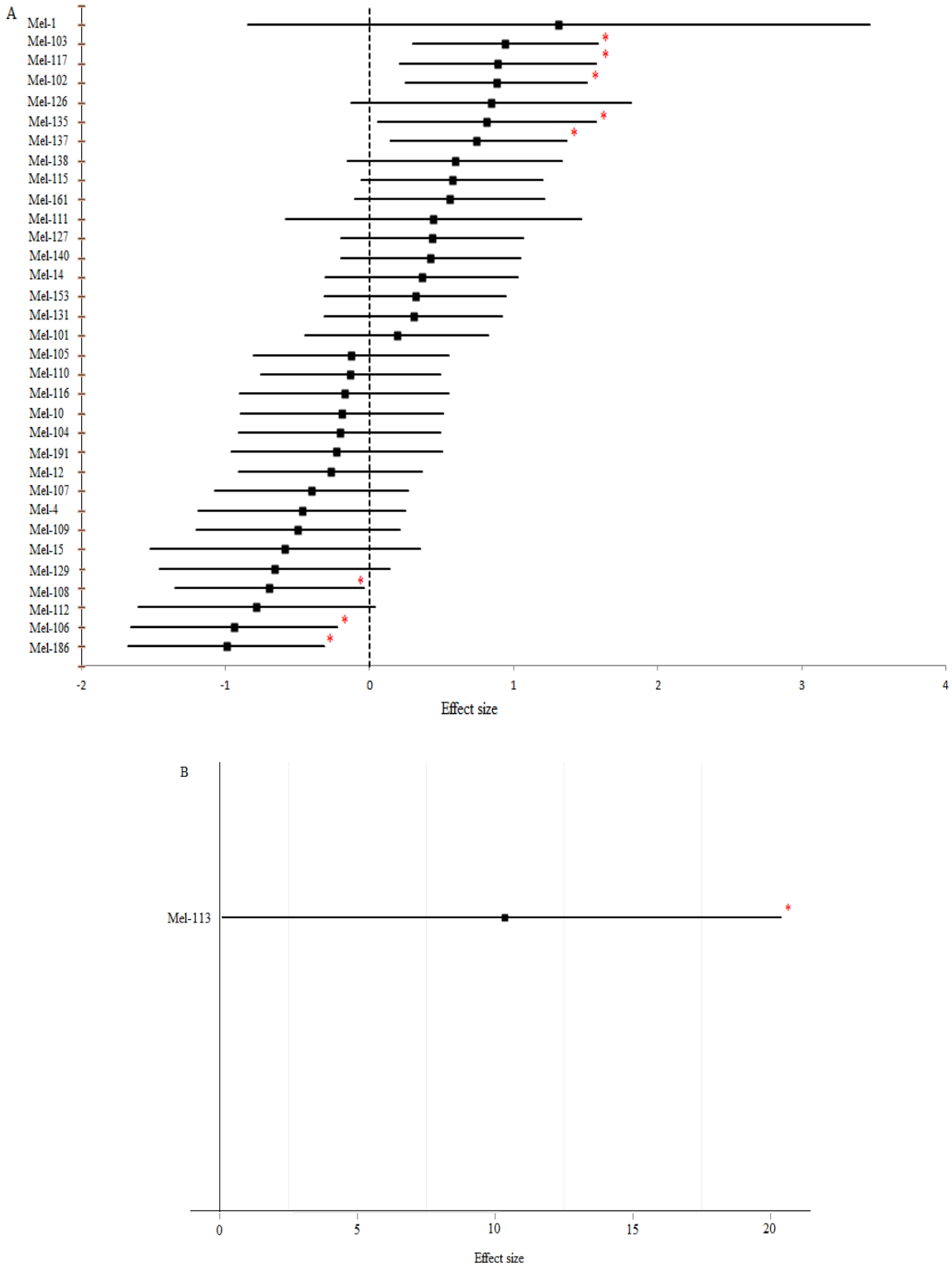


**Figure 3.4** The effect sizes of paternal single-locus heterozygosity on the probability of offspring surviving to age one, including the 95% confidence: (A) 33 loci, (B) locus Mel-113 had an effect size of 10.27* [CI: 0.13, 20.41] and is therefore shown in separate graph. (*) indicates loci with significant effects ($P < 0.050$).

## 3.5 Discussion

Higher first-year survival probability was associated with wetter summers and warmer winters. Cubs with heterozygous fathers (but not mothers) had greater first year survival probability than cubs with less heterozygous fathers, but only in wet years. In the UK, badgers feed predominantly on earthworms (*Lumbricus terrestris*) that are only available at the soil surface under specific conditions, i.e., when the soil is moist (see Macdonald et al., 2010). Low rainfall can thus reduce earthworm availability, impacting on first-year survival probability (Macdonald & Newman, 2002; Macdonald et al., 2010). The paternal heterozygote advantage on first-year survival probability was only apparent under advantageous (wetter) climatic conditions; under stressful (dryer) conditions, I speculate that an elevated, indiscriminate, mortality rate (Macdonald et al., 2010) masked the differential effect. Consequently studies that only investigate HFCs over a narrow range of environmental conditions may miss important effects that only manifest under advantageous conditions (when there is enough variation in fitness; Harrison et al., 2011; this study) or adverse conditions (due to increased magnitude of inbreeding depression, Lesbarreres et al., 2005; Da Silva et al., 2006; Brouwer et al., 2007; Fox et al., 2010). More importantly, very few studies have investigated HFCs using parental as well as individual measures (e.g., Brouwer et al., 2007); in contrast I show that this can lead to effects being missed completely.

### 3.5.1 Age- and sex- specific survival probabilities

Annual survival probabilities have been found to vary in ways that affect the demography of badger populations (Macdonald et al., 2009). I found that cubs survived less well than did adults (corroborating Macdonald & Newman, 2002; Macdonald et al., 2009). First-year

survival probability was not affected by sex, population size or cohort size, but varied considerably between years.

Adult males had significantly lower annual survival probabilities than did adult females. The different survival rates between sexes may be explained by the mitochondrial theory of ageing. Male badgers have a faster rate of reproductive senescence than females (Dugdale et al., 2011b). The mitochondria of males may therefore produce more hydrogen peroxide than females; hydrogen peroxide induces oxidative stress, damaging and ageing cells, which reduces male, relative to female, longevity (Vina et al., 2003).

### 3.5.2 $SH_{Ind}$, f and first-year survival probability

$SH_{Ind}$ did not correlate with first-year survival probability. First-year survival probability was not associated with *f*; however, *f* was included in the plausible models, and small sample sizes may have limited my power to detect this effect. Inbreeding levels may be underestimated with incomplete pedigree information (i.e., inbred individuals will be assigned incorrectly as out-bred, if their ancestors are unassigned). Consequently this will underestimate the severity of, or fail to detect, inbreeding depression (Keller et al., 2002a; Walling et al., 2011) or even generate inbreeding depression incorrectly if there is systematic bias in the inbred individuals that are assigned as out-bred according to fitness (e.g., if longer-lived individuals are less likely to have their grand-parents assigned, and therefore more likely to be assigned as out-bred).

Heterozygosity often correlates with fitness-advantages, but effect sizes are typically small (e.g., Chapman et al., 2009). Correlations in heterozygosity among the loci tested showed that heterozygosity reflected genome-wide heterozygosity and therefore reflected

inbreeding. The correlation between *f* and $SH_{Ind}$ (also with $SH_{Pat}$) was however relatively weak, despite the large number of markers used. Indeed, theory actually predicts that HFCs should be weak, or undetectable, in populations where variance in inbreeding is low (Balloux et al., 2004). The mean and variance of *f* (0.010 and 0.002, respectively) in my dataset was low; hence the weak correlation between *f* and $SH_{Ind}$ is unsurprising (Slate et al., 2004). Indeed, Forstmeier et al. (2012) argued that chance events during Mendelian segregation may lead to an individual's realised inbreeding coefficient (i.e., the actual proportion of the genome that is identical by decent, IBD) deviating substantially from the estimated proportion based on a pedigree (*f*). Deviation between realised and estimated *f* may thus weaken the correlation between $SH_{Ind}$ and pedigree-based *f,* compared to realised *f* (Forstmeier et al., 2012). Nevertheless, $SH_{Ind}$ was informative about inbreeding in my study.

Heterozygosity-linked mortality might also occur at earlier stages of development than I was able to observe, further limiting the power to detect correlation between $SH_{Ind}$ and first-year survival probability. My analyses was limited to post-emergence cubs that had already survived to at least 3 months (for reasons of animal welfare, cubs can be trapped only after weaning). Pre-emergence mortality has been inferred in the study population (36%, extrapolated from ultra-sound, Macdonald & Newman, 2002; mean foetal (1.9 [1.8, 2.0]) versus post-emergence (1.4 [1.3, 1.5]) litter size, Dugdale et al., 2007). This missing fraction, which may be linked to inbreeding, limited the power to detect correlation between $SH_{Ind}$ and first-year survival probability. Nevertheless, these limitations simultaneously afford us a level of minimal confidence in my data – where effects were observed despite reduced statistical power.

The effects of genetic diversity on fitness-related traits have been reported to be more detectable under advantageous conditions (Harrison et al., 2011). Although I detected HFC effects under favourable conditions, using $SH_{Pat}$, I did not detect this relationship using $SH_{Ind}$. Scenarios where HFCs are detected only under stressful environmental conditions, due to increased magnitude of inbreeding depression, have also been reported (Lesbarreres et al., 2005; Da Silva et al., 2006; Brouwer et al., 2007; Fox et al., 2011). Badgers display some founder effects (Pope et al., 2006) and inbreeding (Dugdale, 2007), but I found no evidence of any interaction between a suite of climate variables and $SH_{Ind}$. Again I caution that I cannot reject the possibility that deleterious recessive alleles, causing inbreeding depression on survival, were purged before I was able to trap cubs.

### 3.5.3 Local effects

Paternal single-locus heterozygosity correlated with first-year survival probability better than did multilocus heterozygosity. Potentially, this observed paternal HFC could be due to linkage with dominant, or over-dominant, loci that affect fitness (local effect), rather than to a global relationship with homozygosity due to inbreeding (general effect). For a local effect, markers need to be located close to functional loci (under selection), and both are required to be in linkage disequilibrium (associative over-dominance, Ohta, 1971). Six loci appeared to be linked with first-year survival probability, whereas three loci showed a negative relationship with heterozygosity, i.e., homozygous individuals exhibited superior survival dynamics (Lieutenant-Gossekin & Bernatchez, 2006). Additionally, paternal HFC-environmental interactions were found at four loci; three were associated with greater- and one with lower- first-year survival probability, under favourable environmental conditions. While the occurrence of negative HFCs can be interpreted as evidence for outbreeding depression (Neff, 2004), it could also be due to a null allele

(Dakin & Avise, 2004), although I found no evidence of this (the markers were in Hardy-Weinberg Equilibrium).

Paternal single-local heterozygosity effects on first-year survival probability can arise by both direct and indirect mechanisms. Offspring fitness could be affected directly by the paternal genes e.g., 'Good-genes-as-heterozygosity' (Brown, 1997). The neutrality of microsatellite loci has been challenged recently, based on accumulating evidence that some appear to have functional roles and are located within genes, or expressed regions of the genome (e.g., Li et al., 2002; Da Silva et al., 2006; Kupper et al., 2010). The markers associated with first-year survival probability may therefore be located within genes linked to survival; however, in the absence of a badger genome map I cannot examine the genomic location of these markers.

Differential maternal investment (e.g., maternal care, resource allocation) in their offspring that are fathered by high heterozygous males versus low heterozygous males ('The differential allocation Hypothesis, Burley, 1986) could explain the correlation between paternal heterozygosity and first-year survival probability in badgers. It is also possible that more heterozygous mothers and fathers live in high quality territory (Woodroffe & Macdonald, 2000), thus their offspring have better chance to survive over their first-year. Heterozygous fathers could be bolder (e.g., bolder sticklebacks have higher heterozygosity, Laine et al., 2012) and the expression of this boldness can vary with environment (weaker expression in sticklebacks where there are no predators Dingemanse et al., 2007). Environmental dependent expression of boldness may be linked to fitness (e.g., Dingemanse et al. (2004) showed that offspring survival is linked to parental personality, but this varies between years). Heterozygous male badgers could be bolder and defend

their territory better in wet years that leading to greater offspring survival probability in wet years.

## 3.6 Conclusions

In conclusion, I note that evidence for a paternal heterozygous advantage on first-year probability was only apparent in years with low levels of climate-induced stress. This effect was due to paternal heterozygosity rather than paternal rare alleles, potentially due to population immigrants that produced heterozygous young; when these heterozgous male offspring bred, their offspring had higher survivial probability, than offspring of less heterozygous males, in wet years. Severe stress is indiscriminate, and I posit that under these circumstances all individuals within this badger population were vulnerable and unable to respond (i.e., there was not enough variation in fitness for the effect to be detected). Therefore, previous studies that have not detected HFCs may need re-evaluating in terms of both the range of climatic conditions that they covered and the measures used. Inbreeding was not correlated with first-year survival probability, but small sample sizes reduced the statistical power. In order to maximize their reproductive success, badgers may avoid inbreeding by mating with extra-group partners, but in terms of acquiring an associated heterozygosity advantage this assumes that extra-group males are more heterozygous than within-group males, which has not yet been tested. Paternal heterozygosity advantage and inbreeding depression therefore, have important implications for the evolution of mating strategies in badgers.

### 3.7 Supplementary materials

**Supplementary Table S3.1** Characterisation of the 35 European badger (*Meles meles*) microsatellite loci used. *N* = the total number of individuals that were genotyped, *bp* = base pairs, $H_O$ = observed heterozygosity, $H_E$ = expected heterozygosity.

| Locus | EMBL | *N* | No. of alleles | Expected allele size (bp) | Observed allele size range (bp) | *$H_O$ | *$H_E$ | *Estimated null allele frequency | Multiplex Set | ^Reference source |
|---|---|---|---|---|---|---|---|---|---|---|
| *Mel-1* | AH009955 | 1151 | 3 | 262—274 | 280–286 | 0.09 | 0.09 | 0.00 | 3 | 1 |
| *Mel-4* | AF300711 | 1134 | 3 | 141–147 | 143–147 | 0.38 | 0.43 | 0.05 | 6 | 1 |
| *Mel-10* | AJ309849 | 1163 | 2 | 154 | 160–162 | 0.22 | 0.26 | 0.09 | 1 | 2 |
| *Mel-12* | AJ309053 | 1121 | 4 | 153 | 153–159 | 0.56 | 0.64 | 0.07 | 3 | 2 |
| *Mel-14* | AJ309055 | 1144 | 4 | 188 | 186–196 | 0.67 | 0.72 | 0.04 | 3 | 2 |
| *Mel-15* | AJ309056 | 1126 | 8 | 270 | 244–276 | 0.70 | 0.74 | 0.02 | 3 | 2 |
| *Mel-101* | AJ293349 | 1113 | 4 | 114 | 122–136 | 0.34 | 0.35 | 0.00 | 1 | 3 |
| *Mel-102* | AJ293353 | 1166 | 4 | 187 | 193–199 | 0.59 | 0.61 | 0.01 | 2 | 3 |
| *Mel-103* | AJ293356 | 1165 | 5 | 249 | 251–263 | 0.65 | 0.66 | 0.01 | 1 | 3 |
| *Mel-104* | AJ293352 | 1163 | 7 | 306 | 314–328 | 0.68 | 0.72 | 0.03 | 1 | 3 |
| *Mel-105* | AJ293350 | 1113 | 9 | 129 | 133–151 | 0.65 | 0.69 | 0.03 | 1 | 3 |
| *Mel-106* | AJ293355 | 1166 | 4 | 211 | 221–227 | 0.31 | 0.40 | 0.14 | 1 | 3 |
| *Mel-107* | AJ293359 | 1158 | 5 | 280 | 284–292 | 0.51 | 0.59 | 0.07 | 1 | 3 |
| *Mel-108* | AJ293354 | 1163 | 2 | 313 | 322–326 | 0.24 | 0.26 | 0.03 | 1 | 3 |
| *Mel-109* | AJ293357 | 1161 | 5 | 122 | 106–128 | 0.56 | 0.60 | 0.03 | 1 | 3 |
| *Mel-110* | AJ293360 | 1119 | 4 | 326 | 324–330 | 0.56 | 0.62 | 0.04 | 3 | 3 |
| *Mel-111* | AJ230692 | 1162 | 2 | 126 | 132–138 | 0.19 | 0.18 | -0.01 | 2 | 3 |
| *Mel-112* | AJ230700 | 1139 | 4 | 414 | 418–430 | 0.66 | 0.72 | 0.04 | 2 | 3 |
| *Mel-113* | AJ230713 | 1166 | 5 | 120 | 118–130 | 0.33 | 0.38 | 0.08 | 2 | 3 |
| *Mel-114* | AJ230695 | 1142 | 2 | 222 | 232–234 | 0.01 | 0.01 | 0.00 | 2 | 3 |
| *Mel-115* | AJ230703 | 1154 | 11 | 342 | 330–350 | 0.54 | 0.59 | 0.05 | 2 | 3 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Mel-116* | AJ293351 | 1156 | 8 | 107 | 113–135 | 0.63 | 0.69 | 0.05 | 2 | 3 |
| *Mel-117* | AJ293358 | 1156 | 4 | 184 | 176–194 | 0.57 | 0.59 | 0.01 | 2 | 3 |
| *Mel-126* | AJ293370 | 1144 | 4 | 158 | 164–170 | 0.23 | 0.25 | 0.04 | 4 | 3 |
| *Mel-127* | AJ293368 | 1141 | 4 | 184 | 288–300 | 0.48 | 0.48 | 0.00 | 5 | 3 |
| *Mel-129* | AJ293366 | 1148 | 5 | 213 | 205–219 | 0.61 | 0.62 | 0.00 | 5 | 3 |
| *Mel-131* | AJ293367 | 1143 | 3 | 116 | 116–124 | 0.52 | 0.54 | 0.02 | 7 | 3 |
| *Mel-135* | AJ293375 | 1132 | 4 | 131 | 237–247 | 0.37 | 0.41 | 0.05 | 6 | 3 |
| *Mel-137* | AJ293372 | 1154 | 4 | 119 | 113–121 | 0.49 | 0.50 | 0.01 | 4 | 3 |
| *Mel-138* | AJ293373 | 1104 | 5 | 495 | 489–503 | 0.37 | 0.41 | 0.05 | 7 | 3 |
| *Mel-140* | AJ293374 | 1125 | 4 | 229 | 223–231 | 0.58 | 0.62 | 0.04 | 4 | 4 |
| *Mel-153* | AJ230722 | 1105 | 2 | 309 | 309–313 | 0.40 | 0.43 | 0.04 | 6 | 5 |
| *Mel-161* | AJ293385 | 1070 | 3 | 361 | 355–359 | 0.51 | 0.61 | 0.09 | 5 | 5 |
| *Mel-186* | FR745448 | 923 | 5 | 439 | 431–439 | 0.39 | 0.43 | 0.06 | 4 | 5 |
| *Mel-191* | FR745453 | 1126 | 4 | 273 | 259–275 | 0.25 | 0.27 | 0.05 | 7 | 5 |



* The observed heterozygosity, expected heterozygosity and null allele frequency for each locus were estimated using CERVUS 3.0.3 (Kalinowski, et al., 2007).

^ Reference sources: 1 = Bijlsma et al., 2000; 2 = Domingo-Roura et al., 2003; 3 = Carpenter et al., 2003; 4 = Huck et al., 2008; 5 = Chapter 2

**Supplementary Table S3.2** The number of unsampled candidate mothers and fathers specified (N) in the Restricted and Open parentage analyses using MasterBayes 2.47.

| Cub Cohort | Unsampled Candidate | | | |
|---|---|---|---|---|
| | Restricted Analysis | | Open Analysis | |
| | Mother (N) | Father (N) | Mother (N) | Father (N) |
| 1988 | 1 | 2 | 6 | 2 |
| 1989 | 1 | 4 | 10 | 4 |
| 1990 | 1 | 13 | 9 | 13 |
| 1991 | 1 | 15 | 10 | 15 |
| 1992 | 1 | 15 | 8 | 15 |
| 1993 | 1 | 8 | 10 | 8 |
| 1994 | 1 | 8 | 12 | 8 |
| 1995 | 1 | 12 | 12 | 12 |
| 1996 | 1 | 15 | 12 | 15 |
| 1997 | 1 | 16 | 12 | 16 |
| 1998 | 1 | 12 | 21 | 12 |
| 1999 | 1 | 21 | 23 | 21 |
| 2000 | 1 | 31 | 25 | 31 |
| 2001 | 1 | 28 | 28 | 28 |
| 2002 | 1 | 25 | 25 | 25 |
| 2003 | 1 | 32 | 25 | 32 |
| 2004 | 1 | 22 | 17 | 22 |
| 2005 | 1 | 16 | 10 | 16 |
| 2006 | 1 | 8 | 14 | 8 |
| 2007 | 1 | 15 | 28 | 15 |
| 2008 | 1 | 26 | 36 | 26 |
| 2009 | 1 | 33 | 33 | 33 |
| 2010 | 1 | 24 | 15 | 24 |

**Supplementary Table S3.3** Summary statistics of the pedigree, generated using the pedStatSummary() function in pedantics 1.02, in R 2.13.2

| Parameter | Value |
|---|---|
| Number of records | 989 |
| Maternities | 683 |
| Paternities | 655 |
| Full-sibs | 327 |
| Maternal sibs | 1265 |
| Maternal half sibs | 938 |
| Paternal sibs | 1652 |
| Paternal half sibs | 1325 |
| Maternal grandmothers | 277 |
| Maternal grandfathers | 255 |
| Paternal grandmothers | 370 |
| Paternal grandfathers | 317 |
| Maximum pedigree depth | 7 |
| Founders | 212 |
| Mean maternal sibsip size | 2.996 |
| Mean paternal sibsip size | 3.259 |
| Non-zero F | 42 |
| F > 0.125 | 15 |
| Mean pairwise relatedness | 0.007 |

**Supplementary Table S3.4** Number of mismatches observed between parents assigned in MasterBayes 2.47 and Colony 2.0, and their offspring. Trio = mother-father-offspring.

| **Mismatches (*N*)** | **Trio** | **Mother-Cub** | **Father-Cub** |
|---|---|---|---|
| 0 | 375 | 578 | 573 |
| 1 | 123 | 92 | 65 |
| 2 | 46 | 13 | 15 |
| 3 | 17 | 0 | 2 |
| **Total** | **561** | **683** | **655** |

**Supplementary Table S3.5** Plausible models, and their model selection statistics of the effect of an individual's own ($HL_{Ind}$), maternal ($HL_{Mat}$) and paternal ($HL_{Pat}$) homozygosity by locus on their first-year survival probability ($\Phi$). $T_{sm}$ = mean summer temperature (May–October); $T_{wt}$ = mean winter temperature (November–February); SR = total rainfall (May–October); $HL_{Mat}^2$ and $HL_{Pat}^2$ = quadratic effects; k = number of parameters in the model; $\Delta$ = differences in QAICc from the top model (i.e., model with lowest QAICc); $\omega$ = relative QAICc weight (exp[-0.5 * $\Delta$QAICc], divided by the sum of this quantity for all considered models). Models with $\Delta$QAICc < 7 are presented.

| Model | k | Deviance | QAICc | $\Delta$ | $\omega$ | Model likelihood |
|---|---|---|---|---|---|---|
| ***$HL_{Ind}$ models*** | | | | | | |
| $\Phi(T_{wt})$ | 4 | 5075.3 | 5083.3 | 0.00 | 0.13 | 1.00 |
| $\Phi(T_{wt}, SR)$ | 5 | 5073.5 | 5083.6 | 0.28 | 0.11 | 0.87 |
| $\Phi(T_{wt}, HL_{Ind})$ | 5 | 5074.8 | 5084.8 | 1.48 | 0.06 | 0.48 |
| $\Phi(T_{wt}, SR, HL_{Ind}, HL_{Ind*}SR)$ | 7 | 5070.9 | 5085.0 | 1.69 | 0.06 | 0.43 |
| $\Phi(T_{wt}, SR, HL_{Ind})$ | 6 | 5073.0 | 5085.1 | 1.77 | 0.05 | 0.41 |
| $\Phi(T_{sm}, T_{wt})$ | 5 | 5075.1 | 5085.1 | 1.79 | 0.05 | 0.41 |
| ***$HL_{Mat}$ models*** | | | | | | |
| $\Phi(T_{wt}, SR)$ | 5 | 4234.7 | 4244.7 | 0.00 | 0.11 | 1.00 |
| $\Phi(T_{wt}, SR, HL_{Mat}, HL_{Mat}^2, HL_{Mat*}SR)$ | 8 | 4229.8 | 4245.9 | 1.19 | 0.06 | 0.55 |
| $\Phi(T_{wt}, SR, HL_{Mat}, HL_{Mat}^2)$ | 7 | 4232.1 | 4246.2 | 1.48 | 0.05 | 0.48 |
| $\Phi(T_{sm}, T_{wt}, SR)$ | 6 | 4234.2 | 4246.2 | 1.53 | 0.05 | 0.47 |
| $\Phi(T_{wt})$ | 4 | 4238.5 | 4246.5 | 1.82 | 0.04 | 0.40 |
| $\Phi(T_{wt}, SR, HL_{Mat})$ | 6 | 4234.6 | 4246.6 | 1.90 | 0.04 | 0.39 |
| $\Phi(T_{wt}, SR, HL_{Mat}, HL_{Mat*}SR)$ | 7 | 4232.7 | 4246.7 | 1.99 | 0.04 | 0.37 |
| ***$HL_{Pat}$ models*** | | | | | | |
| $\Phi(T_{wt}, SR, HL_{Pat}, HL_{Pat*}SR)$ | 7 | 3892.9 | 3907.0 | 0.00 | 0.10 | 1.00 |
| $\Phi(T_{sm}, T_{wt}, SR)$ | 6 | 3895.1 | 3907.2 | 0.23 | 0.09 | 0.89 |
| $\Phi(T_{sm}, T_{wt}, SR, HL_{Pat}, HL_{Pat*}SR)$ | 8 | 3891.2 | 3907.3 | 0.36 | 0.08 | 0.83 |
| $\Phi(T_{wt}, SR)$ | 5 | 3897.3 | 3907.4 | 0.42 | 0.08 | 0.81 |
| $\Phi(T_{sm}, T_{wt})$ | 5 | 3897.7 | 3907.7 | 0.76 | 0.07 | 0.68 |
| $\Phi(T_{sm}, T_{wt}, SR, HL_{Pat})$ | 7 | 3894.7 | 3908.8 | 1.83 | 0.04 | 0.40 |
| $\Phi(T_{wt}, SR, HL_{Pat})$ | 6 | 3896.8 | 3908.8 | 1.87 | 0.04 | 0.39 |
| $\Phi(T_{wt}, SR, HL_{Pat}, HL_{Pat}^2, HL_{Pat*}SR)$ | 8 | 3892.8 | 3908.9 | 1.93 | 0.04 | 0.38 |

**Supplementary Table S3.6** Plausible models, and their model selection statistics of the effect of an individual's own ($Ir_{Ind}$), maternal ($Ir_{Mat}$) and paternal ($Ir_{Pat}$) internal relatedness on their first-year survival probability (Φ). $T_{sm}$ = mean summer temperature (May–October); $T_{wt}$ = mean winter temperature (November–February); SR = total rainfall (May–October); $Ir_{Mat}^2$ and $Ir_{Pat}^2$ = quadratic effects; (Φ) = survival probability; k = number of parameters in the model; Δ = differences in QAICc from the top model (i.e., model with lowest QAICc); ω = relative QAICc weight (exp[-0.5 * ΔQAICc], divided by the sum of this quantity for all considered models). Models with ΔQAICc < 7 are presented.

| Model | k | Deviance | QAICc | Δ | $\omega$ | Model likelihood |
|---|---|---|---|---|---|---|
| ***$Ir_{Ind}$ models*** | | | | | | |
| $\Phi(T_{wt})$ | 4 | 5075.3 | 5083.3 | 0.00 | 0.15 | 1.00 |
| $\Phi(T_{wt}, SR)$ | 5 | 5073.5 | 5083.6 | 0.28 | 0.13 | 0.87 |
| $\Phi(T_{wt}, Ir_{Ind})$ | 5 | 5075.0 | 5085.0 | 1.72 | 0.06 | 0.42 |
| $\Phi(T_{sm}, T_{wt})$ | 5 | 5075.1 | 5085.1 | 1.79 | 0.06 | 0.41 |
| ***$Ir_{Mat}$ models*** | | | | | | |
| $\Phi(T_{wt}, SR)$ | 5 | 4234.7 | 4244.7 | 0.00 | 0.12 | 1.00 |
| $\Phi(T_{sm}, T_{wt}, SR)$ | 6 | 4234.2 | 4246.2 | 1.53 | 0.06 | 0.47 |
| $\Phi(T_{wt}, SR, Ir_{Mat}, Ir_{Mat}^2, Ir_{Mat*}SR)$ | 8 | 4230.2 | 4246.2 | 1.53 | 0.06 | 0.47 |
| $\Phi(T_{wt}, SR, Ir_{Mat}, Ir_{Mat}^2)$ | 7 | 4232.3 | 4246.3 | 1.63 | 0.05 | 0.44 |
| $\Phi(T_{wt})$ | 4 | 4238.5 | 4246.5 | 1.82 | 0.05 | 0.40 |
| ***$Ir_{Pat}$ models*** | | | | | | |
| $\Phi(T_{sm}, T_{wt}, SR)$ | 6 | 3895.1 | 3907.2 | 0.00 | 0.11 | 1.00 |
| $\Phi(T_{wt}, SR)$ | 5 | 3897.3 | 3907.4 | 0.19 | 0.10 | 0.91 |
| $\Phi(T_{sm}, T_{wt})$ | 5 | 3897.7 | 3907.7 | 0.53 | 0.09 | 0.77 |
| $\Phi(T_{sm}, T_{wt}, SR, Ir_{Pat})$ | 7 | 3894.5 | 3908.6 | 1.40 | 0.06 | 0.50 |
| $\Phi(T_{wt}, SR, Ir_{Pat})$ | 6 | 3896.7 | 3908.7 | 1.54 | 0.05 | 0.46 |
| $\Phi(T_{wt}, SR, Ir_{Pat}, Ir_{Pat*}SR)$ | 7 | 3894.9 | 3908.9 | 1.73 | 0.05 | 0.42 |
| $\Phi(T_{sm}, T_{wt}, SR, Ir_{Pat}, Ir_{Pat*}SR)$ | 8 | 3893.0 | 3909.1 | 1.93 | 0.04 | 0.38 |

**Supplementary Table S3.7** Number of offspring in the data sets depending on number of parents and grandparents known

| | Both parents known | At least one grandparent known | All four grandparents known |
|---|---|---|---|
| ***f* = 0.0** | 536 | 395 | 85 |
| ***f* = 0.125** | 8 | 8 | 2 |
| ***f* = 0.25** | 17 | 17 | 1 |
| **Total** | 561 | 420 | 88 |

**Supplementary Table S3.8** The interaction effect of paternal heterozygosity ($SH_{pat}$) with rainfall on first-year survival probability ($\Phi$) with and without a measure of an individual's father's rare alleles as a covariate. Alleles that had a frequency of < 5% were defined as rare alleles. For each individual I calculated the number of copies of rare alleles that their father had, divided by the number of alleles that their father was typed for. k = number of parameters; $\Delta$ = difference in QAICc from the top model (i.e., model with lowest QAICc); $\omega$ = relative QAICc weight (exp[-0.5 * $\Delta$QAICc], divided by the sum of this quantity for all considered models). $T_{sm}$ = mean summer temperature (May–October); $T_{wt}$ = mean winter temperature (November–February); SR = total summer rainfall (May–October).

| Model | k | Deviance | QAICc | $\Delta$ | $\omega$ | Model likelihood | $\beta$ (95% confidence intervals) |
|---|---|---|---|---|---|---|---|
| $\Phi$ ($T_{sm}$, $T_{wt}$, SR, $SH_{Pat}$, $SH_{Pat}$ *SR) | 7 | 3891.6 | 3905.7 | 0.00 | 0.59 | 1.00 | $SH_{Pat}$: 1.38 (0.05, 2.71) |
| $\Phi$ ($T_{sm}$, $T_{wt}$, SR, $SH_{Pat}$, $SH_{Pat}$ *SR, rare alleles) | 8 | 3890.3 | 3906.4 | 0.72 | 0.41 | 0.70 | $SH_{Pat}$: 1.45 (0.11, 2.79)<br>rare alleles: -8.63 (-22.94, 5.67) |

# Chapter 4:

# Socio-ecological correlates of extra-group paternity in the European badger (*Meles meles*)

## 4.1 Abstract

Extra-group paternity (EGP) occurs commonly among group-living mammals and plays an important role in the mating systems and the dynamics of sexual selection. The extents to which socio-ecological conditions, such as the number of breeders, influence the rate of EGP within-species are currently under explored. I used genetic paternity and demographic data from 1988 to 2010, to study the pattern of EGP, and the factors driving EGP, in a high-density European badger (*Meles meles*) population. I found the numbers of within-group and neighbouring-group candidate fathers (but not the ratio of within-group candidate fathers to within-group mother/neighbouring-group candidate fathers) had opposing influences on EGP rates in litters - the former had a negative effect while the latter had a positive effect on EGP rates. EGPs were related positively to the average pair-wise relatedness between mothers and within-group candidate fathers, but not to the average, or maximum, within-group heterozygosity of candidate fathers. Although the number of within-group candidate fathers was correlated negatively with resident females' extra-group paternity rates, inbreeding avoidance appears to drive females to attain a high frequency of EGP.

## 4.2 Introduction

Extra-group paternity (EGP) refers to the fathering of offspring (number/proportion) by males from outside of the female's social group (or social pair in socially monogamous species). EGP is a widespread phenomenon, occurring in more than two-third of those mammal species so far investigated (Isvaran & Clutton-Brock, 2007; Soulsbury, 2010) and 90% of birds (Griffith et al., 2002). There is, however, considerable variation in the rates of extra-group paternity across mammalian taxa, where some species within a family exhibit exclusive within-group paternity whereas others do not (e.g., EGP rate in Rodentia: 0–61%, Artiodactyla: 0–38%, Carnivora: 0–80% and Primates: 0–44%; Isvaran & Clutton-Brock, 2007). EGP can play an important role in the mating system and the socio-genetic structuring of populations (e.g., Young et al., 2007; Schubert et al., 2011), yet there is limited understanding of the effects of socio-ecological factors on the frequency of EGP, especially in mammals (see Cohas et al., 2006; Isvaran & Clutton-Brock, 2007) and with regard to within-population variability (Cameron et al., 2011).

Intra-specific variation in the rate of EGP between different individuals within a population may be linked to ecological factors such as (i) the number of breeding males within groups (Isvaran & Clutton-Brock, 2007; Spong et al., 2008), (ii) the number of potential mates outside females social groups (Westneat et al., 1990), (iii) the ratio of within-group males to within-group females (Kokko & Rankin, 2006), and (iv) the ratio of within-group males to neighbouring-group males. There is also the possibility for a null hypothesis strategy, where despite living in (ecologically determined) social groups, these groups may not represent coherent, exclusive, breeding groups – and thus the female may be free to encounter extra-group males while out in extra-territorial foraging grounds, and to mate with them, i.e., random, unconstrained mating. EGPs are expected to decrease as the

number or proportion of males that may monopolise, defend or mate-guard the within-group females increase (van Noordwijk & van Schaik, 2004; Kokko & Rankin, 2006; Isvaran & Clutton-Brock, 2007) and are predicted to increase as the number of neighbouring-group males increase through unrestrained random contact, as well as deliberate attempts to procure matings. The literature, however, is still lacking concerning how these ecological parameters may shape individual mating strategies, especially in mammals (e.g., mammals, Cohas et al., 2006; Isvaran & Clutton-Broack, 2007; birds, Westneat et al., 1990; Griffith et al., 2002; Westneat & Stewart, 2003; reptiles, Uller &Olsson, 2008).

EGP may be an adaptive tactic through which females derive either direct (e.g., fertility assurance (Sheldon, 1994; Vedder, et al., 2011), avoidance of infanticide (Wolff, 1993; Borries et al., 2011) and/or indirect benefits (e.g., 'Good-genes-as-heterozygosity Hypothesis', Brown, 1997; 'Compatible-genes Hypothesis', Neff & Pitcher 2005; Kempenaers, 2007). The 'Good-genes-as-heterozygosity Hypothesis' (Brown, 1997), posits that 'specific' alleles, or 'general' allelic diversity increase fitness, independent of genomic architecture. This predicts that females should value heterozygosity in their offspring and therefore, under certain conditions, in their mates. Any advantage to heterozygous offspring will depend on the relationship between fitness and heterozygosity, and the reported effect sizes of this relationship vary widely (see Britten, 1996; David, 1998; Hansson & Westerberg, 2002; Coltman & Slate, 2003). In studies that show positive effects, heterozygosity has been associated with increased survival rates (Daniels & Walthers, 2000; Cohas et al., 2009; Mainguy et al., 2009), reproductive success (Kruuk et al., 2002; Slate et al., 2004), disease resistance (Coltman et al., 1999; Whiteman et al., 2006; Reid et al., 2007) and developmental stability (reviewed in Kempenaers, 2007). The

'Compatible-gene Hypothesis', proposes that alleles increase fitness in a specific genetic context or via gene–gene interactions (e.g., epistasis, Neff & Pitcher, 2005; Kempenaers, 2007). EGP has been hypothesised to have evolved to avoid inbreeding in animals where inbreeding can be demonstrably costly (Moore & Ali, 1984; Pusey & Wolf, 1996) and this has been demonstrated in some bird studies (e.g., Brouwer et al., 2011). In circumstances where heterozygosity confers fitness benefits to offspring, and where inbreeding can be avoided through extra-group mating, EGP rates would be predicted to correlate with group genetic compositions, such as the degree of relatedness between females and within-group males (Cohas et al., 2006) or the level of heterozygosity of within-group males.

Extra-group (or extra-pair) paternity does, however, occur in many species without advantages being apparent. Indeed, there are risks associated with (extra-group) promiscuity, such as sexually transmitted disease, injury or the possible rejection of extra-group young (Westneat & Stewart, 2003) if extra-group paternity can be detected. Alternatively, EGP could be considered non-adaptive if females mated randomly with whomever they encountered and by chance they mated with extra-group males (Kokko & Rankin, 2006). A non-adaptionist scenario has been explored recently by Forstmeier et al. (2011), to address this puzzling observation. Forstmeier et al. (2011), propose the 'Within-sex genetic correlation Hypothesis' where the genetic basis of a female's response to courtship by her social mate (within-group males) and extra-group males (extra-pair males) is the same.

An alternative plausible resolution for circumstances where promiscuity (pair, or extra-group) appears to be a maladaptive behaviour is that the propensity for males and females to engage in promiscuous mating may be affected by the same set of alleles (Halliday &

Arnold, 1987). There is likely to be strong positive selection for alleles that enhance promiscuous behaviour in males (Albrecht, 2007). If promiscuous behaviour is heritable (Reid et al., 2011), pleiotropic effects on these sexually selected alleles could also cause promiscuous behaviour to evolve in females, despite antagonistic selection (Rice et al., 1992), known as the 'Between-sex genetic correlation Hypothesis' (Halliday & Arnold, 1987; Forstmeier et al., 2011).

In this study I examine the effects of ecological factors and group genetic compositions on extra-group paternity rates, as evidenced by offspring paternity, in a high-density population of European badgers (*Meles meles*), benefiting from 23 years of genetic pedigree data. Badgers provide an informative model species because they live in social groups that vary from 2–7 individuals in low density population (Revilla & Palomares, 2002; Rosalino et al., 2004) and range between 2–10, up to 29 individuals in high-density population (da Silva et al., 1994; Delahay et al., 2000). In this study population, up to seven male and female breeders have been recorded per social-group-year, with a mean (± 95% confidence interval) of 1.9 ± 0.1 successful mothers (Dugdale et al., 2007). Crucially, in high-density populations, around half of all cubs are fathered by extra-group males, primarily from neighbouring groups (Carpenter et al., 2005; Dugdale et al., 2007; Chapter 3) and multiple-paternity do occur (Carpenter et al., 2005; Dugdale et al., 2007).

Badgers exhibit a high degree of natal philopatry (Woodroffe et al., 1995); only 19% of badgers disperse successfully, mainly to neighbouring groups (Macdonald et al., 2008). An individual's breeding social groups and neighbouring-groups are therefore containing close relatives (Dugdale et al., 2008). Under benign environmental conditions (optimal summer rainfall, equating with sufficient food availability; Nouvellet et al., *subm*), cubs that have

fathers with high levels of heterozygosity (i.e., the proportion of markers with heterozygous alleles) had a higher survival probability in their first year than cubs fathered by males with higher homozygosity (Chapter 3). In combination, these factors lead to hypothesise that EGP may confer a selective advantage when within-group males are genetically more similar to resident females and are less heterozygous than extra-group males.

Even without inferring active male defence of females (anti-kleptogamy, Revilla & Palomares, 1999), simply by proportion, a larger number of males in a group may have the potential to decrease EGP rates (see also Spong et al., 2008; meerkats). However since female badgers forage solitarily and are known to transgress and make incursions into neighbouring group ranges (Bohm et al., 2009) and frequently get caught making temporary visits to other groups (given 4 samplings per year; Macdonald et al., 2008), the capacity for within-group males to actively control female access to extra-group males will be limited, although if females mate indiscriminately (polygynandry, Dugdale et al., 2007) then simple extra-group male encounter rate could be influential. Like most mustelids (e.g., Sundqvist et al., 1988), badgers are thought to be induced ovulators (a coital stimulus is required to trigger the ovulation, Mead, 1989), that could restrict their reproduction especially in females living in low densities where the number of males and the opportunity for being choosey are very limited. Thus, female may mate randomly with any males they encounter.

Here I test: [1] the effect of ecological factors on the rate of EGP at individual level (per litter, see Carpenter et al., 2005, Dugdale et al., 2007). In particular I ask: [1a] Does the rate of EGP decrease with the number of within-group males increase or the ratio of

within-group males to within-group breeding females increase in a social group (Kleptogamy defence)? [1b] Does the rate of EGP increase with the number of neighbouring-group males increase (as 86% EGP [paternity assignment was at ≥ 95% confidence] has been attributed to neighbouring-group males; Dugdale et al., 2007)? [1c] Does the rate of EGP decrease with the ratio of within-group to neighbouring-group males increase? I further test for: [2] The effect of group genetic compositions (i.e. offspring heterozygosity benefits) on the rates of EGP: [2a] Does EGP rate increase when the within-group average pair-wise relatedness between within-group breeding females and males is high, indicating inbreeding avoidance, [2b] Does EGP rate correlate negatively with within-group males mean or maximum heterozygosity?.

## 4.3 Materials and methods

### 4.3.1 Study site and field methods

A high-density population of badgers inhabiting Wytham Woods; a 415-ha site situated 5km north west of Oxford, UK (51: 46: 26N; 1: 19: 19W), has been studied intensively since the 1970s (Kruuk, 1978a; Kruuk, 1978b). A detailed description of the study site (e.g., soil, microclimates and vegetation) is provided elsewhere (Morecroft et al., 1998; Savill, 2010).

Since 1987, a systematic capture-mark-recapture regime has attempted to mark all individuals in the population (Macdonald & Newman, 2002; Macdonald et al., 2009). Live-trapping was conducted three to four times per annum; generally over two weeks in June, September and November, with one week of trapping in January of some years (Macdonald et al., 2009). Badgers were caught in mesh-traps, baited with peanuts placed

near the entrances of active setts (Macdonald & Newman, 2002; Macdonald et al., 2009; Tuyttens et al., 1999). Captured badgers were then transferred to holding cages and transported to a central handling facility. Badgers were sedated by an intra-muscular injection using ketamine hydrochloride at 0.2 ml/kg body weight (McLaren et al., 2005). Upon their first capture all badgers were tattooed with a unique individual number on the left inguinal region for permanent individual identification. The sex, age (cub or adult based on body size) and location (sett name) of each badger were recorded. For genetic analysis, hair samples and/or blood from the jugular vein (c. 3 ml) were collected from all individuals upon first capture.

Social group ranges were established using a 'bait-marking' technique approximately every two years (see Kilshaw et al., 2009). The number of social groups occupying this study site has increased steadily (Macdonald et al., 2004b) with a mean of 19 ± 2 (ranges = 14–26) social groups per year (social-group-year, SGY) between 1987 and 2005 (Dugdale et al., 2008).

Macdonald et al. (2008) report that only 19% of badgers with at least 4 captures in this population dispersed (if the 2 most recent captures and at least one or two captures before were within a non-natal group), mainly to neighbouring social groups. This indicates that badgers in this population exhibit high sett-fidelity (often through natal philopatry; see Woodroffe et al., 1995). Temporary group-movements do however occur at quite a high rate (16.4%, Macdonald et al., 2008; based on a maximum of 4 sampling opportunities, from the trapping protocol, per year). By taking into account both permanent dispersal and temporary movements, I define here the social group of residence of each individual per year based on their trapping history according to the following rules:

(a) Badgers first caught as cubs (and thus of known natal group, N = 975) were considered to be resident in their natal group unless, they satisfied the dispersal rules provided by Macdonald et al. (2008) above.

(b) Badgers first caught as adults (N = 421) were assigned to a social group based on the site that trapping revealed them to be affiliated to most frequently, unless clear dispersal events were recorded.

(c) Badgers (unknown natal group affiliation) that exhibited equal affiliation to two or more social groups (N = 14) within their lifetime were assigned to the social group where they were captured initially. Breeding females, however, were allocated to the social group where they were known to have bred most recently.

(d) Badger social groups can undergo fission, i.e., the splitting of a social group into two (or more) new social units (da Silva et al., 1993; Macdonald et al., 2004b), dividing the physical range occupied by the former group (as defined by bait-marking). Clearly, where a subsidiary sett within the former group range splits off and becomes independent from the main sett range, individuals occupying this subsidiary sett range do not move spatially in the process of becoming identified as a new social group in the population records. Badgers gaining a new group affiliation in this way (N = 41), were assigned to the new social group after the group split (with condition they also trapped at least once in the new group before split), unless they were subsequently re-trapped in the former group.

### 4.3.2 Genotyping and parentage analysis

Details of the DNA extraction, microsatellite characterisation and genotyping methods are presented in Dugdale et al., (2007), Chapter 2 and Chapter 3. In summary, I genotyped 1172 individuals at 35 microsatellite loci, of which 813 were cubs born between 1988 and

2010 inclusive (see Chapter 3). Mean observed heterozygosity (± standard error) was estimated at 0.45 ± 0.03, with 4.46 ± 0.34 alleles per locus. Exact tests, performed using GENEPOP 4.0.10 (Raymond & Rousset, 1995), revealed that no locus, or pair of loci, departed consistently from Hardy-Weinberg equilibrium or linkage equilibrium (Chapter 3). Two types of potential genotyping error; allelic drop-out rate ($\varepsilon_1$=0.005) and stochastic error rate ($\varepsilon_2$=0.005), were estimated using PEDANT 1.0 (Johnson & Haydon, 2007).

Parentage was determined sequentially using MasterBayes 2.47 (Hadfield et al., 2006) implemented in the R statistics package 2.12.2 (R Development Core Team 2010) and Colony 2.0 (Wang & Santure, 2009). For detailed description about the selection of candidate parents, and their social group assignment, see Dugdale et al., (2007); for the MasterBayes and Colony analyses see Chapter 3. I was unable to assign a mother and father at 80% confidence to 16% (N = 130) and 19% (N = 158) of the cubs, respectively.

Offspring that were fathered by males from their natal social group in the year of conception were categorised as within-group offspring (WGO, N = 340), whereas offspring fathered by males that did not reside in the natal social group were categorised as extra-group offspring (EGO, N = 315). Cubs where paternity was unknown (N = 158) were excluded from this analysis.

### 4.3.3 Heterozygosity and pair-wise relatedness

Individual standardised heterozygosity was estimated as the proportion of loci that were heterozygous, divided by the population mean heterozygosity for these loci (Coltman et al., 1999) using GENHET 2.2 in R 2.12.2 (Coulon, 2010). The average and maximum standardised heterozygosity of within-group adult males (candidate fathers) was then

derived per social-group-year. Pair-wise relatedness values between females that were assigned maternity, and their potential within-group mates, were estimated using Coancestry 1.0.0.1 (Wang, 2011) and averaged per social-group-year. Two marker-based pair-wise relatedness estimates were computed and compared: The Queller and Goodnight's estimator (QG; Queller & Goodnight, 1989) and Lynch and Ritland's estimator (LR; Lynch & Ritland, 1999). These estimators were chosen because QG is the method most commonly used and simulations show that LR performs well for most population compositions (Csillery et al., 2006).

### 4.3.5 Statistical analyses

I used R 2.13.2 (R Development Core Team 2007) to fit generalised linear mixed models (GLMMs, using the *lmer* function with Laplace approximation in the lme4 package, Bates & Sarkar, 2007). The rate of EGP was investigated at the individual level (i.e., litter level). Initially, I tested whether the distribution of EGO across litter corresponded to an expected distribution generated through binomial processes. I examined the effects of the number of males in proximity to the breeding female, using (i) the number of within-group candidate fathers, (ii) the number of neighbouring-group candidate fathers, (iii) the ratio of within-group candidate fathers to within-group mothers and (iv) the ratio of within-group candidate fathers to neighbouring-group candidate fathers. I considered the effects of the within-group genetic parameters of candidate parents, using (i) the average and maximum heterozygosity of within-group candidate fathers, and (ii) the average pair-wise relatedness of within-group mothers and candidate fathers. I also examined the quadratic effect of pair-wise relatedness between mothers and candidate fathers on EGP. I derived two indices of EGP: the absolute number of individuals fathered by extra-group males and the relative proportion of EGP at litter ($N_{EGO}$/ [$N_{EGO}$ + $N_{WGO}$]). To examine the absolute number of

EGPs within a litter in relation to the explanatory terms, I ran a GLMM, controlling for the total number of cubs per litter, with Poisson error structure and log link function. To investigate the proportion of EGPs per litter, I used a binomial error distribution and a logit link function. Social group and year were included as random effects in all models. I also included female identity as a random effect, because litters produced by the same mother across years are not statistically independent. I reran the models above with the rate of EGP calculated as the number of mate-pairs (either within-group mate-pairs, WGM or extra-group mate-pairs, EGM) rather than the number of offspring (WGO or EGO), because a single copulation could lead to several offspring within a litter and therefore I tested whether this alters the results.

I fitted a maximal model that included all explanatory terms. I eliminated non-significant terms using a backwards selection procedure to derive a minimal adequate model. The significance of including each explanatory term in the model was assessed using a likelihood ratio test (chi-square distribution) by comparing the two nested models (with- and without- the term under consideration). Effect sizes (and their standard error) are based on the final minimal models. Means are given ± standard error.

In order to test EGP per litter (measured as the number of offspring and mate-pairs), I restricted the analysis to social-group-years in which all trapped within-group candidate parents were genotyped and all offspring were assigned both parents (Data $RD_{LTR}$: SGY = 81, no of litters = 137, representing 97 mothers (30 mothers had litters for 2-4 years), 194 cubs: 100 WGO and 94 EGO; mothers had only WGM = 76 [17 had one and 5 had two WGMs], only EGM = 58 [50 had one and 8 had two EGMs] and both WGM + EGM =3 [all had one WGM and one EGM]). I then re-ran the test with an unrestricted dataset that

included social-group-years in which all trapped within-group candidate parents were genotyped and not offspring were assigned both parents (Data $URD_{LTR}$: SGY = 124, no of litters = 220, representing 138 mothers (52 mothers had litters for 2–4 years), 310 cubs: 174 WGO and 136 EGO; mothers had only WGM = 122 [115 had one and 7 had two WGMs], only EGM = 91 [81 had one and 10 had two EGMs] and both WGM + EGM =7 [all had one WGM and one EGM]). The restricted dataset was smaller and thus had reduced statistical power relative to the unrestricted dataset, but including groups with cubs that were not assigned paternity could bias the EGP rate.

## 4.4 Results

### 4.4.1 Patterns of EGP

Nearly half of assigned paternities (48%: 315 of 655 cubs) were extra-group, where the majority were attributable to neighbouring-group males (85%: 268 of 315 cubs). EGP was detected in almost half (47%: 178 of 378, Table 4.1) of litters where all offspring were assigned fathers at 80% confidence (81%: 378 of 467). Mean litter size was 1.46 (CI: 1.43, 1.49; range = 1–5) and the mean number of EGO at litters that contained at least one EGO was 1.40 (CI: 1.32, 1.49; 1–3, mode = 1), 64%, 32% and 4% of litters included one, two and three EGO respectively. No litters were fathered by more than two males. Among the 178 litters that included EGO, 26 were fathered by two males (16 had two extra-group fathers and 10 had one extra-group and one within-group father). The remaining 152 litters were fathered exclusively by one extra-group male. The distribution of EGP among litters differs from what is expected under the binomial distribution (Table 4.1, $\chi^2 = 59.90$, df = 3, $P < 0.001$). This result showed a non-random distribution of EGO over litter in badger, with more females than expected had either many or no EGO (Figure 4.1).

**Table 4.1** Distribution of EGP among litters that include only cubs with both parents assigned. The numbers of litters expected from binomial probabilities are shown in parentheses.

| Litter Size | No. of EGO per litter | | | | |
|---|---|---|---|---|---|
| | **0** | **1** | **2** | **3** | **Total litters** |
| 1 | 132 (124.3) | 107 (114.7) | - | - | 239 |
| 2 | 57 (31.6) | 6 (58.4) | 54 (27.0) | - | 117 |
| 3 | 10 (2.8) | 1 (7.8) | 2 (7.2) | 7 (2.2) | 20 |
| 4 | 1 (0.1) | - | 1 (0.7) | - | 2 |
| Total | 200 (158.8) | 114 (180.9) | 57 (34.9) | 7 (2.2) | 378 |

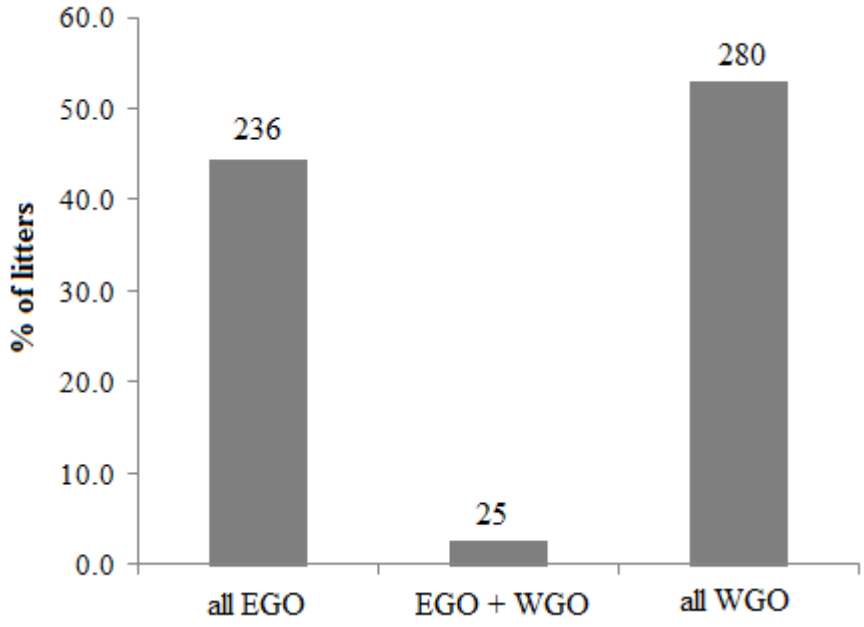


**Figure 4.1** Percentage of litters with only within-group offspring (WGO), only extra-group offspring (EGO) and mixed with both WGO and EGO. Data restricted to litters that include only cubs with both parents assigned (N = 378). Number of offspring within each category are stated on bars.

### 4.4.3 Number of breeders, group genetic compositions and the rate of EGP

EGO occurred in approximately half of the litters (Data $URD_{LTR}$ vs. $RD_{LTR}$: 45% vs. 50%, Figure 4.2). Forty-four percent of females (in Data $URD_{LTR}$; 45% in $RD_{LTR}$) paired with an extra-group male (EGM) with a maximum of two and a mode of 1 mate-pairs. I present results derived from unrestricted datasets (Data $URD_{LTR}$) because restricted models for Data $RD_{LTR}$ did not converge, probably due to small sample size. Since QG and LR pair-wise relatedness estimators were highly correlated (Spearman's Rank correlation coefficient = 0.91, $P < 0.001$) and yielded similar results I used QG estimator for the main analysis (see Supplementary Table S4.1 and S4.2).

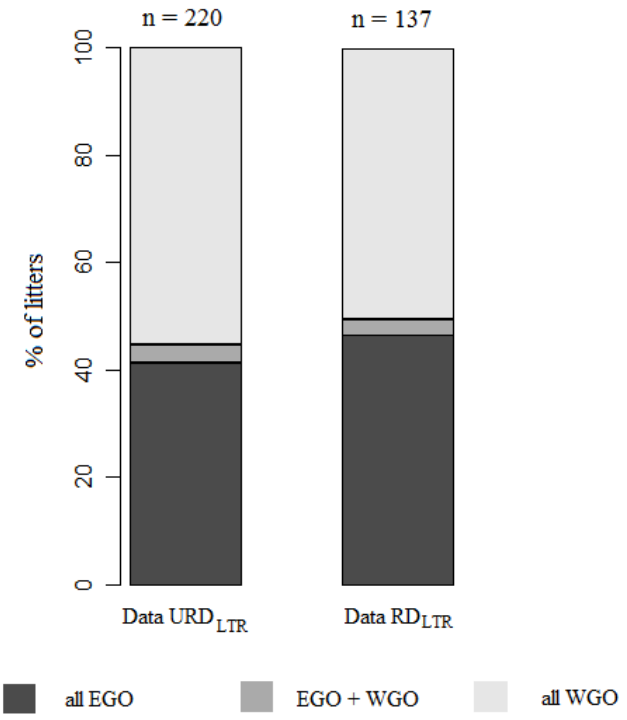


**Figure 4.2** Percentage of litters with cubs that were assigned to extra-group males only (EGO), within-group males only (WGO), and mixed paternity (EGO + WGO) in restricted and unrestricted data.

***Relative proportion and absolute numbers of within-group and extra-group males***

The number of EGO within a litter (Data $URD_{LTR}$) ranged from 1 to 4 with a mean of 1.4 ± 0.1. EGP rates varied among litters, ranging from 33% to 100%. The absolute and relative rates of EGP, measured as the number of EGO and EGM at individual level (per litter) produced similar results (Table 4.2 and 4.4). These results showed that bias in over estimating the number of EGO, such that mating with one EGM may result in two EGOs, did not alter my findings. Higher numbers of within-group candidate fathers correlated with lower numbers of EGO (and EGM) per litter in the relative model (Table 4.3), but not in the absolute model (Table 4.2). The absolute and relative rates of EGP in litters were associated positively with the number of neighbouring-group candidate fathers (Table 4.2 and 4.3). EGP rate was associated neither with the ratio of within-group candidate fathers to within-group mothers, nor with the ratio of within-group candidate fathers to neighbouring-group candidate fathers (Table 4.2 and 4.3).

***Group compositions***

The average pair-wise relatedness between mothers and candidate fathers within-groups had the greatest explanatory power for both the relative and absolute rates of EGP across litters (Table 4.2 and 4.3). Where the average within-group candidate parents' relatedness was greater, the number of EGO (absolute) and the proportion of EGO (relative) were higher (Figure 4.3). Relative and absolute rates of EGP did not correlate with the mean and maximum heterozygosity of within-group candidate fathers (Table 4.2 and 4.3).

**Table 4.2** Parameter estimates from GLMMs analysing *the absolute number of extra-group paternity (EGP) or extra-mate pair* in a litter in relation to group density and composition. Mean SH = Individual standardised heterozygosity and QG = average Queller and Goodnight's pair-wise estimator between mothers and within-group candidate fathers. Unrestricted dataset, $UR_{LTR}$: SGY = 124, no of litters = 220 representing 310 cubs: 174 WGO and 136 EGO.

| No | Explanatory variable | Extra-group paternity | | | Extra-mate pairs | | |
|---|---|---|---|---|---|---|---|
| | | **Estimate ± SE** | **$\chi^2_{[df = 1]}$** | **P** | **Estimate ± SE** | **$\chi^2_{[df = 1]}$** | **P** |
| | **Final model** | | | | | | |
| 1 | Intercept | -2.75 ± 0.40 | - | - | -1.71 ± 0.33 | - | - |
| 2 | Average pair-wise relatedness (QG) | 2.30 ± 0.57 | 15.07 | **<0.001** | 1.99 ± 0.60 | 10.16 | **0.001** |
| 3 | No of neighbouring-group candidate fathers | 0.02 ± 0.01 | 8.39 | **0.004** | 0.02 ± 0.01 | 5.74 | **0.017** |
| | **Rejected terms** | | | | | | |
| 1 | Quadratic effect of QG | -3.90 ± 2.44 | 2.81 | 0.093 | -3.38 ± 2.71 | 1.75 | 0.186 |
| 2 | Average SH within-group candidate fathers or | -0.50 ± 0.89 | 0.32 | 0.574 | -0.04 ± 0.91 | 0.00 | 0.964 |
| | Maximum SH within-group candidate fathers | -0.20 ± 0.68 | 0.09 | 0.770 | 0.00 ± 0.72 | 0.00 | 0.995 |
| 3 | Ratio of within-group candidate fathers to neighbouring-group candidate fathers | 2.31 ± 0.55 | 2.72 | 0.099 | -0.32 ± 0.40 | 0.67 | 0.414 |
| 4 | No of within-group candidate fathers | -0.05 ± 0.03 | 0.20 | 0.656 | -0.05 ± 0.03 | 2.70 | 0.101 |
| 5 | Ratio of within-group candidate fathers to within-group mothers | -0.02 ± 0.03 | 0.74 | 0.391 | 0.01 ± 0.05 | 0.01 | 0.927 |

In the absolute model of extra-group paternity, the random effect for social group, year and mother ID were zero. For extra-mate pairs model, it was zero for year and mother ID but 0.05 ± 0.22 for social group. Significant effects are in bold.

**Table 4.3** Parameter estimates from GLMMs analysing *the relative proportion of extra-group paternity (EGP) or extra-mate pair* in a litter in relation to group density and composition. Mean SH = Individual standardised heterozygosity and QG = average Queller and Goodnight's pair-wise estimator between mothers and within-group candidate fathers. Unrestricted dataset, $UR_{LTR}$: SGY = 124, no of litters = 220 representing 310 cubs: 174 WGO and 136 EGO.

| No | Explanatory variable | Extra-group paternity | | | Extra-mate pairs | | |
|---|---|---|---|---|---|---|---|
| | | **Estimate ± SE** | **$\chi^2_{[df = 1]}$** | **P** | **Estimate ± SE** | **$\chi^2_{[df = 1]}$** | **P** |
| | **Final model** | | | | | | |
| 1 | Intercept | -3.31 ± 1.38 | | | -1.98 ± 0.74 | | |
| 2 | Average pair-wise relatedness (QG) | 10.89 ± 2.78 | 18.66 | **<0.001** | 5.82 ± 1.43 | 17.07 | **<0.001** |
| 3 | No of neighbouring-group candidate fathers | 0.13 ± 0.04 | 13.00 | **<0.001** | 0.07 ± 0.02 | 11.00 | **<0.001** |
| 4 | No of within-group candidate fathers | -0.37 ± 0.13 | 6.95 | **0.008** | -0.19 ± 0.08 | 4.77 | **0.029** |
| | **Rejected terms** | | | | | | |
| 1 | Quadratic effect of QG | -10.86 ± 9.15 | 1.49 | 0.222 | -6.06 ± 5.33 | 1.23 | 0.268 |
| 2 | Average SH within-group candidate fathers or | -0.86 ± 3.49 | 0.06 | 0.812 | -0.11 ± 2.06 | 0.00 | 0.959 |
| | Maximum SH within-group candidate fathers | -1.96 ± 2.81 | 0.44 | 0.507 | -0.70 ± 1.65 | 0.16 | 0.689 |
| 3 | Ratio of within-group candidate fathers to neighbouring-group candidate fathers | -1.44 ± 1.49 | 1.02 | 0.312 | -0.55 ± 0.80 | 0.45 | 0.504 |
| 4 | Ratio of within-group candidate fathers to within-group mothers | 0.08 ± 0.14 | 0.31 | 0.575 | 0.05 ± 0.10 | 0.21 | 0.646 |

In the relative model of extra-group paternity, the random effect for social group, year and mother ID were 4.48 ± 2.12, 1.26 ± 1.12 and 10.15 ± 3.19, respectively. For extra-mate pairs model, they were 1.07 ± 1.03, 0.33 ± 0.58 and 1.23 ± 1.11, respectively.

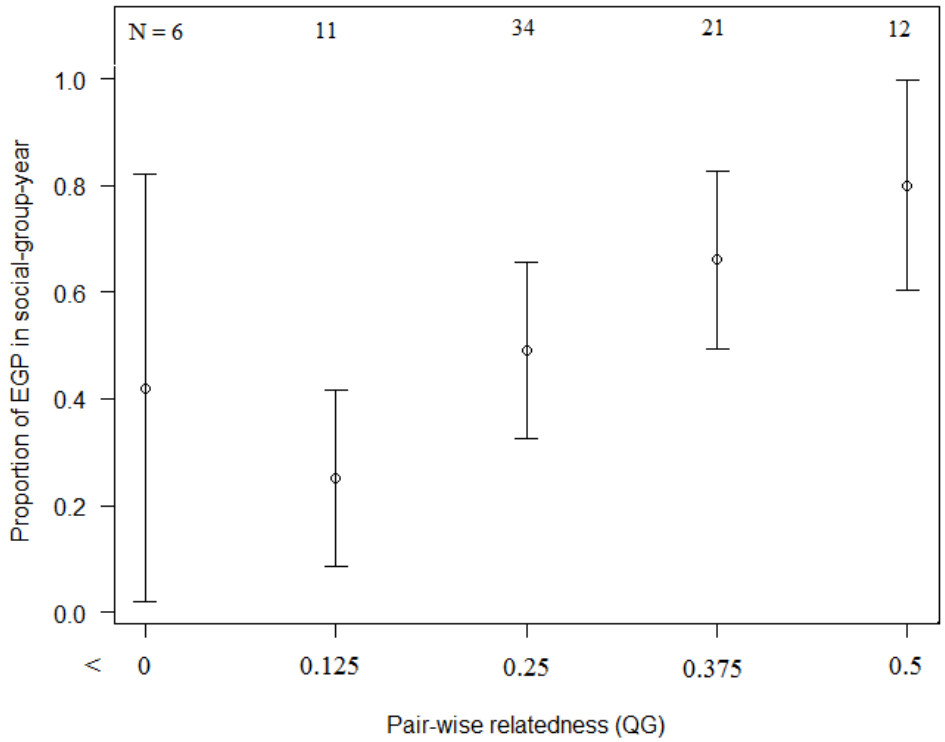


**Figure 4.3** The relationship between the average pair-wise relatedness (Queller and Goodnight's estimator) of within social-group-year candidate parents and the proportion of offspring that are extra-group young at the social group level. For ease of visualisation, pair-wise values were grouped on the x-axis with error bars show ± SE. Numbers along the top of the figure indicate the number of social-group-years in each category.

## 4.5 Discussion

My findings contribute to the contentious debate in evolutionary biology concerning the role of extra-pair (or group) mating, with evidence here of extra social group infidelity. I show that, at the litter level, EGP correlated negatively with the number of within-group candidate fathers, and positively with the number of neighbouring-group candidate fathers. The ratios of within-group fathers to within-group mothers, or to neighbouring-group

candidate fathers, did not correlate with the rate of EGP. While I observed high rates of extra-group paternity, relating to the number of within- and extra-group candidate fathers and within-group genetic composition (pair-wise relatedness), I found no evidence that rates of EGP corresponded to the heterozygosity of within-group males (Arnqvist & Kilpatrick, 2005; Griffith, 2007; Eliassen & Kokko, 2008; Forstmeier et al., 2011). Neither mean nor the maximum level of heterozygosity of within-group candidate fathers correlated with EGP rate in litters.

### 4.5.1 Within-group vs extra-group paternity

Almost half of offspring that were assigned parentage involved infidelity from the parents' social group affiliation. This indicates that the badger social group unit does not correspond to a breeding unit (Carpenter et al., 2005; Dugdale et al., 2007). In meerkats, EGP rates decrease with higher numbers of defending males in a group (Spong et al., 2008). I observed that the number of candidate within-group males had a negative influence on the proportion of the females' offspring sired by extra-group candidate fathers. Similarly, EGP rate correlated positively with the number of neighbouring-group candidate fathers present. While a higher number of within-group males may deter intrusions by extra-group males it is also equally plausible that I am observing a simple density-dependent effect, where females mate *ad libitum* but that lower within-group male presence militates for more matings with extra-group males.

A greater proportion of EGO may result at the litter level if females actively solicit extra-group copulations (EGCs). Female solicitation of extra-group copulations is well documented in birds (e.g., Schwartz et al., 1999; Pryke et al., 2010) and mammals (Wolff & Macdonald, 2004). Female badgers may solicit EGCs to reduce the risk of infanticide.

Circumstantial infanticide by males has been reported in badgers (Kruuk, 1989; Lups & Roper, 1990). By mating with potentially infanticidal males, female may increase the chances of survival of their offspring. Female snub-nosed langurs (*Rhinopithecus* spp) for example, reduce infanticide by mating with the invading males (Ren et al., 2011). It is not known to what extent the solicitation of extra-group matings is led by males or females, or equally, in badgers.

The majority of EGP matings were attributed to candidate fathers from neighbouring-groups. Badger movements between social groups are predominantly temporary and of short distance, i.e., to adjacent groups (Macdonald et al., 2008). Female badgers forage solitarily (Carr & Macdonald, 1986; da Silva et al., 1993), and are known to make transient extra-territorial excursions (Huck et al., 2008; Macdonald et al., 2008; see also Stewart et al., 1997) where they transgress into neighbouring groups (Macdonald et al., 2008) and appear compliant to EGCs, possibly in order to mitigate aggression (Macdonald et al., 2004a; Dugdale et al., 2011a) or due to a fundamental evolved mating strategy, from low(er) density conditions, where the opportunity to copulate is restricted and therefore all opportunities are taken (e.g., Erlinge & Sandell, 1986). Mate guarding (Brotherton & Komers, 2003) has not been observed in badgers – rather non-competitive serial copulations with different partners underscore a freely promiscuous system (Dugdale et al., 2007). Thus I cannot reject the null hypothesis / the simplest possible scenario; however I proceed to include further plausible socio-biological interpretation.

### 4.5.2 Inbreeding avoidance and heterozygosity

Extra-group mating has been proposed as an alternative strategy to avoid inbreeding in a population where dispersal is restricted or does not occur (e.g., Brouwer et al., 2011).

Dispersal and inbreeding avoidance are generally related (see Moore & Ali, 1984). Durant and Hughes (2005) found that in the Australian magpie (*Gymnorhina tibicen*), EGP was inversely related to dispersal rates between two contrasting populations such that high philopatry corresponded with high EGP. Similarly, in some socially monogamous fairy-wrens, *Malurus splendens*, promiscuity provided a potential inbreeding avoidance mechanism (Brooker et al., 1990).

The high incidence of EGP and a negative correlation between EGP and average pair-wise relatedness between mothers and candidate fathers that have been shown in the current study (Figure. 4.3) indicate that female badgers may have evolved extra-group mating strategy to avoid inbreeding. At high density, badger social groups contain close relatives (Dugdale et al., 2008), because dispersal is limited (Pope et al., 2006), and both sexes are recruited into their natal groups. The lifetime reproductive success of inbred badgers in this population is lower than for out-bred (Dugdale, 2007). Thus, female badgers may engage in extra-group copulations preferably, or whenever matings with close relatives is unavoidable, to reduce the probability of producing inbred offspring.

Females may copulate with extra-group males to obtain fitness benefits e.g., heterozygous advantage, good and compatible genes (Jennions & Petrie, 2000). The 'Good-gene-as-heterozygosity Hypothesis' predicts that females may seek extra-group copulation if within-group males have low level of heterozygosity (Brown, 1997). A paternal heterozygosity advantage on first-year survival probability (under most suitable environment conditions) has been observed in this badger population (Chapter 3). This suggests that mating with males with higher levels of heterozygosity brings survival benefits. I would thus predict that when there are heterozygous males within-groups,

females will mate with them, but when only males with low heterozygosity are available, females may benefit from mating outside of their social group. In this study, EGP rates were not related to the average or maximum heterozygosity of within-group candidate fathers. Small sample size for females with mixed paternity litters, however (within-group and extra-group candidate fathers assigned paternity to offspring in the same litter, N = 10), did not allow me to test for the biological significance of differences between within-group and extra-group fathers in terms of heterozygosity.

## 4.6 Conclusions

Despite almost half of offspring-yielding matings taking place between individuals from different, proximate, social groups, I found no evidence for mate selection for genetic quality (i.e., heterozygosity). A positive correlation between the rates of EGP and the average pair-wise relatedness between mothers and within-group candidate fathers, suggesting EGPs may play an important role in reducing inbreeding depression. Most interesting is that within-group versus extra-group parentages do not appear to be opposing mating strategies in badgers; rather these exist within a continuum of polygynandrous mating events. Failure to realise fitness advantages through superior extra-group offspring survival, recruitment or life-time reproductive success would confirm that infidelity in badgers is either socially adaptive, or inevitable, through sex-linked genetic correlation; a 'self-fulfilling prophecy' (Merton, 1968), rather than a selection for superior genetic quality.

## 4.7 Supplementary materials

**Supplementary Table S4.1** Parameter estimates from GLMMs analysing *the absolute number of extra-group paternity (EGP) or extra-mate pairs* in a litter in relation to group density and composition. Mean SH = Individual standardised heterozygosity and LR = average Lynch and Ritland's pair-wise estimator between mothers and within-group candidate fathers. Unrestricted dataset, $UR_{LTR}$: SGY = 124, no of litters = 220 representing 310 cubs: 174 WGO and 136 EGO.

| No | Explanatory variable | Extra-group paternity | | | Extra-mate pairs | | |
|---|---|---|---|---|---|---|---|
| | | Estimate ± SE | $\chi^2_{[df = 1]}$ | P | Estimate ± SE | $\chi^2_{[df = 1]}$ | P |
| | **Final model** | | | | | | |
| 1 | Intercept | -2.74 ± 0.39 | - | - | -1.69 ± 0.31 | - | - |
| 2 | Average pair-wise relatedness (LR) | 2.45 ± 0.56 | 17.07 | **<0.001** | 2.18 ± 0.57 | 12.44 | **<0.001** |
| 3 | No of neighbouring-group candidate fathers | 0.02 ± 0.01 | 1.10 | **0.008** | 0.02 ± 0.01 | 5.01 | **0.025** |
| | **Rejected terms** | | | | | | |
| 1 | Quadratic effect of LR | -4.48 ± 2.44 | 3.89 | 0.050 | -4.80 ± 2.82 | 3.42 | 0.064 |
| 2 | Average SH within-group candidate fathers or | -1.13 ± 0.90 | 1.46 | 0.227 | -0.65 ± 0.95 | 0.47 | 0.492 |
| | Maximum SH within-group candidate fathers | -0.43 ± 0.69 | 0.39 | 0.534 | -0.27 ± 0.75 | 0.02 | 0.900 |
| 3 | Ratio of within-group candidate fathers to neighbouring-group candidate fathers | -0.47 ± 0.37 | 1.78 | 0.182 | -0.29 ± 0.41 | 0.55 | 0.460 |
| 4 | No of within-group candidate fathers | -0.04 ± 0.03 | 1.91 | 0.167 | -0.05 ± 0.03 | 3.30 | 0.069 |
| 5 | Ratio of within-group candidate fathers to within-group mothers | 0.05 ± 0.05 | 0.90 | 0.342 | 0.01 ± 0.06 | 0.01 | 0.921 |

In the absolute model of extra-group paternity, the random effect for social group, year and mother ID were 0.08 ± 0.29, 0.01 ± 0.10 and 0, respectively. For extra-mate pairs model, they were 0.96 ± 0.98, 0.35 ± 0.59 and 1.25 ± 1.12, respectively.

**Supplementary Table S4.2** Parameter estimates from GLMMs analysing *the relative proportion of extra-group paternity (EGP) or extra-mate pairs* in a litter in relation to group density and composition. Mean SH = Individual standardised heterozygosity and LR = average Lynch and Ritland's pair-wise estimator between mothers and within-group candidate fathers. Unrestricted dataset, $UR_{LTR}$: SGY = 124, no of litters = 220 representing 310 cubs: 174 WGO and 136 EGO.

| No | Explanatory variable | Extra-group paternity | | | Extra-mate pairs | | |
|---|---|---|---|---|---|---|---|
| | | **Estimate ± SE** | **$\chi^2_{[df=1]}$** | **P** | **Estimate ± SE** | **$\chi^2_{[df=1]}$** | **P** |
| | **Final model** | | | | | | |
| 1 | Intercept | -3.65 ± 1.41 | | | -2.09 ± 0.73 | | |
| 2 | Average pair-wise relatedness (LR) | 12.83 ± 2.91 | 23.59 | **<0.001** | 6.82 ± 1.47 | 21.93 | **<0.001** |
| 3 | No of neighbouring-group candidate fathers | 0.13 ± 0.04 | 12.08 | **<0.001** | 0.07 ± 0.02 | 9.87 | **0.002** |
| 4 | No of within-group candidate fathers | -0.37 ± 0.14 | 6.71 | **0.010** | -0.18 ± 0.08 | 4.51 | **0.034** |
| | **Rejected terms** | | | | | | |
| 1 | Quadratic effect of LR | -19.90 ± 10.38 | 3.84 | 0.050 | -10.36 ± 5.59 | 3.07 | 0.080 |
| 2 | Average SH within-group candidate fathers or | -2.04 ± 3.34 | 0.09 | 0.765 | -1.05 ± 2.09 | 0.23 | 0.630 |
| | Maximum SH within-group candidate fathers | -1.08 ± 2.19 | 0.00 | 0.999 | -0.27 ± 0.75 | 0.14 | 0.713 |
| 3 | Ratio of within-group candidate fathers to neighbouring-group candidate fathers | -0.79 ± 1.45 | 0.42 | 0.517 | -0.45 ± 0.82 | 0.29 | 0.592 |
| 4 | Ratio of within-group candidate fathers to within-group mothers | 0.08 ± 0.13 | 0.28 | 0.594 | 0.05 ± 0.10 | 0.23 | 0.628 |

In the relative model of extra-group paternity, the random effect for social group, year and mother ID were 4.41 ± 2.10, 1.32 ± 1.15 and 11.41 ± 3.38, respectively. For extra-mate pairs model, they were 0.96 ± 0.98, 0.35 ± 0.59 and 1.25 ± 1.12, respectively.

# Chapter 5:

# Choosing mates for offspring genetic quality: Does extra-group paternity enhance fitness of the European badger (*Meles meles*)?

## 5.1 Abstract

In many group-living species, females often produce offspring fathered by males from outside of their social group (i.e., extra-group paternity, EGP). Why this reproductive strategy is adopted in the mating system of certain species, or under certain socio-ecological conditions remains unclear. The hypothesis that females obtain indirect genetic benefits from EGP has received considerable attention, especially in birds, but the evidence has been equivocal. Here, I test whether extra-group offspring (EGO; offspring sired by extra-group males) exhibit greater fitness (survival and lifetime reproductive success, LRS) than within-group offspring (WGO). EGO had a lower first-year survival probability than WGO, and survived on average for 1.3 years less. Female WGO produced more litters and offspring over their lifetime compared with female EGO. The opposite was observed in males: male EGO produced more offspring in their lifetime than did male WGO. These findings indicate that, for females, the EGP reproductive strategy has a cost, where WGO confer greater lifetime fitness. Sex-specific differences in the LRS advantages in producing EGO or WGO may reflect sexually antagonistic genetic effects, which may determine constraints that maintain the EGP in this population.

## 5.2 Introduction

Over the last decade, the understanding of genetic benefits increasingly informed insights into mate choice evolution (see reviews by Mays & Hill, 2004; Neff & Pitcher, 2005; Roberts et al., 2006; Kempenaers, 2007). In general, individuals are predicted to select for genetic quality in their mating partners when this increases offspring viability and future reproductive success (Ryder et al., 2010), where often females are the more selective sex (e.g., Double & Cockburn, 2000) due to greater investment in offspring (Bateman, 1948).

Two competing, often not mutually exclusive, models have been proposed to explain the effect of mate choice on the genetic quality of offspring, where quality is defined as the contribution of an allele or a genotype to offspring fitness (Kempenaers, 2007);

1) The good allele model: an allele that enhances offspring fitness independent of the genome, and
2) The compatible allele model: a specific male allele, when combined with a specific female allele, enhances offspring fitness in a specific genetic context either via heterozygote advantage or gene–gene interactions (e.g., epistasis, Neff & Pitcher, 2005; Kempenaers, 2007).

Both models predicted mate choice informed by the phenotypic traits of the mating partner, which signal either specific alleles (e.g., advantageous alleles in the traditional 'Good-genes Hypothesis') or general allelic diversity (i.e., heterozygosity).

In the good allele model, one sex selects their mating partner irrespective of their own genotype. In the compatible allele model, however, mate suitability depends largely on the interaction of male and female genotypes; in some cases (e.g., major histocompatibility complex, see Sin et al., 2012), this hypothesis also predicts that genetically dissimilar

mates may produce superior offspring / experience greater fitness (reviewed in Mays & Hill, 2004; Kempenaers, 2007).

Extra-group paternity (henceforth EGP), is a common feature of mating systems, found in approximately 90% of bird species (reviewed by Griffits et al., 2002) and in group-living mammals (e.g., Red foxes, *Vulpes vulpes*, Baker et al., 2004; Alpine marmots, *Marmota marmota*, Goossens et al., 1998; Ethiopian wolves, *Canis simensis*, Sillero-Zubiri et al., 1996; Meerkat, *Suricata suricatta*, Young et al., 2007, see also Isvaran and Clutton-Brock, 2007; Soulsbury, 2010). EGP results in females giving birth to offspring that are fathered by males that live outside of their mating-pair or social group. EGP has major implications for the evolution of mating systems and the dynamics of sexual selection (Isvaran & Clutton-Brock, 2007). Although EGP occurs extensively, the genetic benefits of EGP have been analysed primarily in birds (e.g., Akcay & Roughgarden, 2007; Ryder et al., 2010; Sardell et al., 2011; Schmoll et al., 2011), and such benefits in mammalian mating systems have not been investigated as thoroughly (but see Cohas et al., 2007a & b).

Here I make a quantitative assessment of the within-group and extra-group paternity benefits conferred on offspring fitness, in the European badger (*Meles meles*). The European badger exhibits different social strategies, according to resources and population density over its extensive range (see Newman et al., 2011). In my high-density UK study population, badgers live in groups of up to 29 individuals (da Silva et al., 1994), and exhibit relatively well synchronised parturition, with one litter per year – although there is evidence that the badger is an induced ovultator (Canivenc, 1966), with a post-partum mating peak (though also noted to breed later in the year; Creswell et al., 1992) – delayed implantation leads to synchronised parturition (Yamaguchi et al., 2006).

The large social groups, in which Wytham badgers typically live, usually consist of close kin (Dugdale et al., 2008). Dugdale et al. (2007) report a mean of 5.60 candidate mothers per social-group-year (95% confidence interval, CI [4.82, 6.38]) and 5.80 males (CI [5.02, 6.58]), with up to seven breeding members of both sexes. Badgers have one litter per year with a mean post-emergence litter size of 1.46 (this study, CI [1.43, 1.49]; range = 1–5). Multiple-paternities occurred in 27% of the multi-parous litters, with a maximum of two fathers per litter (this study).

Badgers provide an informative species with which to confirm or refute selection for genetic quality in terms of good, and compatible, alleles, due to their highly polygynadrous mating system, where females mate with several males and males with several females (Dugdale et al., 2007; 2011a), Furthermore, badgers transgress into one another's territories (Macdonald et al., 2008) and cubs may be sired either by males from their mother's social group, or from other groups (Carpenter et al., 2005; Dugdale et al., 2007; Chapter 3). Consequently, rates of EGP reach almost 50% in some high-density badger populations (Carpenter et al., 2005; Dugdale et al., 2007).

I assume mates have either the ability to assess genetic quality in their partner using cues and/or the ability to assess a mate's genotype with respect to their own genotype (e.g., morphological, behavioural, olfactory, see Buesching et al., 2002a & b; Ryder et al., 2010). For example, badger sub-caudal scent contains information on individual specific parameters and group membership (Buesching et al., 2002a & b), and this trait may impact on mate choice.

Here, I use a long-term dataset on the demographics and reproductive patterns (using a genetic pedigree) of a high-density badger population (see Macdonald & Newman, 2002; Macdonald et al., 2009) to investigate the fitness consequences of EGP. I examine whether extra-group offspring (EGO) and within-group offspring (WGO) vary in terms of first-year survival probability or estimated longevity. I consider whether offspring-paternity type is correlated with their lifetime reproductive success (LRS), in terms of the numbers of litters, or offspring, they produce. I test whether estimated longevity and LRS of EGO and WGO depend on sex (there was no sex-specific differences in relative offspring-paternity type first-year survival probability, Chapter 3). Evidence of sex-specific variation in fitness-related traits between EGO and WGO, with any additive genetic basis, would suggest a sex-specific antagonistic effect (i.e., opposing selection pressure between the sexes) that constrain and maintain extra-group reproduction in the population (Foerster et al., 2007; Brommer et al., 2007).

I predict that if extra-group mating increases offspring fitness, EGO will exhibit:

(i) greater first-year survival probability;

(ii) greater lifetime reproductive success; and

(iii) survive to an older age, than WGO.

This informs my evaluation of the selective pressures favouring EGP in badgers. Benefits to EGP are required in order to reject the developing proposition (Chapter 4) that extra-group promiscuity does not confer fitness advantages to parents, from which I would infer either pleiotropic genetic linkage (of genes for extra-pair mating) between the sexes, or mechanisms connected to an adaptionist social function (Forstmeier et al., 2011).

## 5.3 Materials and methods

### 5.3.1 Study species, population and field methods

This study was conducted in Wytham Woods, a 415-ha site, 6 km northwest of Oxford in southern England (51: 46: 26N; 1: 19: 19W). The Wytham badger population inhabits a partly discrete geographical area, which limits, but does not eliminate, the potential for migration into or out of the study area (Macdonald & Newman, 2002). Each year an average of 3% of badgers is estimated to be immigrants (Macdonald & Newman, 2002). Dispersal is restricted, although temporary movements occur (male-biased), most often to neighbouring groups (Macdonald et al., 2008).

Since 1987 a systematic capture-mark-recapture regime has attempted to trap and mark all badgers in the population (Macdonald & Newman, 2002; Macdonald et al., 2009). Trapping was conducted three to four times per annum; typically over two weeks in June, September and November, with one week of trapping in the January of some years (Macdonald et al., 2009). Badgers were caught in mesh-traps baited with peanuts, placed near the entrances of active setts (Tuyttens et al., 1999; Macdonald & Newman 2002; Macdonald et al., 2009). Captured badgers were transferred to holding cages and transported to a central handling facility, where they were sedated by an intra-muscular injection using ketamine hydrochloride at 0.2 ml/kg body weight (McLaren et al., 2005; Thornton et al., 2005). Upon their first capture all badgers were tattooed with a unique individual number on the left inguinal region (Cheeseman & Harris, 1982) for permanent individual identification. Sex, age (cub or adult) and location of capture (sett and social group affiliation were determined through bait-marking surveys, Kilshaw et al., 2009) were

recorded for each badger. For genetic analysis, hair samples and/or blood from the jugular vein (c. 3 ml) were collected from all individuals upon first capture.

### 5.3.2 Genotyping and parentage analyses

Blood or hair samples, collected from 1988 until 2009, were genotyped using 35 microsatellite markers. Details of the microsatellite characterisations and genotyping procedures used for parentage analysis are described in detail elsewhere (Dugdale et al., 2007; Chapter 2 & 3). Briefly, DNA was extracted using a slightly modified Chelex protocol (Chelex® 100, Bio-Rad, Hercules CA, USA: Walsh et al., 1991), then sequenced and analysed using GENEMAPPER® 3.5 software. Of the 1247 badgers that were first caught as cubs (N = 899) and adults (N = 348, unknown age) between 1988 and 2009, 764 (85%) and 256 (74%) were genotyped, respectively.

Parentage analysis was performed using the software MasterBayes 2.47 (Hadfield et al., 2006), implemented in the R statistics package 2.12.2 (R Development Core Team 2010), and Colony 2.0 (Wang & Santure 2009). Parentage was allocated based on those cubs that were able to catch and sample after weaning / independence (at 12-14 weeks; an ethical constraint to ensure animal welfare standards) to which parentage was assigned with at least 80% confidence. Maternity and paternity were assigned to 648 (85%) and 618 (81%) cubs respectively, born between 1988 and 2009. Both parents were assigned to 534 (70%) cubs.

The social group in which an individual was first trapped as a cub was assumed to be its natal social group. Offspring that were fathered by males in their natal social group were categorised as within-group offspring (WGO), whereas offspring fathered by males that

did not reside in their offspring's natal social group in the year of conception (cubs are born one year after conception, due to delayed implantation; see Thom et al., 2004) were categorised as extra-group offspring (EGO). The social group of residence of each individual per year was defined based on their trapping history according to the rules described in detail in Chapter 4. Litters comprised of only WGO were termed within-group litters (WGL); only EGO were termed extra-group litters (EGL); and, litters including both WGO and EGO were denoted as mixed-litters (ML).

### 5.3.3 Statistical analyses

***First-year survival probability***

Cormack-Jolly-Seber models were performed in MARK 6.1 (White & Burnham, 1999), to estimate and compare the first-year survival probability of EGO and WGO born between 1988 and 2009 that were trapped in their first year 1989–2010. The input data consisted of capture-recapture histories for all offspring (e.g., badgers first caught as cubs that were assigned a father, so that the offspring-paternity type [EGO or WGO] could be assigned), over 23 years (1988–2010; Dataset A, the complete dataset, N = 618 offspring [295 EGO and 323 WGO]); data from each trapping session within a year were pooled into one annual capture record. I adopted the basic model infrastructure from Chapter 3 (see also Macdonald et al., 2009), using: two age-classes (cub < 1 years old; adult > 1 years old) with year-dependent survival applied to the cub age-class and consistent, but sex-specific, survival probabilities applied to the adult age-class, with year-dependent recapture probabilities. Estimates of recapture probabilities were established using the primary model, which were then fixed in all models. Dividing the model deviance by the bootstrapped deviance showed that my basic model was slightly over dispersed ($\hat{c}$ = 1.06;

N = 100 replicates); thus, I adjusted the Akaike information criterion (corrected for small sample size [AICc] – Burnham & Anderson, 2002) value accordingly, using quasi-likelihood (QAICc).

I included all effects, shown by previous studies to be associated with first-year survival probability in this population, in my basic model:

(a) standardised total summer rainfall (May–October) (Macdonald et al., 2010; Chapter 3);

(b) standardised mean winter temperature (November–February) (Macdonald et al., 2010; Chapter 3);

(c) paternal multilocus heterozygosity (Chapter 3);

(d) the interaction between paternal multilocus heterozygosity and standardised total summer rainfall (Chapter 3); and

(e) $\log_e(x+1)$ transformed endo-parasitic (coccidial) load (Newman et al., 2001; Nouvellet et al., 2010).

These five effects (a-e) were retained in all models even if they were not statistically significant; however, very few badgers had coccidial load data, limiting this sample size. I therefore ran each model twice (with and without coccidial load e.g., Dataset A [complete dataset] and Dataset $A_{coccid}$ [complete dataset, restricted coccidial load]). By integrating these potentially confounding effects in my models I was able to reduce the unexplained variance in first-year survival of offspring, thereby increasing the power to detect the real effects of the three main tests that I ran.

I first tested the effect of maternal age (Test 1: The effect of maternal age, a continuous effect), which influences first-year offspring survival rates in other mammalian species (e.g., Descamps et al., 2008; Mech & McRoberts 1990). These data for this analysis

(Dataset B, restricted maternal age) were limited to 278 offspring (130 EGO and 148 WGO; from Dataset A) for which the age of their mother was known. Test 1 was also run on badgers from Dataset B that had coccidial load data (Dataset $B_{coccid}$, restricted maternal age and coccidial load: 59 offspring [27 EGO and 32 WGO]).

I then investigated the influence of litter-type; that is, whether WGO or EGO born in different litter types ([I] WGL vs. ML; [II] EGL vs. ML) differed in their first-year survival probability (Test 2: The effect of litter type). My analysis was restricted to litters where all the offspring within a litter had an assigned father (Dataset C, restricted complete litters: [I] N = 281 offspring - 269 WGO in 191 WGL and 12 WGO in 10 ML; [II] N = 233 offspring - 220 EGO in 158 EGL and 13 EGO in 10 ML). Test 2 was also run just for badgers in Dataset C with coccidial load data (Dataset $C_{coccid}$: [I] N = 57 offspring - 55 WGO in 47 WGL and 2 WGO in 2 ML, [II] N = 48 offspring - 43 EGO in 34 EGL and 5 EGO in 4 ML).

I also tested for difference in first-year survival probability according to offspring-paternity type (Test 3: The effect of offspring-paternity type; Dataset A, complete dataset). Test 3 was again run using the same dataset A, but restricted to badgers for which historic parasite data were availability (Dataset $A_{coccid}$).

***Estimated longevity***

Estimated longevity (hereafter longevity), defined in the CMR framework as the age at which an individual's was last captured, was measured for badgers that at least reached sexual maturity (i.e., one year old, Cresswell et al., 1992). Based on a modified 'minimum number alive' technique (Krebs, 1966; see also Macdonald & Newman, 2002), I assessed

longevity of individuals first caught as cubs from cohort 1988 to 1996, allowing sufficient time (1997–2010) for each to achieve a maximal lifespan of up to ca. 15 years (see Dugdale et al., 2011b) i.e., no extant representatives of these cohorts remained after 2008, confirmed as no subsequent recapture in either 2009 or 2010 (Supplementary Figure S5.1; an approach used by Macdonald & Newman, 2002), and 95% of the inter-trap intervals are within 2-years (Dugdale et al., 2007). In order to investigate the differences in longevity between EGO (N = 77) and WGO (N = 125) [Dataset D, complete longevity], I limited the data to individuals first caught as cubs that were assigned both parents. Because this protocol restricted the minimum possible longevity of individuals to 1 year, the number of years that survived after breeding age was transformed, by subtracting one, in order to meet Poisson assumptions. Mean adult survival probability is affected by sex, but not by year, cohort or population size (Chapter 3), thus I included sex as a fixed categorical effect in my model. Offspring-paternity type (EGO or WGO; categorical), and its interaction with sex, were included as fixed effects. Birth year, mother ID and natal group ID were included as random effects, to control for maternal and environmental effects.

***Lifetime reproductive success***

I compared the lifetime reproductive success (LRS) of EGO and WGO ($F_1$ offspring) that were caught as cubs that survived to breeding age and were assigned both parents. I restricted the data to $F_1$ offspring that were assigned parentage themselves in at least one year, or that were assigned at least one $F_2$ grand-offspring (grand-offspring of $F_0$ female). The LRS of these $F_1$ offspring was measured as:

(i) the number of litters (i.e., number of years in which female/male $F_1$ offspring were assigned maternity/paternity) and,

(ii) the number of trapped and genotyped $F_2$ grand-offspring (i.e., offspring of the $F_1$ offspring that survived to independence, at 12 – 15 weeks of age) they were assigned.

LRS analyses were conducted on two data sub-sets. The first dataset (Dataset E, cohorts 1988–1996) comprised individuals born between 1988 and 1996, as all individuals in these cohorts were dead by 2010 (N= 83; EGO: female = 14, male = 17 and WGO: female = 20, male = 32). The second dataset (Dataset E, cohorts 1988-2008) included the complete 1988–1996 cohorts and incomplete 1997–2008 cohorts, i.e., it also included badgers, from cohorts 1997–2008, that were considered to be dead by the end of 2010 (i.e., adults that were not trapped in the subsequent 2 years, and cubs not trapped after 1 year) but excluding longer-lived badgers from these cohorts, that were not dead by 2010 (N = 173; EGO: female = 32, male = 44 and WGO: female = 44, male = 53).

In addition to the fixed and random effects that were used in longevity analysis, I also included the number of other breeding members (males and females) residing at the social-group/year, which is known to affect LRS of cubs born into that group that year as a fixed effect (Dugdale et al., 2010). These were retained in all models (even if they were not significant across the restricted dataset). Dependent LRS measures were (-1)-transformed to meet Poisson assumptions. I analysed LRS for each sex separately.

All analyses for longevity, and reproductive success, were performed in R 2.13.2. I fitted a generalised linear mixed model (GLMM, using the *lmer* function with Laplace approximation of the maximum likelihood in the lme4 package, Bates & Sarkar, 2007) with a Poisson error structure and log-link function for the dependent variable. The significance of including each explanatory term in the model was assessed using a

likelihood ratio test (chi-square distribution) by comparing the two nested models (with and without the term under consideration). All estimates are given as mean ± SE.

## 5.4 Results

***First-year survival probability***

Maternal age had no effect on their offspring's first-year survival probability (Test 1, Table 5.1; Dataset B, restricted maternal age). Maternal age was therefore excluded from further analyses.

Models with the effect of litter-type ([I] WGL vs. ML; [II] EGL vs. ML) had no greater explanatory power than the basic model (QAICc: basic model vs. litter-type model = [I] 1868.41 vs. 1869.93; [II] 1340.39 vs. 1341.79) after including variables (a) to (e) (Test 2, Table 5.1; Dataset C, restricted complete litters). Thus, WGO born in WGL, or EGO born in EGL, had a similar probability of first-year survival as WGO born in ML, or EGO born in ML, respectively.

First-year survival probability was greater for WGO than for EGO (mean survival probability: WGO = 0.74 ± 0.14, EGO = 0.55 ± 0.18; Test 3, Table 5.1; Dataset A, complete dataset). This difference was not apparent when the smaller coccidial dataset was analysed (Table 5.1; Dataset $A_{coccid}$). EGO and WGO had similar coccidial loads (EGO = 1.09 ± 0.21, WGO = 0.94 ± 0.24; N = 60 EGO and 62 WGO; Mann-Whitney U test: $P$ = 0.306).

***Estimated longevity***

Badger longevity ranged from 1 to 14 years with a median of 5 years (restricted to badgers born in cohorts 1988–1996). Sex was a significant predictor of badger longevity; frequency distributions are given in Figure 5.1. Females, on average, survived almost one year longer than did males (female = 5.56 ± 0.39, male = 4.61 ± 0.30, Table 5.2). After controlling for sex, WGO survived around 1.3 years longer than did EGO (WGO = 6.14 ± 0.32, EGO = 4.84 ± 0.40; Table 5.2, Dataset D, complete longevity). There was no interactive effect between offspring-paternity type and sex on longevity.

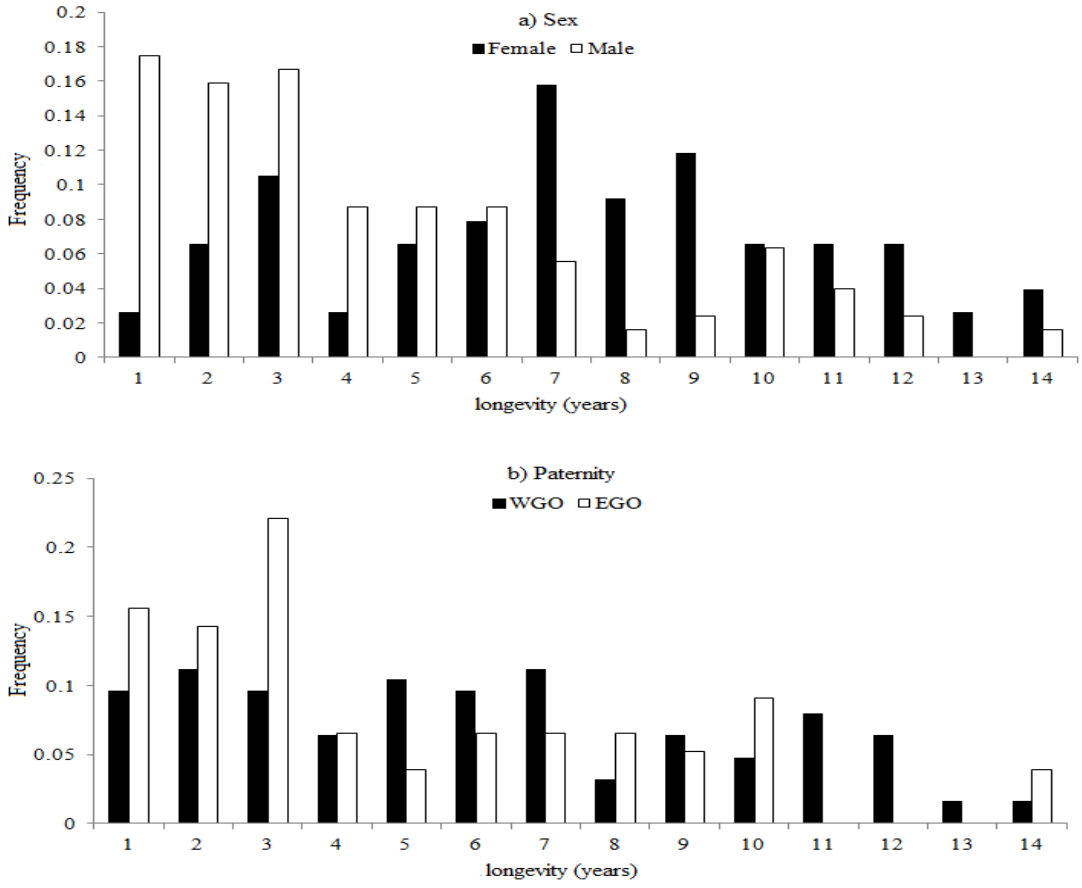


**Figure 5.1** Frequency distributions of longevity in relation to (a) sex (Female = 76, Male = 126) and (b) offspring-paternity type (WGO = 125, EGO = 77) [Dataset D, complete longevity].

**Table 5.1** Tests of the effects of maternal age (Test 1; Dataset B), litter type (Test 2; Dataset C) and offspring-paternity type (Test 3; Dataset A) on first-year survival probability with the inclusion of (a) the standardised total summer rainfall (May–October), (b) standardised mean winter temperature (November–February), (c) paternal multilocus heterozygosity (d) the interaction between paternal multilocus heterozygosity and standardised total summer rainfall and (e) coccidial load. Effect sizes were obtained from the full model.

| | **Effects** | **Without coccidial load** | | | **With coccidial load** | | |
|---|---|---|---|---|---|---|---|
| | | **β (SE)** | **$\chi^2_{[df = 1]}$** | ***P*** | **β (SE)** | **$\chi^2_{[df = 1]}$** | ***P*** |
| Test 1 | Maternal age | 0.01 (0.08) | 0.02 | 0.903 | 0.13 (0.30) | 0.20 | 0.659 |
| | (a) Summer rainfall | -0.47 (1.22) | | | -2.90 (1.88) | | |
| | (b) Winter temperature | 0.10 (0.20) | | | 10.68 (7.63) | | |
| | (c) Paternal heterozygosity | 1.40 (1.01) | | | -8.40 (9.50) | | |
| | (d) Interaction: Paternal heterozygosity*summer rainfall | 0.82 (1.14) | | | -3.69 (6.21) | | |
| | (e) Coccidial load | - | | | -0.27 (0.68) | | |
| Test 2 | Litter type [I] | 1.07 (2.07) | 0.51 | 0.475 | 18.05 (6920.66) | -0.43 | 0.511 |
| | (a) Summer rainfall | -1.40 (1.10) | | | 0.45 (5.10) | | |
| | (b) Winter temperature | 0.17 (0.23) | | | -0.47 (0.54) | | |
| | (c) Paternal heterozygosity | 0.81 (1.14) | | | -4.19 (4.21) | | |
| | (d) Interaction: Paternal heterozygosity*summer rainfall | 1.78 (1.03) | | | 0.44 (4.16) | | |
| | (e) Coccidial load | - | | | 0.44 (0.65) | | |
| | Litter type [II] | 0.75 (1.05) | 0.69 | 0.407 | -6.02 (5.39) | 3.22 | 0.073 |



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| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | (a) Summer rainfall | -0.52 (1.29) | | | 5.32 (6.77) | | |
| | (b) Winter temperature | 0.36 (0.21) | | | -2.36 (1.51) | | |
| | (c) Paternal heterozygosity | 0.69 (1.07) | | | -2.72 (7.72) | | |
| | (d) Interaction: Paternal heterozygosity*summer rainfall | 0.61 (1.23) | | | 0.41 (5.80) | | |
| | (e) Coccidial load | - | | | 0.06 (0.29) | | |
| Test 3 | Offspring-paternity type (WGO vs. EGO) | **-0.86 (0.25)** | **13.08** | ***< 0.001*** | -0.76 (0.76) | 1.05 | 0.306 |
| | (a) Summer rainfall | 0.33 (0.14) | | | -2.19 (0.94) | | |
| | (b) Winter temperature | -1.11 (0.75) | | | 6.67 (5.25) | | |
| | (c) Paternal heterozygosity | 0.75 (0.69) | | | -6.70 (6.24) | | |
| | (d) Interaction: Paternal heterozygosity*summer rainfall | 1.29 (0.71) | | | -1.31 (4.58) | | |
| | (e) Coccidial load | - | | | -0.03 (0.28) | | |

Bold values are significant at $P < 0.05$



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**Table 5.2** Results from a generalised linear mixed model of longevity in relation to sex, offspring-paternity type and their interaction (N = 202; dataset D, complete longevity).

| **Fixed effects** | **β (SE)** | $\chi^2_{[df = 1]}$ | ***P*** |
|---|---|---|---|
| Sex (male) | **-0.47 (0.07)** | **50.41** | **< 0.001** |
| Offspring-paternity type (EGO) | **-0.19 (0.08)** | **5.10** | **0.024** |
| Sex × Offspring-paternity type | -0.01 (0.14) | 0.01 | 0.932 |

Bold values are significant at $P < 0.05$. Fixed effects: sex (female = 0 and male = 1); offspring-paternity type (WGO = 0, EGO = 1). The random effects for birth year, natal group ID and mother ID were: 0.01 ± 0.09, 0.08 ± 0.28 and 0.03 ± 0.17, respectively.

***Lifetime reproductive success***

The proportion of $F_1$ offspring that went on to breed successfully themselves (i.e., with parentage assigned to at least one genotyped $F_2$ grand-offspring) was independent of whether these were EGO or WGO (females: 45.2% of EGO, 45.1% of WGO, $\chi^2_{[df=1]} = 0.02$, $P = 0.89$; males: 34.5% of EGO, 39.0% of WGO, $\chi^2_{[1]} = 0.44$, $P = 0.51$; Dataset E, cohorts 1988–1996). This was not affected by increasing the sample size (females: 40.5% of ego, 42.7% of wgo, $\chi^2_{[df=1]} = 0.02$, $p = 0.88$; males: 35.2% of ego, 39.3% of wgo, $\chi^2_{[1]} = 0.30$, $p = 0.58$; dataset f, cohorts 1988–2008).

Offspring-paternity type had sex dependent effects on LRS when analysed with a smaller, more complete dataset (Dataset E, cohorts 1988–1996; Table 5.3–5.4). When considering a large dataset that included individuals with incomplete life histories (Dataset F, cohorts 1988–2008), there was no significant relationship between LRS and offspring-paternity type, although the direction of the relationship was in the same direction (Table 5.3–5.4). It may be the larger, but incomplete, dataset includes more short-lived individuals that do not show sex effects; consequently this benefit differential effect is only seen among longer-lived individuals.

For females, lifetime number of litters ranged from 1 to 4, with a mean of 2.06 ± 0.18 and for males this ranged from 1 to 6, with a mean of 1.71 ± 0.17 (Dataset E, cohorts 1988-1996, Supplementary Figure S5.2; for Dataset F, cohorts 1988-2008, female: range = 1-6, mean = 2.05 ± 0.14, male: range = 1-6, mean = 1.69 ± 0.11). $F_1$ WGO females were assigned significantly more litters over their lifetime than did $F_1$ EGO (Dataset E, cohorts 1988-1996; Figure 5.2, Table 5.3). The greater lifetime iteroparity of $F_1$ WGO than EGO females was not the result of greater longevity, as these metrics did not correlate (Dataset E, cohorts 1988-1996, $r_s$ = 0.28, $P$ = 0.107; sexes combined). Offspring type did not, however, predict the lifetime number of litters for males (i.e., number of years they bred; see Table 5.4).

The lifetime number of $F_2$ offspring produced by females ranged from 1 to 8, with a mean of 2.85 ± 0.32, and for males from 1 to 13, with a mean of 2.73 ± 0.33 (Dataset E, cohorts 1988-1996, Supplementary Figure S5.3; for Dataset F, cohorts 1988-2008, female: range = 1–9, mean = 2.82 ± 0.23, male: range = 1–15, mean = 2.88 ± 0.25). The number of $F_2$ offspring produced by $F_1$ offspring correlated positively with the longevity (Dataset E, cohorts 1988-1996, $r_s$ = 0.35, $P < 0.001$; sexes combined). $F_1$ WGO females were assigned significantly more $F_2$ offspring over their lifetime than did $F_1$ EGO (Figure 5.3; Table 5.3). $F_1$ EGO males were assigned more $F_2$ offspring than were $F_1$ male WGO (Figure 5.3; Table 5.4).

**Table 5.3** Generalised Linear Mixed Model Results for the offspring-paternity type effect on LRS measured as (i) number of litters and (II) number of $F_2$ offspring using individuals from Dataset E-cohorts 1988–1996 and Dataset F-cohorts 1988–2008: Female Only

| LRS | Effect | Cohorts 1988–1996 EGO = 14 and WGO = 20 | | | | Cohort 1988–2008 EGO = 32 and WGO = 44 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Random effects | Fixed effects | | | Random effects | Fixed effects | | |
| | | Variance ± SD | β ± SE | $\chi^2_{[df = 1]}$ | *P* | Variance ± SD | β ± SE | $\chi^2_{[df = 1]}$ | *P* |
| Number of litters | Year | 0 ± 00 | | | | 0 ± 00 | | | |
| | Mother ID | 0 ± 00 | | | | 0 ± 00 | | | |
| | Natal group ID | 0 ± 00 | | | | 0 ± 00 | | | |
| | Intercept | | 0.88 ± 0.43 | | | | 0.03 ± 0.03 | | |
| | Number of other members | | -0.06 ± 0.04 | 2.56 | 0.110 | | -0.01 ± 0.02 | 0.08 | 0.773 |
| | Offspring-paternity type (EGO) | | **-1.08 ± 0.44** | **6.32** | **0.012** | | -0.25 ± 0.29 | 0.81 | 0.367 |
| Number of $F_2$ offspring | Year | 0 ± 00 | | | | 0 ± 00 | | | |
| | Mother ID | 0 ± 00 | | | | 0 ± 00 | | | |
| | Natal group ID | 0 ± 00 | | | | 0 ± 00 | | | |
| | Intercept | | 1.47 ± 0.40 | | | | 0.70 ± 0.28 | | |
| | Number of other members | | **-0.07 ± 0.04** | **4.26** | **0.039** | | -0.02 ± 0.02 | 1.08 | 0.300 |
| | Offspring-paternity type (EGO) | | **-1.02 ± 0.37** | **8.09** | **0.004** | | -0.35 ± 0.26 | 1.88 | 0.170 |

Significant effects at $P < 0.05$ are in bold. For fixed effect slopes, WPO = 0 and EPO = 1



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**Table 5.4** Generalised Linear Mixed Model Results for the offspring-paternity type effect on LRS measured as (i) number of litters and (II) number of $F_2$ offspring using individuals from Cohorts 1988–1996 (Dataset E) and Cohorts 1988–2008 (Dataset F): Male Only

| LRS | Effect | Cohorts 1988–1996 EGO = 17 and WGO = 32 | | | | Cohort 1988–2008 EGO = 44 and WGO = 53 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Random effects | Fixed effects | | | Random effects | Fixed effects | | |
| | | Variance ± SD | β ± SE | $\chi^2_{[df = 1]}$ | *P* | Variance ± SD | β ± SE | $\chi^2_{[df = 1]}$ | *P* |
| Number of litters | Year | 0.32 ± 0.57 | | | | 0.11 ± 0.33 | | | |
| | Mother ID | 0.46 ± 0.68 | | | | 0.57 ± 0.75 | | | |
| | Natal group ID | 0.35 ± 0.59 | | | | 0.05 ± 0.23 | | | |
| | Intercept | | -0.86 ± 0.60 | | | | -0.79 ± 0.37 | | |
| | Number of other members | | -0.05 ± 0.05 | 0.90 | 0.342 | | -0.02 ± 0.03 | 0.70 | 0.404 |
| | Offspring –paternity type (EGO) | | 1.00 ± 0.50 | 3.26 | 0.071 | | 0.46 ± 0.33 | 1.97 | 0.161 |
| Number of $F_2$ offspring | Year | 0.61 ± 0.78 | | | | 0.19 ± 0.43 | | | |
| | Mother ID | 0.11 ± 0.34 | | | | 0.70 ± 0.84 | | | |
| | Natal group ID | 0.27 ± 0.52 | | | | 0.07 ± 0.27 | | | |
| | Intercept | | 0.09 ± 0.46 | | | | -0.03 ± 0.31 | | |
| | Number of other members | | -0.04 ± 0.03 | 1.62 | 0.203 | | -0.01 ± 0.02 | 0.47 | 0.491 |
| | Offspring-paternity type (EGO) | | **1.22 ± 0.52** | **11.16** | **< 0.001** | | 0.49 ± 0.26 | 3.30 | 0.069 |

Significant effects at $P < 0.05$ are in bold. For fixed effect slopes, WPO = 0 and EPO = 1



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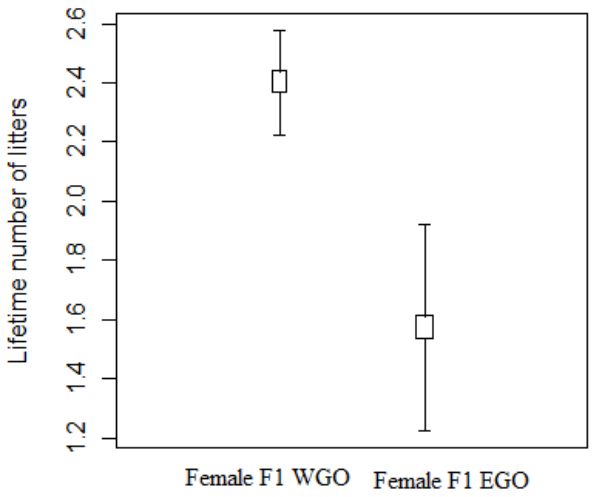


**Figure 5.2** The lifetime number of litters (uncorrected means and SE) for female WGO (N = 20) and EGO (N = 14). (Dataset E).

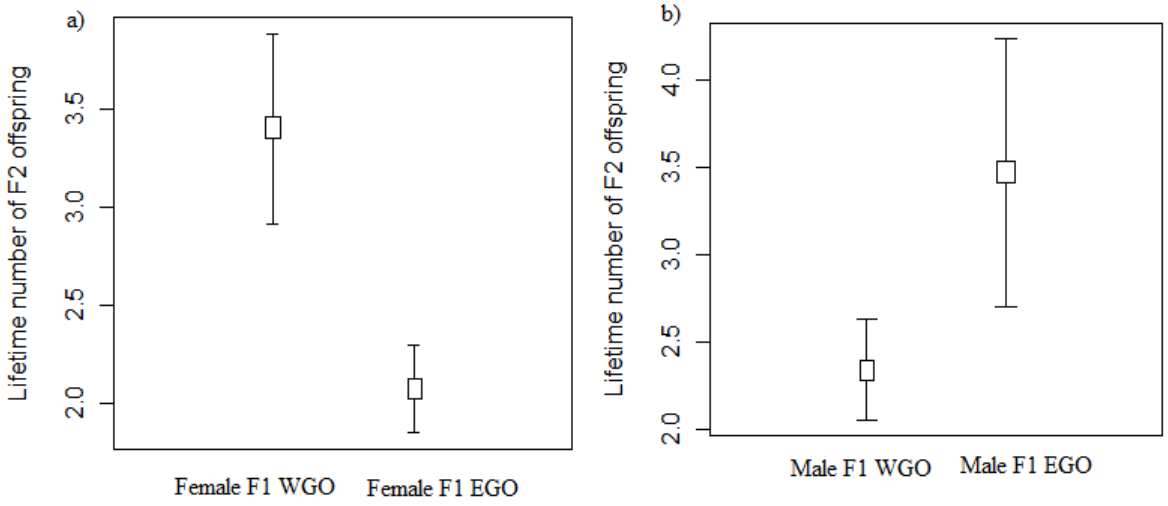


**Figure 5.3** The lifetime number of $F_2$ offspring (uncorrected means and SE) (a) produced by $F_1$ WGO females (N = 20) and $F_1$ EGO females (N = 14) and (b) sired by $F_1$ WGO males (N = 32) and $F_1$ EGO males (N = 17). (Dataset E)

Considering the percentage of each type of $F_2$ grand-offspring (whether $F_2$ EGO or $F_2$ WGO) sired by males, $F_1$ EGO sired 55.3 ± 1.03% of $F_2$ EGO and 44.7 ± 1.03% of $F_2$ WGO (1.96xSE, the confidence intervals did not overlap), whereas $F_1$ WGO sired 50.6 ± 6.45% of $F_2$ EGO and 49.4 ± 6.45% of $F_2$ WGO (Figure 5.4). This indicates that male EGOs may be able to increase their reproductive success through siring extra-group offspring, though these percentages of difference are marginal, given the limitations in my ability to sample the population.

For females, both $F_1$ EGO and $F_1$ WGO produced very slightly more $F_2$ EGO than $F_2$ WGO ($F_1$ EGO: 52.6 ± 7.28% $F_2$ EGO and 47.4 ± 7. 28 $F_2$ WGO; $F_1$ WGO: 51.8 ± 8.21% $F_2$ EGO and 48.2 ± 8.21% $F_2$ WGO) but their confidence intervals overlapped zero

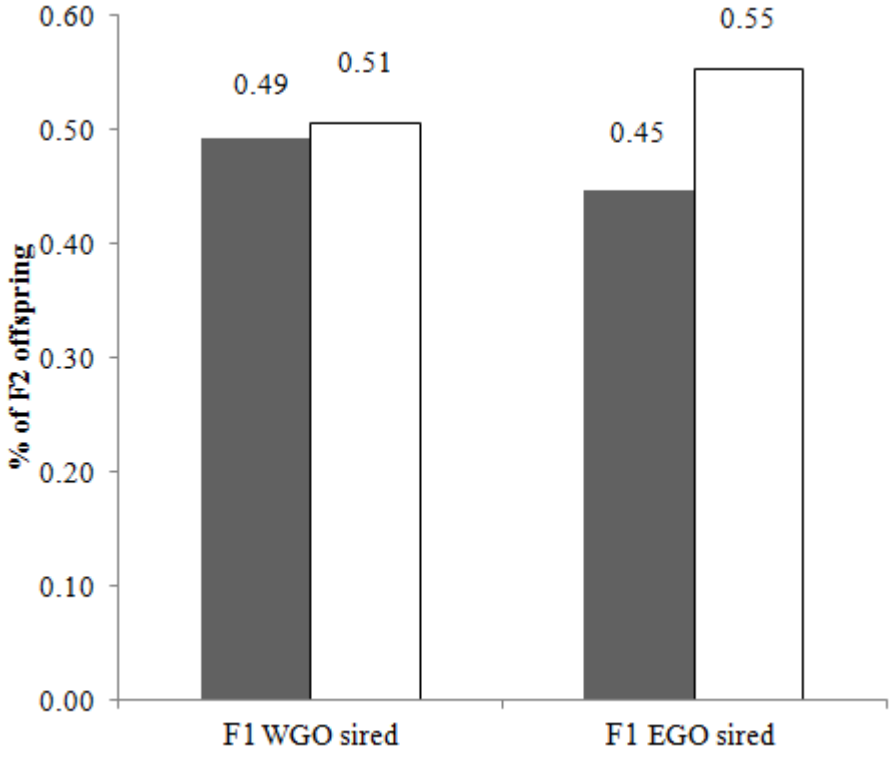


**Figure 5.4** Male extra-group and within-group siring successes. Black bars and white bars represent adult male $F_2$ WGO and $F_2$ EGO respectively.

## 5.5 Discussion

EGP is often associated with offspring fitness advantages and therefore in group-living species should be favoured under circumstances where this strategy produces offspring that exhibit higher fitness, in terms of survival or reproductive success than within-group offspring (e.g., Griffith et al., 2002; Gerlach et al., 2011; Sardell et al., 2011; but see Sardell et al., 2012). Here, there were mixed results: while there was no survival advantage for EGO, EGO exhibited ca. 34% lower survival probability through their first year than did WGO; these EGO on average lived 1.3 years shorter than EGO. There was some evidence for a sex-specific reproductive advantage of EGP in badgers. Female EGO exhibited lower lifetime reproductive success than WGO; however, male EGO exhibited higher lifetime reproductive success than male WGO.

To date, many studies have documented the importance of genetic quality on mate choice (Aparicio et al., 2001; Foerster et al., 2003; Masters et al., 2003; Seddon et al., 2004; Suter et al., 2007; Garcia-Navas et al., 2009); yet, few have exposed the type of mating I see here, where high rates of EGP occur, but with little evidence in support of offspring fitness benefits that would drive EGP (e.g., Sardell et al., 2011 & 2012).

### Survival advantage

For EGO neither first-year survival probability nor longevity was higher than for WGO. First-year mortality is known to occur in badger cubs largely as a consequence of parasitic infections (Newman et al., 2001) and due to stressful weather conditions e.g., low summer rainfall and temperature (i.e., thus low food availability; Macdonald et al., 2010, Table 5.1). In Chapter 3 established that the advantages of paternal heterozygosity in badgers are

apparent only under benign climatic conditions, whereas stressful weather patterns limit access to food (see also Macdonald et al., 2010) and cause indiscriminate mortality. I speculate that these effects could be so strong as to operate independently and indiscriminately of offspring-paternity type, where offspring-paternity type may have a discriminate influence on more subtle parameters than live/die, e.g., health / fitness parameters.

**Sex-specific benefits to EGP mating strategies**

Male $F_1$ EGO badgers had a higher lifetime reproductive success than male $F_1$ WGO (Figure 5.3). In contrast, female $F_1$ EGO had a lower LRS than female $F_1$ WGO. Sex-specific differences in LRS with respect to offspring-paternity type in badgers, support that females may gain an indirect fitness benefit through EGP derived through the greater reproductive success of EGO sons. This study presents the first evidence of sex-specific EGP benefit shown in a mammal species, where this benefit has been reported for birds (e.g., Gerlach et al., 2011).

Forstmeier et al. (2011) point out that within-sex genetic constraint may drive selection for the same alleles in females, as in males, to promote EGP, where female resistance to extra-group males would also confer resistance to within-group males, leading to reduced reproductive success. Therefore, EGP, which can potentially increase the reproductive output of a male per year (i.e., females mated with), may simultaneously result in females pursuing (or being amenable to) a mating strategy that is disadvantageous to their female EGO. My data do support this level of EGP benefit: male F1 EGO did sire more F2 offspring than did male WGO, in comparison; female F1 WGO produced more F2 offspring than did female F1 EGO (Figure 5.3).

Sex-specific, differential fitness-related attributes of EGO versus WGO could potentially reflect a sexually antagonistic effect, such that genes (e.g., good genes or compatible genes) obtained through EGP may be beneficial (i.e., increased reproductive success) when expressed in males, but detrimental when expressed in females. The opposing LRS effects for EGO and WGO shown in males and females in this population, suggest that sexually antagonistic selection may potentially play an important role in the maintenance of EGP in badgers, if the fitness benefits of producing extra-group sons outweigh the costs of producing extra-group daughters (Kokko, 2001).

$F_1$ EGO male badgers sired more $F_2$ EGO than F2 WGO (Figure 5.4). The tendency for $F_1$ EGO male to then sire F2 EGO has several possible explanations that are not mutually exclusive. It has been reported previously that a female's propensity to produce extra-group offspring can be heritable, i.e., the Baldwin effect (Baldwin & Mark, 1896; e.g., Reid et al., 2011). Alternatively, male $F_1$ EGO badger could simply be more attractive as mating partners, i.e., have phenotypic attributes that females can detect as cues favouring a greater production of descendents (i.e., 'Sexy-son Hypothesis', Fisher 1930) or produce more competitive sperm than F1 WGO. In that extra-group matings require females to be receptive and complicit, females may increase their indirect reproductive success by choosing sexually attractive mates, if her sons can inherit their father's attractiveness. Moreover, between-sex genetic constraints (Forstmeier et al., 2011) would lead females that inherited alleles coding for extra-group mating from their fathers to pass these to their offspring, where there would plausibly be a grandson advantage (a 'Promiscuous-grandson Hypothesis', similar to the 'Sexy-son Hypothesis' in Fisher's runaway sexual selection; Fisher, 1930; Weatherhead and Robertson, 1979).

**Do WGO perform better than EGO?**

While I find little evidence to suggest that EGP is adaptive, female badgers in this study population derive greater lifetime reproductive success if they produce more WGO than EGO, especially female WGO, because $F_1$ WGO had better survival (first-year survival probability and longevity) than $F_1$ EGO; also $F_1$ WGO females produced a greater number of litters, and hence offspring, than $F_1$ EGO females.

Understanding mate choice, where individuals appear to express strong preferences despite detecting no/little direct benefits from mate choice, remains a challenge to behavioural ecology (Ryder et al., 2010). Indeed, studies such as Forstmeier et al., (2011) have rationalised a hypothetical framework through which within- and between- sex genetic correlations can drive female promiscuity. This can arise due to plieotropic effects (Forstmeier et al., 2011), despite antagonistic selection whereby males may be selected to be promiscuous whereas females may not be selected for this trait directly, if promiscuity does not increase their reproductive output. My previous research (Chapter 4) has found no clear advantages to EGP in terms of mate selection, aside from weak evidence of mating to avoid pair-wise genetic similarity. Against this background, support for a genetic basis to the high rates of EGP I observed in this badger population could come from benefits in terms of offspring fitness. There is a paradox, that while EGP rates of 48% are evident (Chapter 4; see also Dugdale et al., 2007), $F_1$ WGO performed better in survival and reproduction than did $F_1$ EGO, with the exception that $F_1$ EGO male outperformed $F_1$ WGO male in the number of $F_2$ grand-offspring sired.

This leads to 4, not mutually exclusive, possibilities, where, EGP matings would serve a different or additional function in badger society;

*i) 'Superior natal territory fit Hypothesis'*

There are circumstances where within-group matings would be advantageous: WGO may carry genes that make them a good, or better, fit in their natal territory, with a better chance of breeding successfully (female) and surviving to an older age compared to EGO. WGO may also be more likely to inherit territory / group status from their mothers (Lindstrom, 1986), giving them an advantage over EGO in terms of accessing resources for survival and reproduction. Rather than facilitating genetic quality, EGP may serve to alleviate conflict through inter-social group appeasement and inter-group genetic relationships (see Macdonald et al., 2004a; Delahay et al., 2006).

*ii) 'Uncontrolled / unconstrained mating Hypothesis'*

Isvaran & Clutton-Brock, (2007) report that rates of EGP decline with increasing length of the mating season, which suggests that it is increasingly difficult for males to monopolise their social mates during short mating seasons and when there is high overlap of female oestrus periods. They also found that males are less able to monopolise individual females when there is a higher number of breeding females in a group.

EGP may be accidental, as the unavoidable consequence in badgers of WG-males being unable to monopolise group females through their oestrus period, in a system where inter-group transgression occurs frequently (Macdonald et al., 2008) and foraging grounds may be common between groups (Macdonald et al., 2004a). Female cub numbers are generally equivalent to male cub numbers at the population level in my study population (Dugdale et al., 2003). These females sometimes require repeated intromission in order to ovulate (Ahnlund, 1980), but they do also experience continuing oestrous cycles (Service et al., 2002), that continue during embryonic diapause, i.e., female badgers are capable of superfoetation (conception during pregnancy) (Yamaguchi et al., 2006).

*iii) 'EGO sociological disadvantage Hypothesis'*

A further possibility is that, even if EGO were to exhibit genetic quality advantages, these may be countermanded by the sociological disadvantages of having an extra-group father. Infanticide is known to occur in badger society (Lups & Roper 1990; Cresswell et al., 1992; see also Wolf & Macdonald, 2004). Polygynandrous mating is believed to have evolved in high-density badger populations in an attempt to confuse paternity (Carpenter et al., 2005; Dugdale et al., 2007; Dugdale et al., 2011a). If males are unable to identify their own young, females may mate with males to confuse paternity while applying cryptic female choice to select against certain males after mating. Alternatively, should males be able to recognise that offspring are not their own, or either sex recognise that offspring were not fathered from within their group (see Packer & Pusey, 1984; Lion & van Baalen, 2007), for example through MHC / scent (Sin et al., 2012; Buesching et al*.,* 2003), they may differentially kill EGO over WGO (see Dunn et al., 2012). EGO exhibited 34% poorer first-year survival than did WGO and on average live 1.3 year less; however there is no evidence that group members can detect that they are EGO.

*iv) 'Inbreeding avoidance Hypothesis'*

EGP may reduce inbreeding. When the average within-group candidate parents' relatedness was greater, the number of EGO (absolute) and the proportion of EGO (relative) were higher (Chapter 4). To show that EGP reduces inbreeding requires randomisations evidencing that a reduction in $f$ occurs, rather than that neighbouring individuals are less related than same-group individuals. In high-density populations, as per this study, permanent dispersals are infrequent (Macdonald et al., 2008) and thus EGP (deliberate or accidental) may function to facilitate gene flow between groups (Pope et al., 2006).

## 5.6 Conclusions

The advantages and ecological role of promiscuity depend upon perspective in badgers. It may not inform mating choices based on heterozygosity, i.e., genetic quality (Chapter 4), nor does it confer advantages to female extra-group offspring, although it does to male EGO. This leads me to posit that rather than being under positive selection, this mating strategy is under opposing selection (Foerster et al., 2007), depending on the sex of the resulting offspring. EGP may also occur as an inevitable consequence of the badger's social system at high density, whereas this species predominantly exhibits much smaller group sizes, or mating pairs over the majority of its European continental range (see Newman et al. 2011).

Investigating the evolutionary biology of extra-group mating strategies thus requires a comprehensive theoretical framework, more extensive than simply testing for heterozygosity-fitness advantages, where genetic advantages must be weighed against the implied concept of freedom of choice and sociological corollaries of producing young with expatric fathers.

## 5.7 Supplementary materials

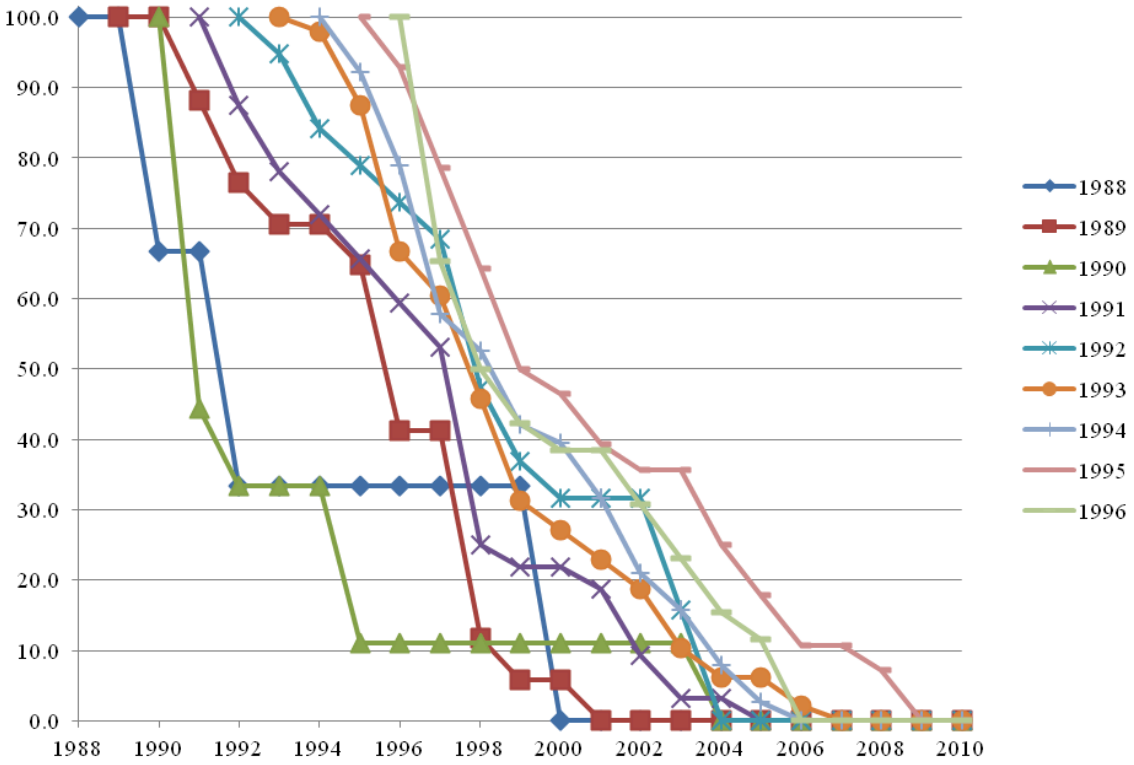


**Figure S5.1** The percentage of badgers recaptured over the period of 1988-2010 for those individuals that survived to breeding age from cohorts 1988 to 1996 (N = 202).

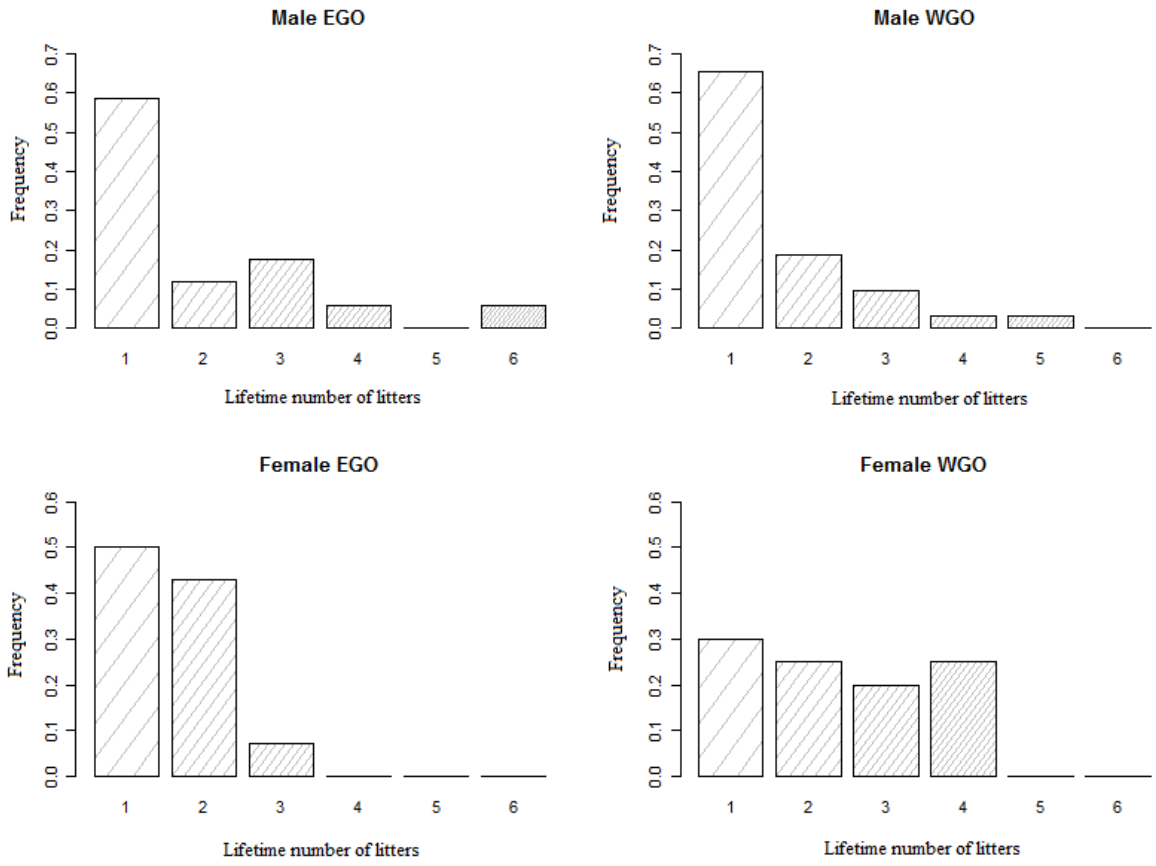


**Figure S5.2** Frequency of the lifetime number of litters in relation to offspring-paternity type for male only and female only. The raw data are based on $F_1$ offspring born between 1988 and 1996 (EGO: female = 14, male = 17 and WGO: female = 20, male = 32).

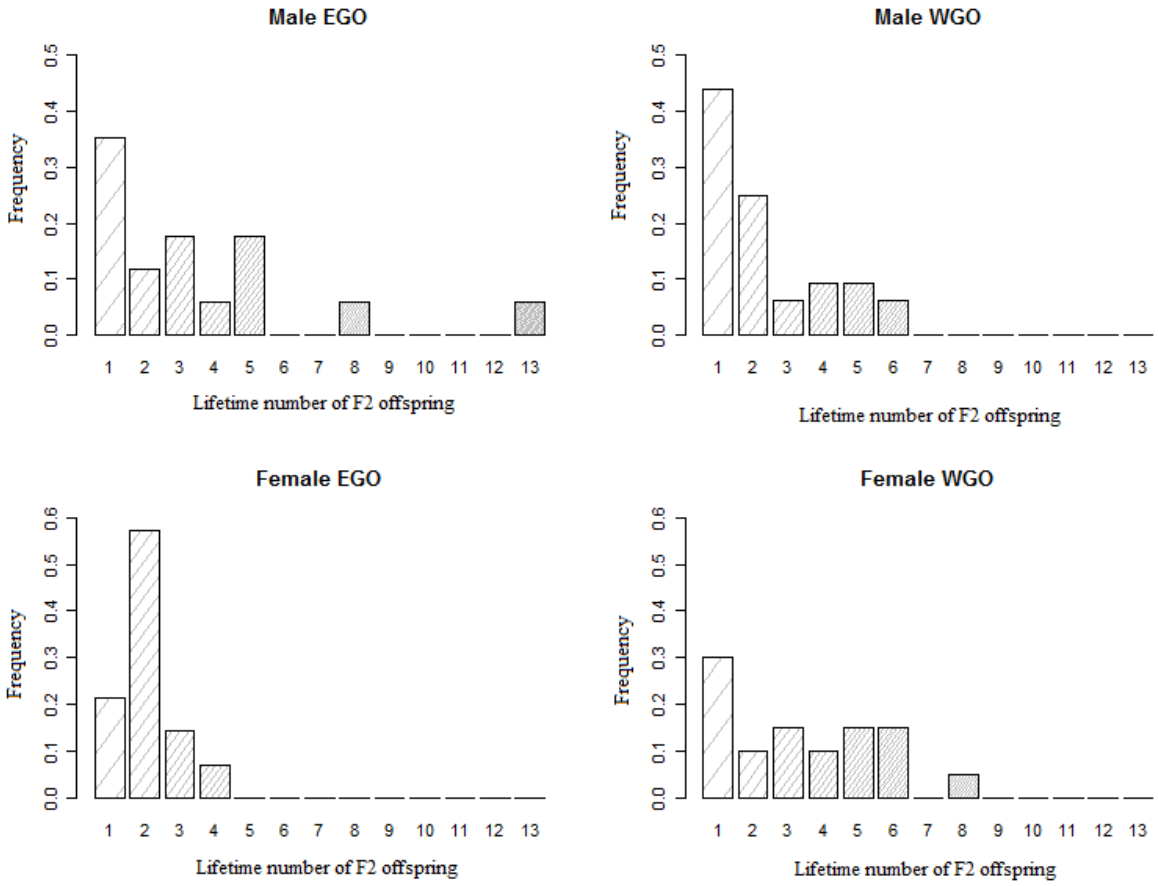


**Figure S5.3** Frequency of the lifetime number of $F_2$ offspring in relation to offspring-paternity type for both sexes combined together, male only and female only. Data are based on $F_1$ offspring born between 1988 and 1996 (EGO: female = 14, male = 17 and WGO: female =20, male = 32

# Chapter 6:

# Causes and consequences of extra-group paternity: a synthesis

## 6.1 Introduction

Darwin's theory of natural selection states that behavioural, morphological, and physiological traits are selected in a way that they maximise the survival and/or reproductive success of individuals (Darwin, 1859; Williams, 1966; Endler, 1986). The molecular detection of extra-group/extra-pair mating (i.e., mating outside of their social mate bonds), although paradoxical, is a widespread phenomenon, which has changed the understanding of the mating system and sexual selection radically (Griffith, et al., 2002; Westneat & Stewart, 2003; Isvaran & Clutton-Brock, 2007; Reid, 2012). Extra-group/extra-pair mating can incur costs (e.g. through reduced paternal care or sexual disease transmission), thus why individuals engage in this mating strategy is often unclear and this has puzzled evolutionary biologists for decades (e.g., Daly, 1976; Westneant & Stewart, 2003).

My thesis aimed to contribute to the understanding of the driving factors and adaptive benefits of extra-group paternity (EGP), in a high-density group-living badger population. I examine the effects of socio-ecological conditions that may promote extra-group matings in badger. I show that EGPs are associated with sex-specific fitness benefits and may therefore be selected/ maintained through sex-specific antagonistic effects.

## 6.2. The evolution of extra-group paternity (EGP) in European badger

In group-living species, it is straightforward to understand how extra-group mating strategy (resulting in extra-group paternity, EGP) can be adaptive for males (Sheldon, 1994; Whittingham & Dunn, 1998) as this may increase the reproductive success of males (i.e., siring additional offspring outside of their social group); the adaptive advantages of EGP

however, are less apparent for females and these have been debated in the literature (see Jennions & Petrie, 2000; Arnqvist & Kirkpatrick, 2005; Akçay & Roughgarden, 2007; Griffith, 2007). The over-arching conclusion as to what benefits females that mate with extra-group males may derive is through indirect genetic benefits via increased offspring's genetic quality (Jennions & Petrie, 2000; Griffith et al., 2002).

In the European badger, in a high-density population in lowland UK, I observed a polygynandrous genetic mating system (Dugdale et al., 2007 & 2011a; Chapter 4), where approximately 48% of cubs were sired by extra-group males (Chapter 4), primarily from neighbouring groups, with 27% of litters having multiple-paternity (i.e., litters consisting of offspring fathered by more than one male, Chapter 4). Extra-group paternity rates were extremely variable, ranging from 33–100%, but were higher when a greater number of neighbouring males were present relative to the number of within-group males (Chapter 4). Unusually, among mammals, in badgers both sexes exhibit natal philopatry (Woodroffe et al., 1995), females are thus more related to within-group males than to neighbouring-group males (Dugdale et al., 2008). Temporary group movements do occur at relatively high rates in both sexes (Macdonald et al., 2008), but their function has hitherto been unresolved. Females, therefore, may potentially gain indirect fitness benefits through mating with less-related extra-group males facilitated through temporary movements (of either sex). Furthermore, cubs that have heterozygous fathers have increased survival in good years, in line with the 'Good-genes-as-heterozygosity Hypothesis' (Chapter 3). There are also sex-dependent fitness benefits to extra-group male but not extra-group female offspring (Chapter 5). These findings enable us to consider key questions in the evolution of promiscuity:

***Do females seek for more heterozygous extra-group males?***

Individuals may benefit by choosing more heterozygous mates ('Good-gene-as-heterozygosity Hypothesis', Brown, 1997) that will maximise (or optimise) the heterozygosity of their offspring. Such benefit would arise if individual heterozygosity correlates positively with fitness under circumstances that are often found in the natural populations. When such heterozygosity-fitness correlation (HFC) exists, females are predicted to seek EGPs if mating with within-group mates produces less heterozygous offspring than mating with extra-group mates.

Badger cubs that had fathers with high levels of heterozygosity had greater first-year survival probability in wet years compared to cubs that were fathered by males with low level of heterozygosity (Chapter 3). Despite this paternal heterozygosity-fitness correlation, neither the average nor the maximum of within-group heterozygosity of candidate fathers were affected by the number/proportion of offspring fathered by extra-group males within a litter (Chapter 4).

These findings lead me to propose that EGP in badgers did not evolve in order to seek out mates that have higher levels of heterozygosity. Further investigation, however, is required, for example to identify why females may breed with both within-group and extra-group males, producing a mixed paternity litter, containing both EGO and WGO. Testing whether these within-group males are less heterozygous than extra-group males is necessary in order to reject 'Good-gene-as-heterozygosity Hypothesis'. The small number of females with litters containing both WGO and EGO (N =10) in this study did not permit this.

***Does EGP mitigate inbreeding in badgers?***

EGP has been hypothesised to have evolved to avoid inbreeding in mammals (e.g., Dugdale, 2007; Sillero-Zubiri et al., 1996), where inbreeding depression is demonstrably costly (Moore & Ali, 1984; Pusey & Wolf, 1996). Badger groups consist of closely related individuals (Dugdale et al., 2008), thus the potential for inbreeding is very high. I observed that as the average pair-wise relatedness between mothers and within-group candidate fathers increases, the number/proportion of EGPs within a litter increased (Chapter 4), suggesting that inbreeding may be costly and therefore badgers may adopt an extra-group mating strategy to reduce inbreeding.

Inbreeding has been reported to incur reproductive costs in badgers (Dugdale, 2007). Inbred badgers have been found to have a lower lifetime breeding success than non-inbred badger (Dugdale, 2007). In terms of first-year survival probability, an individual's inbreeding coefficient was not influential (Chapter 3). A number of studies have reported that, within a species, inbreeding depression can be detected in some fitness-related traits, but not in others, and sometimes become more pronounced under stressful conditions (e.g., Lacy & Horner, 1997; Fox et al., 2010; Valtonen et al., 2011). Inbreeding is thus potentially costly to badgers in terms of their reproduction success, but not with regard to first-year survival probability.

***How do the benefits of EGP and paternal heterozygosity relate to environmental conditions?***

The effects of covariates such as parasitic infections (Newman et al., 2001) or stressful weather conditions e.g., low summer rainfall and temperature (i.e., low food availability; Macdonald et al., 2010, Chapter 3) may also affect the relative costs and benefits of EGP

(and heterozygosity). I observed that under relatively benign conditions, subtle advantages could be effective on survival, whereas in relatively harsh years mortality was indiscriminate (i.e., no extent of paternal genetic advantage will allow an animal to survive without food, ultimately). This demonstrates that it is important to analyse HFCs under a range of environmental conditions to ensure that the full picture is detected.

***Do extra-group offspring (EGO) perform better than within-group offspring (WGO)?***

Females may obtain fitness benefits if EGO performs better in their survival and reproduction than WGO. Here the results are mixed. I demonstrate that EGO had ca. 34% lower first-year survival probability than did WGO, and on average EGO lived 1.3 years less than WGO (Chapter 5). Female EGO exhibited lower lifetime reproductive success than female WGO (Chapter 5). There are circumstances where WGO are fitter than EGO. WGO may carry genes that make them a good, or better, fit in their natal territory, giving them an advantage over EGO in terms of accessing local resources for survival and reproduction. WGO may also be less vulnerable to infanticide by within-group males compared to EGO if males able to recognise EGO, for example through MHC / scent (Buesching et al., 2003; Sin et al., 2012). However, male EGO produced more offspring than did male WGO. Consequently, if sons inherit their father's traits, females may increase their reproductive success by choosing males which will ultimately produce more descendents, where females can detect these 'sexy' phenotypic traits, through cues (i.e., Sexy-son Hypothesis, Fisher, 1930); or under circumstances where these males produce more competitive sperm (Jennions & Petrie, 2000).

These sex-specific, differential fitness-related attributes of EGO versus WGO could potentially result from an antagonistic sex-effect, such that genes (e.g. good genes or

compatible genes) arising through EGP may be beneficial (i.e., increased reproductive success) when expressed in males, but detrimental when expressed in females. Based on the opposing LRS effects for EGO and WGO shown by males and females in this population, I propose that this antagonistic selection may play an important role in the maintenance of EGP in badgers; provided the fitness benefits of producing extra-group sons outweighs the costs of producing extra-group daughters (Kokko, 2001).

***Socio-ecological correlates of EGP***

The spatio-temporal distribution of extra-group mating opportunities is an important factor affecting the intensity of sexual selection (Shuster & Wade, 2003). Here EGP correlated negatively with the number of within-group candidate fathers, and positively with the number of neighbouring-group candidate fathers (Chapter 4). While it is possible (but somewhat implausible, given the inter-group overlap in foraging ranges and high rates of inter-group trespass in this species) that a higher number of within-group males may deter intrusions by extra-group males it is also equally plausible that this is a simple density-dependent effect, where females mate *ad libitum*, but that lower within-group male presence militates for more matings with extra-group males.

EGP may indeed be accidental in badgers, as the unavoidable consequence of within-group males being unable to monopolise group females through their oestrus period. Even without inferring active male defence of females (anti-kleptogamy, Revilla & Palomares, 1999), simply by proportion, a larger number of males in a group has the potential to decrease EGP rates (Spong et al., 2008). Since female badgers forage solitarily and are known to transgress and make incursions into neighbouring group ranges (Bohm et al., 2009; occasionally being caught temporarily in other groups, Macdonald et al., 2008), the

capacity for within-group males to actively control female access to extra-group males will be limited; if females mate indiscriminately (polygynandry, Dugdale et al., 2007) then simple extra-group male encounter rate could be influential.

A further consideration is that EGP may confer fundamental sociological advantages to the mating generation, such as if females are induce ovulators (Yamaguchi et al., 2006), or confusing paternity both within the home group and within the neighbouring group, to safeguard against non-paternal male infanticide (Lups & Roper, 1990; Cresswell et al., 1992; Agrell et al., 1998) in this polgynadrous system.

**6.3 General conclusions**

Survival and fecundity are the two components of fitness. Any variation in behavioural, morphological, and physiological traits that influence fitness also has the potential to influence the population growth rate as well as the genetic composition of the extant population (Nussey et al., 2007). The behavioural patterns that determine the dynamics of mating systems and in turn the compositions of social groups and populations are fundamental to socio-ecology (Rubenstein & Kealey, 2010) and lay the foundations of conservation biology.

Mustelid species consistently, but not universally, exhibit intra-sexual territoriality, where individuals maintain territories only with respect to members of the same sex (Erlinge & Sandell, 1986; Yamaguchi & Macdonald, 2003), with male spacing affected by female spacing, and access to mates, as well as by the distribution of available food resources (Helldin, 1999; Zalewski, 2007). In accord with its phylogenetic heritage, Meles fundamentally exhibits life-history and ecological similarities with other mustelids, and in

low density populations in continental Europe exhibits similar mating systems (see Revilla & Palomares, 2002; for a full review consult Roper, 2010).

That the European badger exhibits such a different mating system when group-living at high-density, as seen in this study, presents a puzzle – clearly the evolved stability of the mating strategy in this species (Maynard-Smith, 1974) is adaptable, and we are observing a response to alter socio-spatial dynamics. Under low density conditions, finding a mate, any mate, may have been difficult. In such, ecological circumstances mate choice (female choice) would have been at best restricted to the territorial male that over-lapped her smaller range. We posit, therefore, that selection would not have acted to make females, 'choosey', but rather would have favoured liberal mate choice and promiscuity (Schwartz et al., 1999; Pryke et al., 2010). Crammed into more recent, high-density, group-living ecological circumstances, and possibly equipped with a tendency not to be highly selective of mating partners, translates into high levels of fairly indiscriminate promiscuity in badgers. This mating strategy, under these circumstances may be anachronistic and maladaptive, and interestingly we observed that while 71% of individuals engaged in promiscuous EGP, there was a significant percentage (29%) that were never observed to be promiscuous and only ever bred within-group.

In this sense, these badgers in the Wytham study population may provide an opportunity to observe how mating systems evolve in mammalian societies, where the advantages of EGP may continue to shift should this species evolve further co-operative behaviours as it adapts to ecological circumstances fundamentally changed from those experienced by its (recent) ancestors.

## 6.4 Future directions for studies on extra-group paternity

The development in new molecular technologies and analytical tools for assigning paternity accurately and relatedness estimates among breeding pairs will allow for more detailed studies into the patterns and mechanisms of extra pair paternity, and help to resolve many unanswered questions as to why females or males, to some extent, select extra-group mates. Here I mention briefly some specific lines for future enquiry;

Which sex solicits EGPs?

- It is currently debated whether extra-group copulations are primarily driven by the interests of males or females, and which sex solicits and controls the extra-group mate choice. Female solicitation of extra-group copulations (EGCs) is documented in birds (e.g., Schwartz et al., 1999; Pryke et al., 2010) and in some mammals (see Wolff & Macdonald, 2004). It is unknown in badgers which sex solicits extra-group mating given that both male and female badgers have means, motive and opportunity, that is, they are both solitarily foragers (Carr & Macdonald, 1986; da Silva et al., 1993), and are both known to make temporary extra-territorial excursions (Huck et al., 2008; Macdonald et al., 2008; see also Stewart et al., 1997), where they visit neighbouring groups (Macdonald et al., 2008). On-going detailed and high-resolution tracking, using RFID tags, is helping to resolve this question (Dyo et al., 2012), as camera trapping and video surveillance (Stewart et al., 1997) requires too much man power to elucidate the behavioural component of which sex is choosy and solicits matings.

What other indirect genetic benefits may there be to EGPs?

- Further investigation is required into other indirect genetic hypotheses that may explain the high-rates of EGP in badgers. The ‘Genetic diversity within litters’

Hypothesis (Williams, 1975; Yasui, 1998) predicts that females may increase the genetic diversity within a single litter of cubs through extra-group matings and mixed paternity strategy, as documented in badgers (Carpenter et al., 2005; Dugdale et al., 2007; Chapter 4). A larger sample size of litters with both WGO and EGO will facilitate this line of enquiry in badgers.

Environmental dependency of the benefits of EGPs

- Spatial and temporal variation in (seasonal) food availability and climatic conditions (Macdonald et al., 2010) are likely to influence the rate and benefits of extra-group paternity (Cameron et al., 2011), where we have already noted that the benefits of paternal heterozygosity are only expressed in benign years; mortality being indiscriminate in harsh years.

The trait based mechanism of mate-choice

- The relationship between extra-pair paternity and male behavioural and or/ morphological traits has been studied in many birds (Westneat & Stewart, 2003). For mammals it still remains to be fully established whether there any difference in behavioural or morphological traits between extra-group and within-group fathers. This will be necessary in order to understand how male morphological traits/secondary sexual characters relate to mate choice and in determining variation in EGP in badgers.

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# Appendix 1:

# Pathogen burden, co-infection and major histocompatibility complex variability in the European badger (*Meles meles*)

[1] A modified version of this appendix is currently being prepared as: Sin, Y.W., Annavi, G., Dugdale, H.L., Newman, C., Macdonald, D.W. & Burke, T. Pathogen burden, co-infection and major histocompatibility complex variability in the European badger (*Meles meles*).

## A1.1 Abstract

The genes of the major histocompatibility complex (MHC) are involved in host-pathogen co-evolution. It is generally believed that pathogen-mediated selection maintains the extreme diversity in MHC genes through a heterozygous advantage, rare-allele advantage or fluctuating selection. The advantage of heterozygotes being able to recognize and bind a wider range of antigens may only be realized when multiple pathogens are considered simultaneously. Therefore, we investigated the prevalence (infected or not), infection intensity and co-infection status of 13 pathogens, and examined their relationships to MHC constitution in a wild population of European badgers (*Meles meles*). MHC heterozygosity was not correlated with co-infection status. In contrast, there were individual MHC alleles that conferred resistance or susceptibility to trypanosomes presence, Mustelid herpes virus infection intensity, *Yersinia* presence and intensity, and *Campylobacter* intensity. Our results thus indicate rare-allele advantage and/or fluctuating selection, but not heterozygote advantage, to be the selective force shaping the MHC diversity in this species.

## A1.2 Introduction

The MHC is a diverse gene family that plays a crucial role in the adaptive immune system (Hedrick, 1994). MHC genes encode cell surface glycoproteins that are vital in both humoral and cell-mediated immune responses, as MHC molecules bind and present antigens to T cells and trigger an immune cascade (Swain, 1983). Because of this critical role in the immune system, MHC genes are under constant selective pressures due to challenges from parasites and pathogens (hereafter, both are referred to as "pathogens") (Jeffrey & Bangham, 2000; Piertney & Oliver, 2006). It is generally believed that balancing and diversifying selection, through an arms race between pathogens and hosts, maintains the extreme diversity in MHC genes. This pathogen-mediated selection is proposed to operate through a heterozygote advantage, rare-allele advantage and fluctuating selection (Spurgin & Richardson, 2010).

The rare-allele advantage hypothesis proposed that rare alleles within the population are likely to offer greater protection to pathogens than common alleles, and so have a selective advantage (Takahata & Nei, 1990; Slade & McCallum, 1992). Through a rare-allele advantage, specific pathogens drive a cyclic change in the frequency of MHC alleles, known as negative frequency-dependent selection. A heterozygote advantage (Doherty & Zinkernagel, 1975; Penn *et al.*, 2002) presumes that MHC heterozygous individuals are able to recognize and bind more antigens than homozygous individuals, and therefore trigger immune responses against a wider variety of pathogens. We use "heterozygote advantage" in the broad sense, where heterozygotes on average exhibit a fitness advantage over homozygotes (Penn *et al.*, 2002) due to dominance or overdominance. The fluctuating selection hypothesis proposes that the spatial and temporal fluctuation in pathogen types and abundance may maintain the diversity at the MHC (Hedrick, 2002; Spurgin & Richardson, 2010). The pathogen fluctuation may lead to the intensity of directional selection at MHC genes to fluctuate, hence different MHC alleles are selected for at different time or spatial point.

The majority of studies of MHC-pathogen relationships have reported associations between specific resistant/susceptible MHC alleles and pathogens, e.g. in fish (Langefors *et al.*, 2001), mammals (Harf & Sommer, 2005; Meyer-lucht & Sommer, 2005) and birds (Bonneaud *et al.*, 2006; Loiseau *et al.*, 2011), while a heterozygote advantage has limited supported (but see Thursz *et al.*, 1997; Penn *et al.*, 2002; Froeschke & Sommer, 2005). The association between specific alleles and pathogens could be due to frequency-dependent selection (Takahata & Nei, 1990); however, there may also be a frequency-dependent component to a heterozygote advantage (Apanius *et al.*, 1997). Both rare-allele and heterozygote advantages favour rare alleles and also maintain large numbers of alleles, making it difficult to distinguish between them. The advantage of being able to recognize and bind a wider range of antigens may only be realized when multiple pathogens are considered simultaneously (Hughes & Nei, 1992; McClelland *et al.*, 2003). MHC heterozygotes may have a lower number of co-infecting pathogens than homozygotes, rather than it being due to rare alleles (McClelland *et al.*, 2003). Furthermore, the limited reported evidence of a heterozygosity advantage may be due to studies analysing few pathogens and therefore not examining the overall selection pressure to which animals are exposed. Hence the relationship between the MHC and pathogens can only be tested properly by examining multiple pathogens.

The relationship between the MHC and pathogens may be context-dependent and only emerge under particular environmental conditions (Bernatchez & Landry, 2003; Sommer, 2005; Piertney & Oliver, 2006). Therefore it is more evolutionarily meaningful to examine the association between MHC and pathogens in natural populations. Here we examine the associations between MHC class I and class II gene variation and multiple pathogens in a wild population of European badgers (*Meles meles*). Because of the analytical complications that arise from high allelic diversity (Richardson *et al.*, 2005), a population with a relatively low number of MHC alleles offers great potential for identifying the ecological consequences underlying MHC variation (Oliver *et al.*, 2009). Only one DRB locus is polymorphic with three putatively functional sequences (Sin *et al.*, 2012a; Sin *et al.* in prep), while seven putatively functional MHC class I sequences were also identified in this badger population (Sin *et al.*, 2012b). It thus provides an ideal opportunity to

study MHC-pathogen relationships, as it does not have a high MHC diversity. Additionally, European badgers are infected by a wide range of pathogens, e.g. virus [mustelid herpes virus (MHV); (King *et al.*, 2004)], bacteria [*Salmonella*; (Wilson *et al.*, 2003)], protozoa [*Trypanosoma pestanai* (Macdonald *et al.*, 1999); *Eimeria melis* and *Isospora melis* (Newman *et al.*, 2001)], helminths (*Capellaria*, *Strongyloides*, other nematodes), and invertebrate ectoparasites [badger fleas (*Paraceras melis*), badger lice (*Trichodectes melis*) and ticks (*Ixodes hexagonus*); (San, 2007)]. Studying such a range of pathogens facilitates a comprehensive understanding of MHC-pathogen association in wild animal population, compared to MHC-pathogen studies in other species that usually focus on single or a few taxa (Paterson *et al.*, 1998; Froeschke & Sommer, 2005; Harf & Sommer, 2005; Meyer-lucht & Sommer, 2005; Schad *et al.*, 2005). In Addition, both pathogens presence/absence and infection intensity were examined to obtain a more complete picture of pathogen-mediated selection (Westerdahl *et al.*, 2012).

This study tests whether badger MHC diversity is driven by pathogen-mediated selection through a heterozygosity advantage or either rare-allele advantage or fluctuating selection. We test whether: (1) particular MHC alleles will confer individuals with lower or higher pathogen burden; and (2) MHC heterozygotes will exhibit lower rates of co-infection than homozygotes. Associations between specific pathogens and particular MHC alleles suggest a role for either rare-allele advantage or fluctuating selection. While associations between MHC heterozygosity and number of co-infecting pathogens suggest a role for heterozygote advantage (Spurgin & Richardson, 2010).

## A1.3 Materials and methods

### Study population and sample collection

This study was conducted on a high-density badger population (44 badgers/km$^2$; Macdonald and Newman 2002) in Wytham Woods (a 4 km$^2$ deciduous woodland in Oxfordshire, UK; 51°46'26N, 1°19'19W). Trapping events have usually been undertaken four times a year since 1987 (Macdonald & Newman, 2002), generally over two week in June (spring), September (summer)

and November (autumn), with one week of trapping in January (winter) of some years (Macdonald *et al.*, 2009). Badgers were caught in mesh-traps baited with peanuts placed near the entrances of active setts (Macdonald & Newman, 2002; Macdonald *et al.*, 2009). Captured badgers were then transferred to holding cages and transported to a central handling facility. Badgers were then sedated by an intra-muscular injection using ketamine hydrochloride at 0.2 ml/kg body weight (McLaren *et al.*, 2005). Upon their first capture all badgers were tattooed with a unique individual number on the left inguinal region for permanent individual identification. The sex, age group/maturity (cub or adult based on body size), weight, body length and location (sett name) of each badger were recorded. These protocols were subject to ethical review and were performed under Natural England Licence (currently 20104655) and UK Home Office Licence (PPL 30/2835).

During trapping DNA samples were collected: guard hairs were plucked, and approximately 3 ml of blood was taken by jugular venipuncture using a vacutainer containing EDTA. Blood samples were stored at -20°C, and hair samples in 80% ethanol at room temperature, until DNA isolation was performed. Faecal samples were collected following administration of an enema consisting of 7.5 ml warm soapy water per kg bodyweight (Newman *et al.*, 2001). Faecal samples were preserved individually as two sub-samples. The first sub-sample was preserved using 2.5% aqueous potassium dichromate ($K_2Cr_2O_7$) at $4^oC$ for later examination using faecal flotation technique (Foreyt, 2001), and the second sub-sample was stored at -20°C until DNA isolation was performed. The blood and faecal samples for pathogen screening were collected from individuals trapped June 2009-January 2010

## Pathogen screening

*Trypanosome and Mustelid Herpes Virus*

DNA from 200 µl whole blood samples was isolated using QIAamp DNA Blood kit (QIAGEN, Hilden, Germany) and eluded in 100 µl $ddH_2O$. A quantitative real-time PCR (qPCR) approach was used to determine the presence and quantity of trypanosome [*Trypanosoma pestanai*; number

of samples (n) = 360] and mustelid herpes virus (n = 361). qPCR primers were designed for regions in the 18S rRNA gene from *T. pestanai* (forward: 5'-GTCCAGCGAATGAACGAAATTAA; reverse: 5'-AGGGCAGTTGTTCGTCAGAAG) and DNA polymerase gene from mustelid herpes virus (MHV) (forward: 5'-GGAGAGTGCTGACCGATGGA; reverse: 5'-AAAAGCCTGGAATTGGATCAATAA; 150bp) using the software StepOne 2.1 [Applied Biosystems (ABI), Foster City, CA, USA]. qPCR was performed in a 20 µl reaction mix consisted of: 10 µl SYBR Green PCR Master Mix (ABI), 0.4 µl (for trypanosome) or 0.1 µl (for MHV) of each forward and reverse primer [200nM, trypanosme; 50 nM, MHV], 4.2 µl (trypanosome) or 4.8 µl (MHV) of RNase-free water, and 5 µl DNA sample. Amplification and real-time fluorescence detection were performed with StepOnePlus PCR Systems (ABI). Each real-time PCR assay contained serial dilutions of $10^7$ to $10^1$ plasmid standards, which contained the PCR products amplified by each primer set (cloning methods are described in Sin et al. 2012), to produce the calibration curve. All standards, samples and controls were run in triplicate on the same plate. The thermal cycling conditions were: 95°C for 10 mins, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. A melting curve analysis was added at the end to confirm the identity of the product. The number of pathogen DNA copies was calculated from the Ct values and standard curves and expressed as copies per 1 µl of extracted DNA.

*Enteric bacteria (Salmonella, Yersinia and Campylobacter)*

DNA from faecal samples was isolated using QIAamp DNA Stool kit (Qiagen), according to the manufacturer's instructions. A semi-quantitative PCR approach was used to determine the presence and quantity of three bacteria:,*Salmonella*, *Yersinia* and *Campylobacter* (n = 150). PCR primers were designed for regions in the *ompC* gene from *Salmonella* [forward: 5'-ATCGCTGACTTATGCAATCG; reverse: 5'-GTTGCTGATGTCCTTACCTTTAG; (355bp)], *pla* gene from *Yersinia* (forward: 5'-GCTTTATGACGCAGAAACAGGA; reverse: 5'-AACCAGCCTTTCACATTGAGGT; 270bp) and 16S rRNA gene from *Campylobacter* (forward: 5'-GGATGACACTTTTCGGAG; reverse: 5'-AATTCCATCTGCCTCTCC; 246bp). PCR was performed in a 10 µl reaction mix consisted of 0.6 µl of forward and reverse primer (100 µM), 2.7

µl of $ddH_2O$, 0.1 µl BSA (ABI), 200 µM of each dNTP, 1× PCR buffer (containing $MgCl_2$; Qiagen), 2 units of HotStarTaq (Qiagen) and 1 µl of DNA. The thermal cycling conditions were: initial denaturation at 95°C for 15 mins, followed by 38 cycles at 94°C for 30 s, 63°C (*Salmonella*) or 52°C (*Campylobacter*) for 30 s, and 72°C for 30 s, with a final extension at 72°C for 10 mins. Touchdown PCR was performed for *Yersinia*; cycles started at 65°C and dropped by 1°C per cycle until the remaining 29 cycles continued at 55°C. The reactions were performed in triplicate. PCR products were resolved by electrophoresis on 1.5% agarose gel, visualized by ethidium bromide staining, and analyzed by GeneTools (SynGene).

*Enteric parasites (Coccidia and helminths)*

The faecal flotation technique (Foreyt, 2001) was used to assess the presence and intensity of gastro-intestinal parasites present in faecal samples (n = 275), including coccidia (*Eimeria melis* and *Isospora melis*) and helminths (nematodes in general and *Strongyloides*). Following the quantitative methods of Newman *et al*. (2001), two slides per faecal sample were screened by microscopy for coccidia oocysts, helminth eggs and larvae (MAFF, 1986; Parker & Duszynski, 1986; Lloyd & Smith, 1997) and the mean intensity of infection (coccidian oocysts per gram of pelletted faecal material; Anwar *et al.*, 2000) was then calculated. While absolute rate of infection can only be established by necropsy, assessing gastro-intestinal parasitoses from faecal oocyst / egg counts, as an indirect method, provides a reliable analogue (Seivwright *et al.*, 2004). Not least, this technique allows estimation of the parasite burden in "live hosts" (Hall, 1982; Michael & Bundy, 1989).

*Ecto-parasites (fleas, lice, ticks)*

Ecto-parasites were counted when handling sedated badgers (n = 418). The number of badger fleas (*Paraceras melis*) was counted during a cursory examination of 20 sec, by parting the fur and examining the back and underside (Cox *et al.*, 1999). The total number of ticks (*Ixodes hexagonus*) on the body, and the number of badger lice (*Trichodectes melis*) in a 4 $cm^2$ skin surface of inguinal region were also counted.

*Co-infection*

The number of species simultaneously co-infecting each badger was evaluated, only for badgers in which all 13 pathogens were screened. The 13 pathogens were detected in blood samples (trypanosome, MHV), faecal samples (*Eimeria*, *Isospora*, *Capellaria*, nematodes, *Strongyloides*, *Salmonella*, *Yersinia* and *Campylobacter*), and on external skin (badger fleas, badger lice and ticks). Co-infection status was estimated for 120 time-points from 90 individuals.

## MHC and microsatellite genotyping

Genomic DNA was isolated using the GFX Genomic Blood DNA Purification Kit (Amersham Biosciences, Little Chalfont, UK), following the scalable method in the manufacturer's protocol, or from a minimum of 20 hairs with visible follicles using a Chelex protocol (Walsh *et al.*, 1991). We used published primers to amplify exon3 and exon 2 regions that encode the antigen-binding domain in MHC class I and class II DRB genes respectively (Sin *et al.*, 2012a; Sin *et al.*, 2012b). Previous studies indicated the presence of at least two DRB loci and two class I loci (Sin *et al.*, 2012a; Sin *et al.*, 2012b), with four and seven putatively functional sequences identified by transcription analysis. The MHC sequences were separated by reference strand-mediated conformation analysis (RSCA) according to Sin *et al*. (in prep). We use the number of alleles within an individual as a measure to reflect MHC heterozygosity across multiple loci (Richardson *et al.*, 2005; Westerdahl *et al.*, 2005). The allele number of class II DRB gene actually indicates the heterozygosity of the polymorphic locus, as another locus was monomorphic for *Meme-DRB*02* (Sin *et al*. in prep).

Neutral variation should also be measured to control for potential confounding effects of demographic processes on MHC structure (Spurgin & Richardson, 2010). Therefore all individuals were also genotyped at 35 microsatellites loci (detailed in Annavi *et al.*, 2011) and the standardized multilocus heterozygosity of each individual was calculated (Coltman *et al.*, 1999; Annavi et al. in prep).

## Data analysis

*Multi-model inference*

Factors known to influence pathogens in the wild, such as seasonality, can be controlled for by employing linear models that allow the inclusion of multiple explanatory variables and random factors (Paterson *et al.*, 1998; Oliver *et al.*, 2009). Multi-model inference can then be used to find out the explanatory variables that are most supported in the model to show an effect (Burnham & Anderson, 2002; Anderson, 2008; Symonds & Moussalli, 2011). We performed our analysis using R 2.15.0 (R Core Development Team 2012) and packages lattice (Sarkar, 2008), lme4 (Bates & Maechler, 2009), MuMIn (Barton, 2009) and AICcmodavg (Mazerolle, 2011). An initial data exploration (Zuur *et al.*, 2009) was conducted to check the distribution and spread of the data, identify outliers and examine relationships between variables. The infection intensities of trypanosome, MHV, *Eimeria*, *Salmonella*, *Yersinia*, *Campylobacter* and lice were transformed by $\log_{10}(\text{intensity} + 1)$ to correct for heterogeneity of variance.

To reduce the number of factors included in the same model, the multi-model inference was divided into two steps. In step one, we first tested whether different life-history factors affected the: (1) prevalence (presence/absence) of a particular pathogen, (2) pathogen infection intensity (infected individuals only, as non-infection can be due to non-exposure or resistance), or (3) number of species co-infecting a badger simultaneously. We fitted linear mixed models (LMM: non-count intensity) and generalized linear mixed models [GLMM: prevalence (binomial error, logit link), and coinfection or ecto-parasite counts (Poisson error, log link)]. The models included six fixed effects: three categorical [maturity (cub or adult), sex and season] and three continuous effects (age, weight/length ratio and standardized microsatellite heterozygosity). The number of fleas was also included in the trypanosome infection model, as *P. melis* is the vector of *T. pestanai* (Lizundia *et al.*, 2011). We controlled for individuals with multiple samples by including individual identity as a random effect. We also included social group identity as a random effect. All possible models were evaluated using information theoretic procedures (Burnham & Anderson, 2002; Anderson, 2008; Symonds & Moussalli, 2011). Model selection was based on Akaike's

information criterion corrected for sample size (AICc; Akaike 1973). Models that are better supported by the data result in lower AICc values. Multi-model inference (Burnham & Anderson, 2002) was performed for models with ΔAICc < 2. All variables in each supported model with ΔAICc < 2 were retained for use in step 2.

In step two, we investigated (1) whether the presence of specific MHC alleles were associated with pathogen prevalence, pathogen burden and co-infection, and (2) whether MHC heterozygosity were associated with co-infection. We included them as fixed effects in each model (containing the significant variables from step 1). All alleles [three class II DRB: *Meme-DRB*01*, *-DRB*03*, and *-DRB*04* (Sin *et al.*, 2012a); five class I: *Meme-MHCI*01*, *-MHCI*02*, *-MHCI*03*, *-MHCI*04*, and *-MHCI*07* (Sin *et al.*, 2012b)] were included in the same model except for *Meme-DRB*02* and *Meme-MHCI*05* that were present in all individuals and *Meme-MHCI*06* that always present with *Meme-MHCI*07* (see Sin *et al*. in prep), and were thus excluded from the analyses. Parameter estimates (estimate with shrinkage also reported, which is a weighted average that zeros are assigned for models where the parameter does not appear, thus lessens the bias due to model selection), and their unconditional standard errors (a conservative measure as it accounts for model uncertainty) and 95% confidence intervals, were then estimated by model averaging (Anderson, 2008). This allows model uncertainty to be included in both model evaluation and derivation of parameter estimates. The relative importance of a factor was defined as the sum of Akaike weights for all models containing the predictor factor (Burnham & Anderson, 2002). The factor with the largest sum was inferred to be the most influential.

### A1.4 Results

Five MHC alleles were associated with variation in the prevalence and/or intensity of four pathogens (trypanosome, MHV, *Yersinia* and *Campylobacter*). No association was identified between MHC alleles and *Eimeria*, *Salmonella*, *Yersinia*, fleas and lice.

Multimodel inference indicated the presence of trypanosomes was influenced by the number of fleas, maturity, sex and the presence of *Meme-DRB*03* and *Meme-MHCI*03* (Table 1). Individuals that carried *Meme-MHCI*03* were less likely to be infected by trypanosomes, while individuals with *Meme-DRB*03* had a higher probability of carrying trypanosomes. Infection intensity was only associated with age (Table 2). Age, maturity, season, weight/length ratio, and the presence of *Meme-DRB*01* and *Meme-MHCI*03* were correlated with the intensity of MHV infection (Table 3). Individuals with *Meme-DRB*01* had higher MHV intensity and individuals with *Meme-MHCI*03* had lower MHV intensity. The presence of *Eimeria* was affected by maturity and age (Table 4), and the infection intensity was affected by maturity and season (Table 5). The presence of *Salmonella* was associated with maturity and season (Table 6), and *Salmonella* infection intensity was related to standardized microsatellite heterozygosity (Table 7). *Meme-MHCI*04* and season had an effect on the presence of *Yersinia* (Table 8), whereas *Yersinia* intensity was affected by *Meme-DRB*01* and *Meme-DRB*04* (Table 9). Individuals that carried *Meme-MHCI*04* were more likely to be infected by *Yersinia*, and the infection intensity of *Yersinia* was higher for individuals with *Meme-DRB*01* and lower for individuals with *Meme-DRB*04*. None of the tested explanatory variables were related with *Campylobacter* presence (Table 10), but *Meme-MHCI*03* (marginally), season and sex were associated with *Campylobacter* infection intensity (Table 11). Individuals that carried *Meme-MHCI*03* had lower infection intensity of *Campylobacter*. The presence of fleas was affected by maturity, season and weight/length ratio (Table 12), and the load of flea was affected by season (Table 13). The presence of lice was affected by season, sex, weight/length ratio and age (marginally) (Table 14), and the load of lice was affected by sex, weight/length ratio and age (marginally) (Table 15). Both ectoparasites did not associate with MHC alleles.

Individual badgers were simultaneously co-infected with between two and eleven different pathogens (mean = 6.0, SE ± 0.075). Coinfection was not associated with the presence/absence of individual MHC alleles or MHC heterozygosity, and there was no association between the number of co-infecting species and any of the tested explanatory variables (Table 16). By excluding

ectoparasites, we examined the number of coinfecting internal pathogens (range = 2-9, mean = 4.4, SE ± 0.065), and again there was no association with the explanatory variables.

**Table 1** The presence or absence of trypanosome (*Trypanosoma pestanai*) in badgers: Parameter estimates with their unconditional standard error (SE) and relative importance after model averaging. 95% confidence intervals that do not overlap zero are in bold.

| Parameter | Estimate (with shrinkage) | Estimate | Unconditional SE | 95% Confidence interval | | Relative importance^ |
|---|---|---|---|---|---|---|
| (Intercept) | 0.900 | 0.900 | 0.719 | -0.509 | 2.309 | |
| Fleas | 0.054 | 0.054 | 0.028 | 0.000 | 0.108 | 1.00* |
| Maturity (adult) | 1.370 | 1.370 | 0.356 | 0.673 | 2.067 | 1.00* |
| Sex (female) | -1.217 | -1.217 | 0.338 | -1.879 | -0.555 | 1.00* |
| DRB*03 (present) | 1.088 | 1.151 | 0.520 | 0.131 | 2.170 | 0.95 |
| MHCI*03 (present) | -1.200 | -1.269 | 0.483 | -2.215 | -0.322 | 0.95 |
| DRB*01 (present) | -0.363 | -0.906 | 0.751 | -2.378 | 0.566 | 0.40 |
| MHCI*01 (present) | -0.251 | -0.639 | 0.422 | -1.466 | 0.187 | 0.39 |
| MHCI*07 (present) | -0.318 | -1.046 | 0.803 | -2.620 | 0.528 | 0.30 |
| MHCI*04 (present) | 0.125 | 0.656 | 0.517 | -0.357 | 1.668 | 0.19 |
| DRB*04 (present) | 0.025 | 0.470 | 0.607 | -0.719 | 1.659 | 0.05 |
| MHCI*02 (present) | 0.017 | 0.326 | 0.639 | -0.927 | 1.579 | 0.05 |

* Included in all models

^ Relative importance is the sum of Akaike weights for all models containing the parameter

**Table 2** The infection intensity of trypanosome (*Trypanosoma pestanai*) in badgers: Parameter estimates with their unconditional standard error (SE) and relative importance after model averaging. 95% confidence intervals that do not overlap zero are in bold.

| Parameter | Estimate (with shrinkage) | Estimate | Unconditional SE | 95% Confidence interval | | Relative importance^ |
|---|---|---|---|---|---|---|
| (Intercept) | 2.855 | 2.855 | 0.351 | 2.274 | 3.650 | |
| Age | -0.112 | -0.112 | 0.036 | **-0.184** | **-0.042** | 1.00* |
| DRB*03 (present) | 0.632 | 0.756 | 0.403 | -0.061 | 1.519 | 0.84 |
| MHCI*03 (present) | -0.421 | -0.631 | 0.343 | -1.314 | 0.032 | 0.67 |
| DRB*01 (present) | 0.197 | 0.503 | 0.332 | -0.161 | 1.140 | 0.39 |
| DRB*04 (present) | 0.172 | 0.420 | 0.300 | -0.191 | 0.986 | 0.41 |
| MHCI*02 (present) | 0.134 | 0.433 | 0.338 | -0.230 | 1.093 | 0.31 |
| MHCI*04 (present) | 0.023 | 0.254 | 0.253 | -0.252 | 0.739 | 0.09 |
| MHCI*07 (present) | -0.016 | -0.384 | 0.624 | -1.607 | 0.839 | 0.04 |
| MHCI*01 (present) | -0.010 | -0.254 | 0.304 | -0.851 | 0.342 | 0.04 |

* Included in all models

^ Relative importance is the sum of Akaike weights for all models containing the parameter

**Table 3** The infection intensity of Mustelid herpes virus in badgers: Parameter estimates with their unconditional standard error (SE) and relative importance after model averaging. 95% confidence intervals that do not overlap zero are in bold.

| Parameter | Estimate (with shrinkage) | Estimate | Unconditional SE | 95% Confidence interval | | Relative importance^ |
|---|---|---|---|---|---|---|
| (Intercept) | 1.920 | 1.920 | 0.142 | 1.655 | 2.213 | |
| Age | -0.028 | -0.028 | 0.010 | **-0.049** | **-0.009** | 1.00* |
| Maturity (adult) | -0.491 | -0.491 | 0.091 | **-0.669** | **-0.314** | 1.00* |
| DRB*01 (present) | 0.298 | 0.298 | 0.091 | **0.119** | **0.475** | 1.00 |
| MHCI*07 (present) | 0.133 | 0.199 | 0.122 | -0.049 | 0.428 | 0.67 |
| MHCI*03 (present) | -0.125 | -0.125 | 0.051 | **-0.226** | **-0.028** | 1.00 |
| Season (Summer) [a] | -0.023 | -0.023 | 0.048 | -0.116 | 0.072 | 1.00* |
| Season (Autumn) [a] | 0.010 | 0.010 | 0.057 | -0.101 | 0.124 | " |
| Season (Winter) [a] | -0.364 | -0.364 | 0.117 | **-0.593** | **-0.133** | " |
| Weight/length ratio | -0.049 | -0.049 | 0.014 | **-0.076** | **-0.022** | 1.00* |
| MHCI*01 (present) | 0.034 | 0.090 | 0.063 | -0.054 | 0.191 | 0.38 |
| MHCI*02 (present) | 0.040 | 0.113 | 0.091 | -0.092 | 0.266 | 0.36 |
| DRB*03 (present) | 0.006 | 0.069 | 0.079 | -0.086 | 0.224 | 0.09 |
| DRB*04 (present) | -0.009 | -0.120 | 0.096 | -0.309 | 0.068 | 0.07 |

[a] Spring was the reference category
* Included in all models
^ Relative importance is the sum of Akaike weights for all models containing the parameter

**Table 4** The presence or absence of *Eimeria* in badgers: Parameter estimates with their unconditional standard error (SE) and relative importance after model averaging. 95% confidence intervals that do not overlap zero are in bold.

| Parameter | Estimate (with shrinkage) | Estimate | Unconditional SE | 95% Confidence interval | | Relative importance^ |
|---|---|---|---|---|---|---|
| (Intercept) | 1.683 | 1.683 | 0.618 | 0.471 | 2.895 | |
| Age | -0.191 | -0.191 | 0.067 | **-0.322** | **-0.060** | 1.00* |
| Maturity (adult) | -1.219 | -1.219 | 0.442 | **-2.085** | **-0.353** | 1.00* |
| DRB*03 (present) | -0.337 | -0.522 | 0.306 | -1.121 | 0.078 | 0.65 |
| Season (Summer)[a] | -0.560 | -0.560 | 0.321 | -1.189 | 0.070 | 1.00 |
| Season (Autumn)[a] | -0.427 | -0.427 | 0.389 | -1.189 | 0.336 | " |
| MHCI*07 (present) | 0.218 | 0.809 | 0.690 | -0.543 | 2.161 | 0.27 |
| DRB*01 (present) | 0.185 | 0.712 | 0.639 | -0.541 | 1.964 | 0.26 |
| MHCI*02 (present) | -0.042 | -0.464 | 0.561 | -1.564 | 0.637 | 0.09 |
| MHCI*04 (present) | 0.019 | 0.233 | 0.350 | -0.453 | 0.919 | 0.08 |
| MHCI*01 (present) | 0.019 | 0.239 | 0.376 | -0.498 | 0.976 | 0.08 |

[a] Spring was the reference category
* Included in all models
^ Relative importance is the sum of Akaike weights for all models containing the parameter

**Table 5** The infection intensity of *Eimeria* in badgers: Parameter estimates with their unconditional standard error (SE) and relative importance after model averaging. 95% confidence intervals that do not overlap zero are in bold.

| Parameter | Estimate (with shrinkage) | Estimate | Unconditional SE | 95% Confidence interval | | Relative importance^ |
|---|---|---|---|---|---|---|
| (Intercept) | 2.509 | 2.509 | 0.256 | 1.996 | 2.998 | |
| Maturity (adult) | -1.036 | -1.036 | 0.162 | **-1.350** | **-0.714** | 1.00* |
| Season (Summer)[a] | -0.564 | -0.564 | 0.164 | **-0.887** | **-0.244** | 1.00* |
| Season (Autumn)[a] | -0.613 | -0.613 | 0.221 | **-1.050** | **-0.182** | " |
| MHCI*01 (present) | -0.054 | -0.281 | 0.220 | -0.712 | 0.150 | 0.19 |
| MHCI*04 (present) | 0.021 | 0.174 | 0.208 | -0.235 | 0.582 | 0.12 |
| MHCI*07 (present) | 0.025 | 0.234 | 0.348 | -0.448 | 0.916 | 0.11 |
| DRB*04 (present) | -0.021 | -0.194 | 0.287 | -0.756 | 0.368 | 0.11 |
| DRB*01 (present) | 0.027 | 0.262 | 0.439 | -0.599 | 1.123 | 0.10 |
| DRB*03 (present) | -0.011 | -0.109 | 0.194 | -0.489 | 0.271 | 0.10 |

[a] Spring was the reference category
* Included in all models
^ Relative importance is the sum of Akaike weights for all models containing the parameter

**Table 6** The presence or absence of *Salmonella* in badgers: Parameter estimates with their unconditional standard error (SE) and relative importance after model averaging. 95% confidence intervals that do not overlap zero are in bold.

| Parameter | Estimate (with shrinkage) | Estimate | Unconditional SE | 95% Confidence interval | | Relative importance^ |
|---|---|---|---|---|---|---|
| (Intercept) | -0.750 | -0.750 | 0.566 | -1.859 | 0.360 | |
| Maturity (adult) | -1.525 | -1.525 | 0.525 | **-2.553** | **-0.496** | 1.00* |
| Season (Summer)[a] | 2.669 | 2.669 | 0.565 | **1.562** | **3.777** | 1.00* |
| Season (Autumn)[a] | 2.737 | 2.737 | 0.571 | **1.619** | **3.855** | " |
| MHCI*03 (present) | -0.252 | -0.672 | 0.504 | -1.659 | 0.315 | 0.37 |
| DRB*03 (present) | 0.081 | 0.689 | 0.554 | -0.396 | 1.775 | 0.12 |
| MHCI*01 (present) | 0.054 | 0.459 | 0.508 | -0.537 | 1.454 | 0.12 |
| MHCI*04 (present) | -0.072 | -0.379 | 0.450 | -1.260 | 0.503 | 0.19 |
| MHCI*07 (present) | -0.035 | -0.398 | 0.754 | -1.876 | 1.081 | 0.09 |
| MHCI*02 (present) | -0.033 | -0.377 | 0.798 | -1.941 | 1.186 | 0.09 |

[a] Spring was the reference category
* Included in all models
^ Relative importance is the sum of Akaike weights for all models containing the parameter

**Table 7** The infection intensity of *Salmonella* in badgers: Parameter estimates with their unconditional standard error (SE) and relative importance after model averaging. 95% confidence intervals that do not overlap zero are in bold.

| Parameter | Estimate (with shrinkage) | Estimate | Unconditional SE | 95% Confidence interval | | Relative importance^ |
|---|---|---|---|---|---|---|
| (Intercept) | 2.856 | 2.856 | 0.332 | 2.185 | 3.486 | |
| MHCI*03 (present) | -0.105 | -0.206 | 0.110 | -0.396 | 0.034 | 0.51 |
| SH[a] | -0.673 | -0.673 | 0.294 | **-1.286** | **-0.133** | 1.00* |
| Weight/length retio | -0.030 | -0.030 | 0.021 | -0.073 | 0.011 | 1.00* |
| MHCI*04 (present) | -0.074 | -0.213 | 0.135 | -0.457 | 0.071 | 0.35 |
| DRB*01 (present) | 0.039 | 0.233 | 0.168 | -0.156 | 0.502 | 0.17 |
| DRB*03 (present) | -0.029 | -0.171 | 0.110 | -0.376 | 0.054 | 0.17 |
| MHCI*07 (present) | 0.043 | 0.276 | 0.225 | -0.156 | 0.725 | 0.15 |
| DRB*04 (present) | -0.011 | -0.184 | 0.164 | -0.506 | 0.138 | 0.06 |
| MHCI*01 (present) | 0.007 | 0.119 | 0.131 | -0.137 | 0.375 | 0.06 |

* Included in all models

^ Relative importance is the sum of Akaike weights for all models containing the parameter

[a] Standardised microsatellite heterozygosity

**Table 8** The presence or absence of *Yersinia* in badgers: Parameter estimates with their unconditional standard error (SE) and relative importance after model averaging. 95% confidence intervals that do not overlap zero are in bold.

| Parameter | Estimate (with shrinkage) | Estimate | Unconditional SE | 95% Confidence interval | | Relative importance^ |
|---|---|---|---|---|---|---|
| (Intercept) | -1.222 | -1.222 | 0.543 | -2.285 | -0.158 | |
| MHCI*07 (present) | -1.004 | -1.421 | 0.878 | -3.142 | 0.300 | 0.71 |
| MHCI*04 (present) | 0.979 | 0.979 | 0.452 | **0.093** | **1.864** | 1.00 |
| Season (Summer)[a] | 1.233 | 1.233 | 0.449 | **0.353** | **2.112** | 1.00* |
| Season (Autumn)[a] | 1.146 | 1.146 | 0.451 | **0.261** | **2.031** | " |
| DRB*04 (present) | 0.315 | 0.869 | 0.559 | -0.226 | 1.964 | 0.36 |
| MHCI*02 (present) | 0.045 | 0.535 | 0.730 | -0.896 | 1.966 | 0.08 |
| MHCI*03 (present) | -0.023 | -0.276 | 0.387 | -1.034 | 0.483 | 0.08 |
| DRB*03 (present) | -0.019 | -0.247 | 0.398 | -1.026 | 0.532 | 0.08 |
| MHCI*01 (present) | 0.022 | 0.294 | 0.503 | -0.692 | 1.280 | 0.08 |

[a] Spring was the reference category

* Included in all models

^ Relative importance is the sum of Akaike weights for all models containing the parameter

**Table 9** The infection intensity of *Yersinia* in badgers: Parameter estimates with their unconditional standard error (SE) and relative importance after model averaging. 95% confidence intervals that do not overlap zero are in bold.

| Parameter | Estimate (with shrinkage) | Estimate | Unconditional SE | 95% Confidence interval | | Relative importance^ |
|---|---|---|---|---|---|---|
| (Intercept) | 1.208 | 1.208 | 0.243 | 0.719 | 1.671 | |
| DRB*01 (present) | 0.479 | 0.512 | 0.217 | **0.072** | **0.922** | 0.94 |
| DRB*04 (present) | -0.289 | -0.373 | 0.173 | **-0.695** | **-0.016** | 0.77 |
| MHCI*04 (present) | -0.082 | -0.315 | 0.201 | -0.717 | 0.073 | 0.26 |
| MHCI*01 (present) | 0.063 | 0.223 | 0.181 | -0.124 | 0.585 | 0.28 |
| MHCI*07 (present) | 0.131 | 0.514 | 0.378 | -0.230 | 1.253 | 0.25 |
| MHCI*02 (present) | -0.018 | -0.275 | 0.213 | -0.691 | 0.142 | 0.06 |

^ Relative importance is the sum of Akaike weights for all models containing the parameter

**Table 10** The presence or absence of *Campylobacter* in badgers: Parameter estimates with their unconditional standard error (SE) and relative importance after model averaging. 95% confidence intervals that do not overlap zero are in bold.

| Parameter | Estimate (with shrinkage) | Estimate | Unconditional SE | 95% Confidence interval | | Relative importance^ |
|---|---|---|---|---|---|---|
| (Intercept) | 0.539 | 0.539 | 0.238 | 0.073 | 1.006 | |
| DRB*04 (present) | 0.076 | 0.504 | 0.483 | -0.443 | 1.451 | 0.15 |
| DRB*03 (present) | 0.134 | 0.535 | 0.487 | -0.419 | 1.489 | 0.25 |
| MHCI*03 (present) | -0.141 | -0.547 | 0.485 | -1.499 | 0.404 | 0.26 |
| MHCI*07 (present) | -0.069 | -0.563 | 0.657 | -1.851 | 0.726 | 0.12 |
| MHCI*01 (present) | 0.040 | 0.342 | 0.444 | -0.528 | 1.213 | 0.12 |

^ Relative importance is the sum of Akaike weights for all models containing the parameter

**Table 11** The infection intensity of *Campylobacter* in badgers: Parameter estimates with their unconditional standard error (SE) and relative importance after model averaging. 95% confidence intervals that do not overlap zero are in bold.

| Parameter | Estimate (with shrinkage) | Estimate | Unconditional SE | 95% Confidence interval | | Relative importance^ |
|---|---|---|---|---|---|---|
| (Intercept) | 1.040 | 1.040 | 0.158 | 0.692 | 1.312 | |
| MHCI*03 (present) | -0.115 | -0.231 | 0.122 | **-0.471** | **0.006** [a] | 0.50 |
| Season (Summer)[b] | -0.446 | -0.446 | 0.118 | **-0.675** | **-0.214** | 1.00* |
| Season (Autumn)[b] | -0.214 | -0.214 | 0.111 | **-0.431** | **0.004** [a] | " |
| Sex (female) | 0.311 | 0.311 | 0.123 | **0.063** | **0.544** | 1.00* |
| DRB*03 (present) | -0.094 | -0.221 | 0.125 | -0.469 | 0.022 | 0.43 |
| MHCI*01 (present) | 0.053 | 0.203 | 0.145 | -0.072 | 0.497 | 0.26 |
| MHCI*02 (present) | -0.034 | -0.232 | 0.222 | -0.667 | 0.202 | 0.15 |
| DRB*01 (present) | -0.023 | -0.170 | 0.177 | -0.515 | 0.177 | 0.13 |
| MHCI*04 (present) | -0.006 | -0.102 | 0.126 | -0.348 | 0.144 | 0.06 |

* Included in all models
^ Relative importance is the sum of Akaike weights for all models containing the parameter
[a] Marginally significant
[b] Spring was the reference category

**Table 12** The presence or absence of fleas in badgers: Parameter estimates with their unconditional standard error (SE) and relative importance after model averaging. 95% confidence intervals that do not overlap zero are in bold.

| Parameter | Estimate (with shrinkage) | Estimate | Unconditional SE | 95% Confidence interval | | Relative importance^ |
|---|---|---|---|---|---|---|
| (Intercept) | 2.025 | 2.025 | 0.770 | **0.517** | **3.534** | |
| Maturity (adult) | 1.866 | 1.866 | 0.456 | **0.972** | **2.760** | 1.00* |
| Season (Summer)[a] | 1.799 | 1.799 | 0.347 | **1.118** | **2.479** | 1.00* |
| Season (Autumn)[a] | 1.728 | 1.728 | 0.411 | **0.922** | **2.533** | " |
| Season (Winter)[a] | -0.108 | -0.108 | 0.680 | -1.441 | 1.225 | " |
| Weight/length retio | -0.263 | -0.263 | 0.089 | **-0.439** | **-0.088** | 1.00* |
| DRB*01 (present) | -0.128 | -0.559 | 0.653 | -1.838 | 0.722 | 0.23 |
| MHCI*07 (present) | -0.058 | -0.340 | 0.692 | -1.696 | 1.017 | 0.17 |
| MHCI*01 (present) | 0.020 | 0.122 | 0.348 | -0.561 | 0.805 | 0.16 |

[a] Spring was the reference category
* Included in all models
^ Relative importance is the sum of Akaike weights for all models containing the parameter

**Table 13** The infection intensity of fleas in badgers: Parameter estimates with their unconditional standard error (SE) and relative importance after model averaging. 95% confidence intervals that do not overlap zero are in bold.

| Parameter | Estimate (with shrinkage) | Estimate | Unconditional SE | 95% Confidence interval | | Relative importance^ |
|---|---|---|---|---|---|---|
| (Intercept) | 1.678 | 1.678 | 0.139 | 1.405 | 1.951 | |
| MHCI*04 (present) | 0.126 | 0.186 | 0.115 | -0.039 | 0.411 | 0.68 |
| Season (Summer)[b] | 0.195 | 0.195 | 0.070 | **0.057** | **0.332** | 1.00* |
| Season (Autumn)[b] | 0.145 | 0.145 | 0.076 | **-0.003** | **0.293**[a] | " |
| Season (Winter)[b] | -0.545 | -0.545 | 0.272 | **-1.078** | **-0.012** | " |
| DRB*01 (present) | 0.030 | 0.160 | 0.178 | -0.189 | 0.509 | 0.18 |
| DRB*03 (present) | 0.009 | 0.093 | 0.104 | -0.110 | 0.297 | 0.10 |
| MHCI*02 (present) | 0.026 | 0.154 | 0.171 | -0.181 | 0.489 | 0.17 |
| MHCI*07 (present) | 0.014 | 0.167 | 0.225 | -0.274 | 0.607 | 0.08 |
| DRB*04 (present) | -0.005 | -0.066 | 0.143 | -0.346 | 0.215 | 0.07 |
| MHCI*03 (present) | 0.003 | 0.043 | 0.095 | -0.144 | 0.229 | 0.07 |

* Included in all models

^ Relative importance is the sum of Akaike weights for all models containing the parameter

[a] Marginally significant

[b] Spring was the reference category

**Table 14** The presence or absence of lice in badgers: Parameter estimates with their unconditional standard error (SE) and relative importance after model averaging. 95% confidence intervals that do not overlap zero are in bold.

| Parameter | Estimate (with shrinkage) | Estimate | Unconditional SE | 95% Confidence interval | | Relative importance^ |
|---|---|---|---|---|---|---|
| (Intercept) | 3.270 | 3.270 | 0.637 | 2.021 | 4.519 | |
| Age | 0.084 | 0.084 | 0.045 | **-0.004** | **0.172**[a] | 1.00* |
| Season (Summer)[b] | 0.673 | 0.673 | 0.269 | **0.145** | **1.201** | 1.00* |
| Season (Autumn)[b] | 0.556 | 0.556 | 0.309 | -0.049 | 1.162 | " |
| Season (Winter)[b] | 1.082 | 1.082 | 0.743 | **-0.375** | **-2.539** | " |
| Sex (female) | -0.593 | -0.593 | 0.244 | **-1.070** | **-0.116** | 1.00* |
| Weight/length retio | -0.273 | -0.273 | 0.058 | **-0.386** | **-0.161** | 1.00* |
| DRB*04 (present) | 0.195 | 0.553 | 0.408 | -0.247 | 1.353 | 0.35 |
| MHCI*02 (present) | 0.053 | 0.369 | 0.437 | -0.487 | 1.225 | 0.14 |
| MHCI*01 (present) | -0.029 | -0.265 | 0.360 | -0.970 | 0.439 | 0.11 |
| DRB*03 (present) | 0.010 | 0.089 | 0.243 | -0.388 | 0.566 | 0.11 |
| MHCI*04 (present) | -0.011 | -0.103 | 0.281 | -0.654 | 0.449 | 0.11 |

* Included in all models

^ Relative importance is the sum of Akaike weights for all models containing the parameter

[a] Marginally significant

[b] Spring was the reference category

**Table 15** The infection intensity of lice in badgers: Parameter estimates with their unconditional standard error (SE) and relative importance after model averaging. 95% confidence intervals that do not overlap zero are in bold.

| Parameter | Estimate (with shrinkage) | Estimate | Unconditional SE | 95% Confidence interval | | Relative importance^ |
|---|---|---|---|---|---|---|
| (Intercept) | 1.056 | 1.056 | 0.067 | 0.934 | 1.195 | |
| Age | 0.007 | 0.007 | 0.004 | **-0.001** | **0.016**[a] | 1.00* |
| MHCI*02 (present) | 0.032 | 0.068 | 0.046 | -0.021 | 0.160 | 0.47 |
| Sex (female) | -0.097 | -0.097 | 0.027 | **-0.150** | **-0.042** | 1.00* |
| Weight/length retio | -0.037 | -0.037 | 0.006 | **-0.049** | **-0.025** | 1.00* |
| MHCI*04 (present) | 0.009 | 0.039 | 0.030 | -0.020 | 0.099 | 0.23 |
| MHCI*03 (present) | -0.003 | -0.022 | 0.026 | -0.076 | 0.026 | 0.14 |
| DRB*01 (present) | 0.006 | 0.041 | 0.048 | -0.052 | 0.135 | 0.14 |
| MHCI*07 (present) | -0.006 | -0.045 | 0.064 | -0.174 | 0.079 | 0.13 |
| MHCI*01 (present) | -0.001 | -0.020 | 0.032 | -0.083 | 0.042 | 0.06 |

* Included in all models

^ Relative importance is the sum of Akaike weights for all models containing the parameter

[a] Marginally significant

**Table 16** The association of (a) presence or absence of alleles or (b) MHC genes heterozygosity and co-infection status in badgers: Parameter estimates with their unconditional standard error (SE) and relative importance after model averaging. 95% confidence intervals that do not overlap zero are in bold.

| Parameter | Estimate (with shrinkage) | Estimate | Unconditional SE | 95% Confidence interval | | Relative importance^ |
|---|---|---|---|---|---|---|
| (a) | | | | | | |
| (Intercept) | 1.814 | 1.814 | 0.068 | 1.681 | 1.947 | |
| MHCI*03 (present) | -0.046 | -0.117 | 0.088 | -0.289 | 0.055 | 0.40 |
| DRB*04 (present) | 0.017 | 0.093 | 0.107 | -0.118 | 0.303 | 0.18 |
| MHCI*01 (present) | 0.007 | 0.078 | 0.093 | -0.104 | 0.260 | 0.09 |
| MHCI*07 (present) | -0.019 | -0.119 | 0.167 | -0.447 | 0.210 | 0.16 |
| DRB*03 (present) | 0.001 | 0.005 | 0.112 | -0.214 | 0.224 | 0.15 |
| DRB*01 (present) | 0.004 | 0.054 | 0.115 | -0.172 | 0.279 | 0.07 |
| (b) | | | | | | |
| (Intercept) | 1.828 | 1.828 | 0.161 | 1.512 | 2.143 | |
| Class I heterozygosity | -0.018 | -0.064 | 0.066 | -0.194 | 0.065 | 0.29 |
| Class II DRB heterozygosity | 0.006 | 0.032 | 0.076 | -0.117 | 0.181 | 0.19 |

^ Relative importance is the sum of Akaike weights for all models containing the parameter

## A1.5 Discussion

Associations between specific pathogens and particular MHC alleles were found in this population. Some alleles conferred resistance or susceptibility to a few different pathogens. For example, *Meme-MHCI*03* provided resistance to trypanosomes, MHV and *Campylobacter*. While *Meme-DRB*01* was a susceptibility allele for MHV and *Yersinia*. By the examination of infection intensity, alleles that provided quantitative resistance (e.g. *Meme-DRB*04*) could also be identified in addition to qualitative resistance alleles (Westerdahl *et al.*, 2012), thus providing a more complete picture of MHC-pathogen associations. These associations between certain MHC alleles and pathogen resistance or susceptibility are in concordance with the prediction of rare-allele advantage hypothesis (Takahata & Nei, 1990), whereby MHC alleles that have been the target of recent pathogen evasion will be susceptible and become less frequent, and rare or new alleles will become resistant to newly mutated pathogens and increase in frequency. In addition to the host-pathogen co-evolution and cyclic change in allele frequencies, the presence of susceptible alleles could be due to simultaneous resistance to a pathogen and susceptibility to others (Penn & Potts, 1999). On the other hand, fluctuating selection can also produce the same effect on MHC-pathogen association. These pathogen-MHC allele associations were independent of genome-wide heterozygosity, therefore they were not a by-product of confounding effects of demographic processes on MHC structure (Spurgin & Richardson, 2010).

Both class I and class II alleles were associated with particular pathogens in this study. MHC class II molecules principally bind exogenous antigens and are only expressed on antigen-presenting cells, such as B cells and macrophages (Hughes & Yeager, 1998). MHC class I molecules are responsible primarily for intracellular antigen binding and are expressed on the surface of all nucleated somatic cells (Bjorkman & Parham, 1990), while cross-presentation also allows class I molecules to process exogenous antigens (Heath & Carbone, 2001; Ackerman & Cresswell, 2004). Our investigation found that class I alleles were associated with both intracellular pathogens (e.g. MHV) and extracellular pathogens (e.g. *T. pestanai*, *Campylobacter* and *Yersinia*). Class II DRB

alleles also associated with extracellular pathogens (e.g. *T. pestanai* and *Yersinia*) and intracellular pathogens (e.g. MHV).

We found no support for MHC heterozygoiy being associated with fewer co-infecting pathogens. Other studies have also investigated and found no association between MHC heterozygosity and pathogen resistance (e.g.Langefors *et al.*, 2001; Harf & Sommer, 2005; Meyer-lucht & Sommer, 2005; Schad *et al.*, 2005; Tollenaere *et al.*, 2008) but demonstrated associations between individual MHC alleles and specific pathogens. The majority of MHC-pathogen studies considered only one or a few pathogens, and it might be possible that evidence of heterozygous advantage could be found by including more pathogens (Langefors *et al.*, 2001). We examined thirteen pathogenic species from a wide variety of taxa to establish the co-infection status. Still, MHC heterozygosity did not influence the co-infection status.

There were other factors associated with burden of different pathogen species in addition to the presence of MHC alleles. Flea (*P. melis*) burden correlated positively with trypanosome (*T. pestanai*) prevalence, consistent with the finding that *P. melis* is the vector of *T. pestanai* in European badgers (Lizundia *et al.*, 2011). Adults exhibited a higher prevalence of fleas and this may be the reason that they had a higher prevalence of trypanosomes than did cubs. Age class correlated with certain pathogens; adults exhibited lower MHV intensity, prevalence of *Salmonella*, and prevalence and intensity of *Eimeria*, than did cubs. Cubs (<1 years old) are still under-going immunological development, hence they are more vulnerable to pathogens. This is consistent with Anwar *et al.* (2000) and Newman *et al.* (2001) who report that cubs have a much higher burden of *Eimeria*. Similarly, MHV intensity was also higher in cubs than in adults, where cubs may be first exposed to MHV by vertical transmission (Jones, 2001; Corey & Wald, 2009), rather than through sexual transmission. Due to immunological memory, the immune systems of adults should be better able to clear these pathogens than that of cubs. In addition to age-class effects, absolute age was also associated with lower intensities of trypanosome and MHV and lower prevalence of

*Eimeria* in older individuals, but higher prevalence and intensity of lice on them (with marginal significance).

Weight/length ratio, as an index of body-condition was associated with lower flea and lice prevalence and lower MHV and lice intensity. Higher ratios indicate individuals in better body-condition, they may be better able to allocate resources to combating pathogens (Macdonald *et al.*, 2002; Montes *et al.*, 2011). Alternatively, individuals with higher levels of infection may suffer compromised metabolic budgets, and thus loose condition as a consequence. Flea intensity affects growth and body size in great tits (*Parus major*) (Fitze *et al.*, 2004), while juvenile gerbils (*Gerbillus andersoni*) parasitized by natural levels of fleas lost body mass faster and gained body mass slower than uninfected individuals (Hawlena *et al.*, 2006b), leading to compromised survival probability (Hawlena *et al.*, 2006a). Demonstrably, variation in pathogen burdens can impact on host health and survival traits, which highlight the importance of having advantageous alleles to mount the most effective immune response, enhancing host fitness (Brouwer *et al.*, 2010).

Our study provides evidence for the association of pathogens and MHC alleles in a wild population of mammals. We found no evidence for a MHC heterozygote advantage under any co-infection status. Thus rare-allele advantage and/or fluctuating selection are likely to be the selective force shaping the MHC diversity in this species instead. These results, however, do not preclude other proposed selection mechanisms, such as mating preferences or post-copulatory mate choice, and apply only to those pathogens investigated here. Future studies will be needed to evaluate the importance of these mechanisms in MHC evolution in this and other species.

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# Appendix 2:

# MHC-based mate choice: Female preference for MHC-similar male European badgers (*Meles meles*)

[2] A modified version of this appendix is currently being prepared as: Sin, Y.W., Annavi, G., Dugdale, H.L., Newman, C., Burke, T. & Macdonald, D.W. MHC-based mate choice: Female preference for MHC-similar male European badgers (*Meles meles*).

## A2.1 Abstract

The major histocompatibility complex (MHC) plays a crucial role in the immune system, and has been shown in some species as a target by which individuals choose mates in order to optimize the fitness of their offspring, potentially mediated by olfactory cues. Under the genetic compatibility hypothesis, females are predicted to choose mates with dissimilar MHC, to enhance the resistance of their offspring to pathogens. Studies on MHC-based mate choice in wild mammals are under-represented currently. We investigated whether mate choice based on compatibility of the MHC class I genes and MHC class II DRB genes occurred in a population of wild European badgers (*Meles meles*). We also compared the MHC with neutral microsatellite markers to distinguish MHC-specific effects from genome-wide effects. We found evidence for MHC-assortative mating for within-group and neighbouring-group matings, there was an overall effect on amino acid distance, for both class I genes and class II DRB genes. We also found considerable annual fluctuations in the occurrence of MHC-based mate choice. MHC similarity was the apparent actual target, as no genome-wide based inbreeding was found in those mating randomizations.

## A2.2 Introduction

Indirect genetic benefits have been proposed as the mechanism underpinning mate selection, especially in case where direct benefits have not been detected (Zelano & Edwards, 2002). In theory, females should select mates based on 'good genes' or genetic compatibility (Trivers, 1972; Neff & Pitcher, 2005; Kempenaers, 2007). The good genes hypothesis predicts that all breeding females in the population show consistent patterns of choice, irrespective of their own genotypes (Neff & Pitcher, 2005). While compatibility, which contributes non-additive genetic variance (Puurtinen *et al.*, 2005), requires individuals to assess the genotype of potential mates by self-reference to their own genotype.

The major histocompatibility complex (MHC) has the potential to be the target of this compatibility assessment (Penn & Potts, 1998; Mays & Hill, 2004). Evidence for a role of the MHC in mate choice has been accumulating (Bernatchez & Landry, 2003) since it was discovered in mice (Yamazaki *et al.*, 1976). The MHC is a diverse gene family that plays a crucial role in the adaptive immune system, as it encodes cell surface glycoproteins that bind and present antigens to T cells and trigger an immune cascade (Swain, 1983). The MHC may therefore provide the basis on which individuals discriminate mating partners, in order to optimize the fitness of their offspring (Penn & Potts, 1999; Penn *et al.*, 2002; Milinski, 2006). MHC genes are under constant selective pressures as a consequence of challenges from parasites and pathogens (Jeffrey & Bangham, 2000; Piertney & Oliver, 2006). This arms race between pathogens and hosts maintains the extreme diversity in MHC genes. MHC-related reproductive mechanisms, such as mating preferences, selective fertilization and abortion, could also contribute to generating the MHC diversity (Ziegler *et al.*, 2005). MHC genotypes have been posited to be detectable as olfactory cues (Penn, 2002; Leinders-Zufall *et al.*, 2004; Milinski *et al.*, 2005), or through other traits (e.g. ornamentation; vonSchantz *et al.*, 1996; vonSchantz *et al.*, 1997; Dunn *et al.*, 2012), thus allowing the MHC to be an important target for indirect genetic mate choice benefits.

The evolution of MHC-based mate choice is expected to favour the combination of MHC that confers the highest resistance to pathogenic invasions. Under the genetic compatibility hypothesis, females are predicted to exhibit preferences for mates possessing complementary genotypes to their own (Trivers, 1972; Tregenza & Wedell, 2000; Neff & Pitcher, 2005). Because of codominance in the nature of MHC expression, individuals with a greater MHC diversity may have an advantage when facing a wide array of pathogens (Hughes & Nei, 1992; McClelland *et al.*, 2003). Choosing mates with dissimilar MHC can maximize the MHC diversity, and consequently enhance disease resistance among offspring. MHC-disassortative mating has been found in mice (Potts *et al.*, 1991), humans (Havlicek & Roberts, 2009), other primates (Schwensow *et al.*, 2008a; Setchell *et al.*, 2010), reptiles (Olsson *et al.*, 2003; Miller *et al.*, 2009) and salmons (Landry *et al.*, 2001; Consuegra & Garcia-de-Leaniz, 2008; Neff *et al.*, 2008). In contrast, a preference for MHC-similar males has also been found in mice (Yamazaki *et al.*, 1978), humans (Roberts *et al.*, 2005) and tiger salamanders (Bos *et al.*, 2009). Mate choice based on MHC is more likely to happen in species that have a high probability of inbreeding (Jordan & Bruford, 1998), lack direct mating benefits (e.g. male care), and have offspring with uncertain paternity (Zelano & Edwards, 2002).

The European badger (*Meles meles*) is well suited to investigate MHC-based mate choice in the wild. In high-density populations, *M. meles* is group living (Newman *et al.*, 2011) and has a polygynandrous mating system (i.e. they do not have one exclusive social mate; Dugdale *et al.*, 2007; Dugdale *et al.*, 2011) with high levels of extra-group paternity (Carpenter *et al.*, 2005; Dugdale *et al.*, 2007) and low fecundity (i.e. mean of 1-2 cubs once a year; Macdonald & Newman, 2002; Carpenter *et al.*, 2005; Dugdale *et al.*, 2007). European badgers have delayed implantation, which uncouples mating and parturition (Thom *et al.*, 2004), and a mating season with a post-parturition peak in late-February-March, but with some females ovulating subsequently later into the summer (Woodroffe, 1995; Yamaguchi *et al.*, 2006; Roper, 2010). As female badgers are potentially capable of superfoetation (conception during pregnancy; Yamaguchi *et al.*, 2006) and embryo reabsorption (Yamaguchi *et al.*, 2006), in combination this extends the opportunity for females to select the most suitable mates. This mate-choice may be facilitated by the fact that

badgers have a sub-caudal scent gland that encodes individual-specific information (Buesching *et al.*, 2002), which may provide an olfactory cue for MHC genotype (Yamazaki *et al.*, 1979; Brown *et al.*, 1987; Ziegler *et al.*, 2005). These are components that may underscore pre- & post-copulatory MHC-based mate choice. Furthermore, badgers are under selective pressure from a wide array of pathogens (Jeffrey & Bangham, 2000; Piertney & Oliver, 2006). Given this mating system, together with their life-history characteristics (Macdonald *et al.*, 2009) and lack of paternal care (Dugdale *et al.*, 2010), we predict that badgers are strong candidates to have evolved mate choice for indirect genetic benefits.

MHC-based choice can be involved in extra-pair mating, for example, females may seek extra-pair matings if their social partners have low MHC diversity or similar MHC (Richardson *et al.*, 2005; Schwensow *et al.*, 2008b) where this results in offspring with greater MHC heterozygosity (Potts *et al.*, 1991). In this badger population, approximately 50% of paternities were assigned to extra-group males, the majority of which were neighbouring males (Carpenter *et al.*, 2005; Dugdale *et al.*, 2007). In addition, within-group males are more related to females than neighbouring males (Dugdale *et al.*, 2008). Given mate-choice based on inbreeding avoidance, we therefore analysed within-group and extra-group parent pairs separately. We predict that MHC-based mate choice may play a stronger role in extra-group parentage.

We investigated the genetic compatibility hypothesis by examining whether badgers choose mates based on MHC similarity (class II DRB and class I), taking into account both the number of shared MHC alleles and functional differences between alleles. We investigated whether MHC-based mating is associated with extra-group mating by performing the analyses separately for within-group and neighbouring-group pairs. To distinguish MHC-specific effects from genome-wide effects, we compared MHC with that of potentially neutral microsatellite markers. If assigned parent pairs are less related than random expectation this would indicate inbreeding avoidance. If they are more related than random expectation this would indicate outbreeding avoidance. Mating

associated with MHC but random mating at microsatellite loci would instead indicate that the MHC is the actual target of mate choice.

## A2.3 Materials and methods

### Study site and sample collection

A dense (44 badgers/km$^2$; Macdonald & Newman 2002) population of badgers was studied in Wytham Woods, a 4 km$^2$ deciduous woodland in Oxfordshire, UK (51°46'26N, 1°19'19W). Trapping events have usually been undertaken four times a year since 1987 (Macdonald & Newman, 2002), generally over two week in June, September and November, with one week of trapping in January of some years (Macdonald *et al.*, 2009). Badgers were caught in mesh-traps baited with peanuts placed near the entrances of active setts (Macdonald & Newman, 2002; Macdonald *et al.*, 2009). Captured badgers were then transferred to holding cages and transported to a central handling facility. Badgers were then sedated by an intra-muscular injection using ketamine hydrochloride at 0.2 ml/kg body weight (McLaren *et al.*, 2005). Upon their first capture all badgers were tattooed with a unique individual number on the left inguinal region for permanent individual identification. The sex, age group/maturity (cub or adult based on body size) and location (sett name) of each badger were recorded. These protocols were subject to ethical review and were performed under Natural England Licence (currently 20104655) and UK Home Office Licence (PPL 30/2835). During trapping DNA samples were collected: guard hairs were plucked and stored in 80% ethanol, and approximately 3 ml of blood was taken by jugular venipuncture and collected in a vacutainer containing EDTA. Blood samples were stored at -20°C, and hair samples in 80% ethanol at room temperature, until DNA isolation was performed.

Social group ranges were established using bait-marking techniques every two years (Delahay *et al.*, 2000; Macdonald *et al.*, 2008; Kilshaw *et al.*, 2009), the mean number of social groups in which badgers were present each year was 19 ± 2 (range = 14–26; Dugdale *et al.*, 2008). The majority of badgers in this population exhibit high group-fidelity; only 19% of individuals

dispersed to other social groups (Macdonald *et al.*, 2008). Temporary group-movements do however occur at quite a high rate (16.4%, Macdonald *et al.* 2008; based on a maximum of 4 sampling times per year). Taking into account both permanent dispersal and temporary movements, we defined the social group of residence of an individual using the following criteria: (a) badgers were assigned to the social group in which they were first trapped; and (b) a badger was resident within a social group if the 2 most recent captures were made within the same social group.

**DNA extraction**

Genomic DNA was isolated using the GFX Genomic Blood DNA Purification Kit (Amersham Biosciences, Little Chalfont, UK), following the scalable method in the manufacturer's protocol, or from a minimum of 20 hairs with visible follicles using a Chelex protocol (Walsh *et al.*, 1991).

**MHC gene amplification**

Sin *et al.* (2012c) has shown that the DRB gene is the most variable among the four MHC class II genes studied in badgers, with four putatively functional sequences identified from both gDNA and cDNA. Seven putatively functional MHC class I sequences have also been identified from both gDNA and cDNA in the same population (Sin *et al.*, 2012b). The variable DRB and class I genes thus are good targets for investigation of MHC-based mate choice in this species. Their intermediate, but not too high, variability also allows inference of loci number and haplotypes. We used published primers to amplify the regions that encode the antigen-binding domain in class I (Meme-MHCIex3, for the exon 3; Sin *et al.* 2012b) and class II DRB (Meme-DRBex2, for the exon 2; Sin *et al.* 2012c) genes from all individuals. Previous studies indicated the presence of at least two DRB loci and two class I loci (Sin *et al.*, 2012c; Sin *et al.*, 2012b). Using these primers on 10–30 ng of gDNA, PCR amplification was performed in a 10-μl reaction mix that also contained: 0.5 μM of each primer, 200 μM of each dNTP, 1 X PCR buffer (containing $MgCl_2$; Qiagen), and 1 units of HotStarTaq (Qiagen). The PCR cycle began with incubation at 94°C for 15 min, followed

by 35 incubation cycles at 94°C for 30 s, 59°C for 30 s, and 72°C for 60 s, ending with an extension step at 72°C for 10 min.

**MHC genotyping**

The MHC was genotyped using reference strand-mediated conformation analysis (RSCA) following Arguello *et al.* (1998). Fluorescent-labeled reference strands (FLRs) were generated using alleles from closely related species (American badger *Taxidea taxus*, polecat *Mustela putorius*, stoat *Mustela erminea* and mink *Neovison vison*) and based on phylogenetic analyses. FLRs were generated by PCR using cloned alleles as templates and a 5'-FAM-labelled primer. Cloning and sequencing details are described in Sin *et al.* (2012b, 2012c). The same PCR protocol, detailed in the MHC gene-amplification section, was used for making FLRs except that the primer proportion was altered to 0.5 μM FAM-labelled primer (Meme-DRBex2R or Meme-MHCIex3F) and 0.1 μM unlabeled primer (Meme-DRBex2F or Meme-MHCIex3R). Nine FLRs were trialled for each of the class II DRB and the class I genes to determine a subset that could best resolve all sequences in the control samples. A final sets of three FLRs derived from the American badger, polecat and stoat clones and three FLRs derived from American badger and mink were used for the class II DRB genes and the class I genes, respectively.

All the resulting FLRs were diluted 1:5 in dd$H_2O$ before use in the hybridization reactions. To form heteroduplexes, 2 μl diluted FLR and 2 μl test sample PCR product were mixed and incubated in a thermal cycler at 95°C for 10 min, cooled down to 55°C at 1°C/s, hybridized at 55°C for 20 min, cooled down to15°C for 1 min and put on ice for 30 min. The plate was stored at 4°C until required. Subsequently, 3 μl of hybridization product was mixed with 7.82 μl water and 0.18 μl Genescan Rox-500 size standard [Applied Biosystems (ABI), Foster City, CA, USA] in a 96-well plate. The samples were then run on an ABI 3100 Genetic Analyzer, 4% Genescan non-denaturing polymer (ABI), and data collected using matrix Dye set D. The running protocol was an injection voltage of 8 kV, injection time 15 s, run voltage of 15 kV, run temperature of 18°C. The heteroduplex peaks were identified using GENEMAPPER 3.7 (ABI) and their motilities were

estimated relative to the internal ROX size standard. Control alleles from cloned plasmids were included in each run to control for variation between runs. Peaks with the same motility across different FLRs were designated as identical putative alleles. All seven class I alleles and four class II DRB alleles identified were sequenced (Sin *et al.*, 2012c; Sin *et al.*, 2012b). We successfully genotyped 1149 and 1126 individuals for MHC class II DRB genes and MHC class I genes respectively.

**Microsatellite typing and parentage analyses**

989 individuals were genotyped at 35 microsatellites loci (amplification conditions described in Annavi *et al.*, 2011). Details of candidate parent rules, genotyping procedures and parentage analysis are described elsewhere (Dugdale *et al.*, 2007; Annavi *et al.*, subm.). Briefly, parentage and sibships were assigned with 80% confidence (accounting for genotyping error and unsampled individuals: see Dugdale *et al.*, 2007; Annavi *et al.*, subm.) using MasterBayes 2.47 (Hadfield *et al.*, 2006) implemented in R 2.12.2 (R Development Core Team 2010), and Colony 2.0 (Wang & Santure, 2009), respectively. Candidate males were all breeding males (older than one year old) present in the year (May/June − Jan) before the cub was born (due to delayed implantation; Yamaguchi *et al.*, 2006), and candidate mothers were mothers present in the year the cub was born. Candidates were based on trapping history according to the following rules: (a) individuals trapped in that year; (b) individuals were included for an extra 2 years after the their last capture if their death date was unknown, to minimize error from badgers that were present but not caught; (c) and individuals were included for the whole period between two trappings. Only six years [1993 (class I data set: $N = 36$; class II data set: $N = 47$), 2004 (class I: $N = 32$; class II: $N = 33$), 2005 (class I: $N = 52$; class II: $N = 55$), 2008 (class I: $N = 27$; class II: $N = 27$), 2009 (class I: $N = 30$; class II: $N = 30$) and 2010 (class I: $N = 23$; class II: $N = 23$)] with high number of cubs that were assigned parents were used in this study. The loci were in Hardy–Weinberg equilibrium and linkage equilibrium (see Dugdale *et al.*, 2007; Annavi *et al.*, subm.). Parents that were from the same social group were categorised as within-group mates, whereas parents that were from the different social group were categorised as extra-group mates.

We use mate choice/preference in a broad sense that comprise both pre- and post-copulatory mate choice throughout this passage. Assigned parent pair here means badger pair that give birth to a cub with parentage assigned but not a pair that only mated behaviourally.

**Data analysis**

To examine whether assigned parent pairs have more (or less) similar MHC genes than would be expected under random mating, we examined MHC compatibility using both the extent of allele-sharing and the magnitude of the differences between genotypes. According to the heterozygote advantage hypothesis, it is beneficial to have alleles that are different (Potts & Wakeland, 1990). On the other hand, two alleles differing by more amino acids than two less different alleles could potentially recognize and bind a wider range of antigens. The allele-sharing value is the number of alleles shared between mates. To incorporate the functional similarity between alleles in the analysis, we also calculated the amino acid distance from pairwise combinations of alleles in parent pairs (Landry *et al.*, 2001). We used the average amino acid distance (Landry *et al.*, 2001; Forsberg *et al.*, 2007; Miller *et al.*, 2009) because individuals might carry different numbers of alleles and hence the number of pairwise comparisons could be different between parent pairs. We calculated the amino acid distance for both the whole exon sequences (exon 2 for DRB and exon 2+3 for class I; Sin *et al.* 2012b, 2012c) and just the antigen-binding sites (ABS) in these exons only (Sin *et al.*, 2012c; Sin *et al.*, 2012b). The ABS is the basis of antigen recognition, so it may only be variation in this region that is functionally important.

Since most extra-group mating occurred between neighbouring groups (DRB data set: 178/210 = 84.8%, class I data set: 173/205 = 84.4%), the analyses for extra-group mating were restricted to assigned parent pairs from neighbouring groups only and random pairs were assigned from neighbouring groups only during randomization. By performing a randomization test (Landry *et al.*, 2001), we compared the mean allele-sharing value and amino acid distance value for the assigned parent pairs with the frequency distribution of mean values generated from 1000 simulations of the same number of random parent pairs. Specifically, all assigned parent pairs

established from parentage analysis were disassociated and females were reassembled to candidate males at random 1000 times with replacement in order to obtain a distribution of values under random mating. The analyses were performed separately for within-group and neighbouring-group pairs in addition to separate analysis within different years. Females were then paired with a random male that was selected from the same social group for within-group analyses and only from neighbouring-groups for neighbouring-group analyses. The *p*-value was calculated as the proportion of the total number of iterations greater or smaller than the observed mean (Fisher, 1935). A 2-tailed test with $\alpha = 0.05$ was applied, with any values that fall out of this 97.5-2.5% CI are significant. All statistical analyses were performed in R v. 2.15.0 (R Core Development Team 2012).

To ensure that any observed MHC-based mating was not as a consequence of selection on wider genetic diversity, we also examined mating pattern at neutral markers. We calculated the genetic similarity of parent pairs based on 35 microsatellite loci (Annavi *et al.*, 2011) using Queller & Goodnight's pairwise relatedness measure *R* (Queller & Goodnight, 1989). The average pairwise relatedness of observed mated pairs was compared with 1000 simulations of randomly chosen pairs.

We assessed overall significance, across years, using Fisher's method of combining probabilities (Sokal & Rohlf, 1994). Theoretically the data points are independent, as they were different reproductive events with different candidate males and females presented each year. Furthermore, females are able to prevent males from mounting them (59/257 = 23% of mounting events are failed mountings; Dugdale *et al.*, 2011) so they won't be forced to reproduce with the same males each year.

## A2.4 Results

### Major histocompatibility complex screening

MHC class II DRB genotypes were determined for 520 assigned parent pairs and MHC class I genotypes for 501 parent pairs. Among them, six class II DRB and twelve class I genotypes were identified. Four class II DRB and seven class I gene sequences were identified in total; all were identified and sequenced in Sin *et al.* (2012b, 2012c) and shown to be putatively functional sequences. Three DRB and five class I haplotypes (Fig. 1) were inferred from parentage assignments, based on the assumption of Mendelian inheritance.

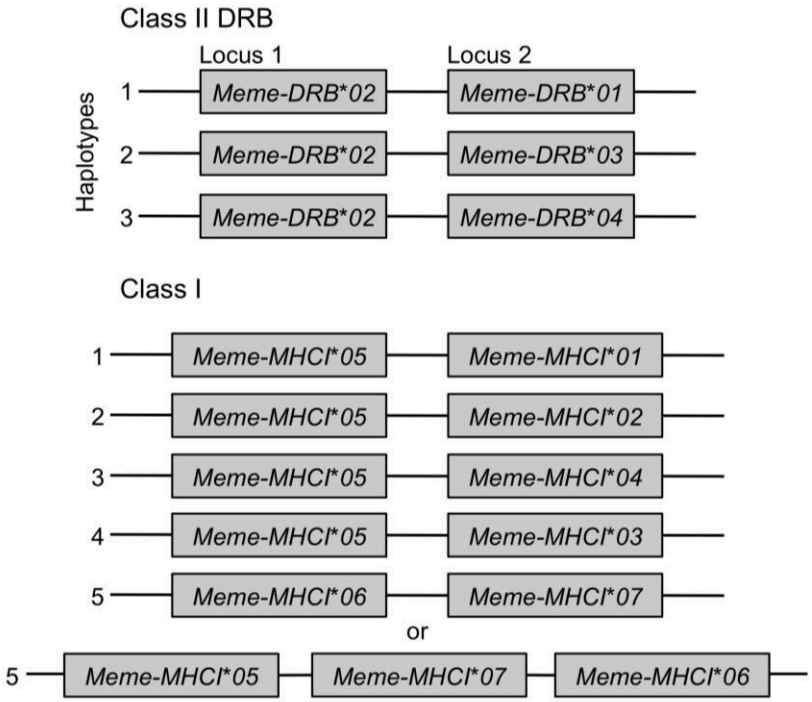


**Fig. 1** Three MHC class II DRB haplotypes and five MHC class I haplotypes in the study population of European badger (*Meles meles*). For MHC class I haplotype 5, it was not certain whether it comprises *Meme-MHCI*05* or not, because *Meme-MHCI*05* was present in all examined individuals and the frequency of haplotype 5 was low in the population and no homozygote was identified.

## MHC-based mate choice

### MHC class I

*Allele sharing*

Assigned within-group pairs shared fewer alleles than expected from random mating in two of six tested years: 1993 ($N$ = 24; $p$ = 0.015; Fig. 2a) and 2010 ($N$ = 12; $p$ < 0.001; Fig. 2f). Similarly, assigned neighbouring-group parents also shared fewer alleles than random neighbouring-group pairs 2005 ($N$ = 28; $p$ = 0.015; Fig. 2c), but they shared more alleles in 1993 ($N$ = 12; p = 0.025; Fig. 2a). There was an overall significant effect of assigned within-group pairs shared less alleles than random within-group pairs (Fisher's method of combining probabilities: $\chi^2_{12}$ = 25.70, P < 0.025; Table 1). In contrast, there was no difference in allele sharing between assigned neighbouring-group pairs and random neighbouring-group pairs (Fisher's method of combining probabilities: $\chi^2_{12}$ = 14.09, P > 0.05; Table 1).

*Amino acid distance of all sites*

Assigned within-group pairs had smaller amino acid distances (all sites) than random within-group pairs in 2009 ($N$ = 20; $p$ < 0.001; Fig. 2e). The same pattern was observed when only considering neighbouring-group pairs in 1993 ($N$ = 12; $p$ = 0.023; Fig. 2a). There was an overall significant effect of assigned within-group pairs had smaller amino acid distances (all sites) than random within-group pairs (Fisher's method of combining probabilities: $\chi^2_{12}$ = 21.59, P < 0.05; Table 1), and an overall significant effect of assigned neighbouring-group pairs had smaller amino acid distances (all sites) than random neighbouring-group pairs (Fisher's method of combining probabilities: $\chi^2_{12}$ = 24.88, P < 0.025; Table 1).

*Amino acid distance of ABS only*

The amino acid distance (ABS only) of assigned within-group pairs were smaller than that of random within-group pairs for in 2009 ($N$ = 20; $p$ < 0.001; Fig. 2e). The relationship was in the same direction, but only differed from random at a less conservative $\alpha$ = 0.1 level, for neighbouring-group pairs in 1993 ($N$ = 12; $p$ = 0.028; Fig. 2a). There was an overall significant

effect of assigned within-group pairs had smaller amino acid distances (ABS only) than random within-group pairs (Fisher's method of combining probabilities: $\chi^2_{12} = 21.55$, $P < 0.05$; Table 1), and an overall significant effect of assigned neighbouring-group pairs had smaller amino acid distances (ABS only) than random neighbouring-group pairs (Fisher's method of combining probabilities: $\chi^2_{12} = 21.04$, $P < 0.05$; Table 1).

**MHC class II DRB**

*Allele sharing*

The allele sharing of assigned within-group pairs was lower than expected under random mating in 2005 ($N = 27$; $p = 0.021$; Fig. 3c) and 2010 ($N = 12$; $p = 0.007$; Fig. 3f). There was an overall significant effect of assigned within-group pairs shared less alleles than random within-group pairs (Fisher's method of combining probabilities: $\chi^2_{12} = 23.0$, $P < 0.05$; Table 1). In contrast, there was no difference in allele sharing between assigned neighbouring-group pairs and random neighbouring-group pairs (Fisher's method of combining probabilities: $\chi^2_{12} = 17.15$, $P > 0.05$; Table 1).

*Amino acid distance of all sites or ABS only*

For within-group pairs, the amino acid distance of assigned pairs were smaller than that of random pairs in 2005 ($N = 27$; all sites: $p = 0.034$; ABS only: $p = 0.039$; Fig. 3c) and in 2008 ($N = 19$; all sites: $p = 0.033$; Fig. 3d) using less conservative $\alpha$=0.1. The amino acid distance (both all sites and ABS only) of assigned neighbouring-group pairs were smaller than that of random within-group pairs in 1993 ($N = 15$; all sites: $p = 0.007$; ABS only: $p = 0.001$; Fig. 3a), 2004 ($N = 15$; all sites: $p = 0.01$; ABS only: $p = 0.009$; Fig. 3b) and 2005 ($N = 28$; all sites: $p = 0.003$; ABS only: $p < 0.001$; Fig. 3c). There was an overall significant effect of assigned within-group pairs had smaller amino acid distances than random within-group pairs (Fisher's method of combining probabilities: all sites: $\chi^2_{12} = 23.6$, $P < 0.025$; ABS only: $\chi^2_{12} = 21.79$, $P < 0.05$; Table 1), and an overall highly significant effect of assigned neighbouring-group pairs had smaller amino acid distances than

random neighbouring-group pairs (Fisher's method of combining probabilities: all sites: $\chi^2_{12}$ = 34.22, P < 0.001; ABS only: $\chi^2_{12}$ = 40.75, P < 0.001; Table 1).

**Relatedness**

The relatedness of assigned within-group pairs was significantly lower than would be expected from random within-group mating in 1993 (DRB: $N$ = 32, $p$ = 0.024; class I: $N$ = 24, $p$ = 0.002; Fig. 2a & 3a), 2008 (DRB: $N$ = 19, $p$ = 0.001; class I: $N$ = 19, $p$ < 0.001; Fig. 2d & 3d) and 2010 (DRB: $N$ = 12, $p$ = 0.001; class I: $N$ = 12, $p$ < 0.001; Fig. 2f & 3f), in both DRB and class I data set. While the relatedness of assigned neighbouring-group pairs were significantly higher than random neighbouring-group pairs in 2010, in both DRB and class I data set (DRB: $N$ = 11, $p$ = 0.012; class I: $N$ = 11, $p$ = 0.015; Fig. 2f & 3f). There was an overall significant effect of assigned within-group pairs less related than random within-group pairs (Fisher's method of combining probabilities: class I data set: $\chi^2_{12}$ = 46.21, P < 0.001; DRB data set: $\chi^2_{12}$ = 43.32, P < 0.001; Table 1). In contrast, there was no difference in relatedness between assigned neighbouring-group pairs and random neighbouring-group pairs (Fisher's method of combining probabilities: class I data set: $\chi^2_{12}$ = 9.59, P > 0.05; DRB data set: $\chi^2_{12}$ = 10.34, P > 0.05; Table 1).

**Table 1** Overall significance of MHC-based mating and inbreeding avoidance across six years (1993, 2004, 2005, 2008, 2009 and 2010), calculated using Fisher's method of combining probabilities (Sokal & Rohlf 1994). p-values are combined for lower allele sharing, smaller amino acid (a.a.) distance (all site and ABS only) and lower Queller & Goodnight's index of pairwise relatedness (R) of assigned parent pairs to random pairs. Significant p-values are shown in bold.

| MHC gene | Parent pairs | Allele sharing | a.a. distance (all) | a.a. distance (ABS) | Q&G's relatedness |
|---|---|---|---|---|---|
| Class I | Within-group | **$\chi^2_{12}$ = 25.70 p<0.025** | **$\chi^2_{12}$ = 21.59 p<0.05** | **$\chi^2_{12}$ = 21.55 p<0.05** | **$\chi^2_{12}$ = 46.21 p<0.001** |
| | Neighboring-group | $\chi^2_{12}$ = 14.09 p>0.05 | **$\chi^2_{12}$ = 24.88 p<0.025** | **$\chi^2_{12}$ = 21.04 p<0.05** | $\chi^2_{12}$ = 9.59 p>0.05 |
| Class II DRB | Within-group | **$\chi^2_{12}$ = 23.00 p<0.05** | **$\chi^2_{12}$ = 23.60 p<0.025** | **$\chi^2_{12}$ = 21.79 p<0.05** | **$\chi^2_{12}$ = 43.32 p<0.001** |
| | Neighboring-group | $\chi^2_{12}$ = 17.15 p>0.05 | **$\chi^2_{12}$ = 34.22 p<0.001** | **$\chi^2_{12}$ = 40.75 p<0.001** | $\chi^2_{12}$ = 10.34 p>0.05 |

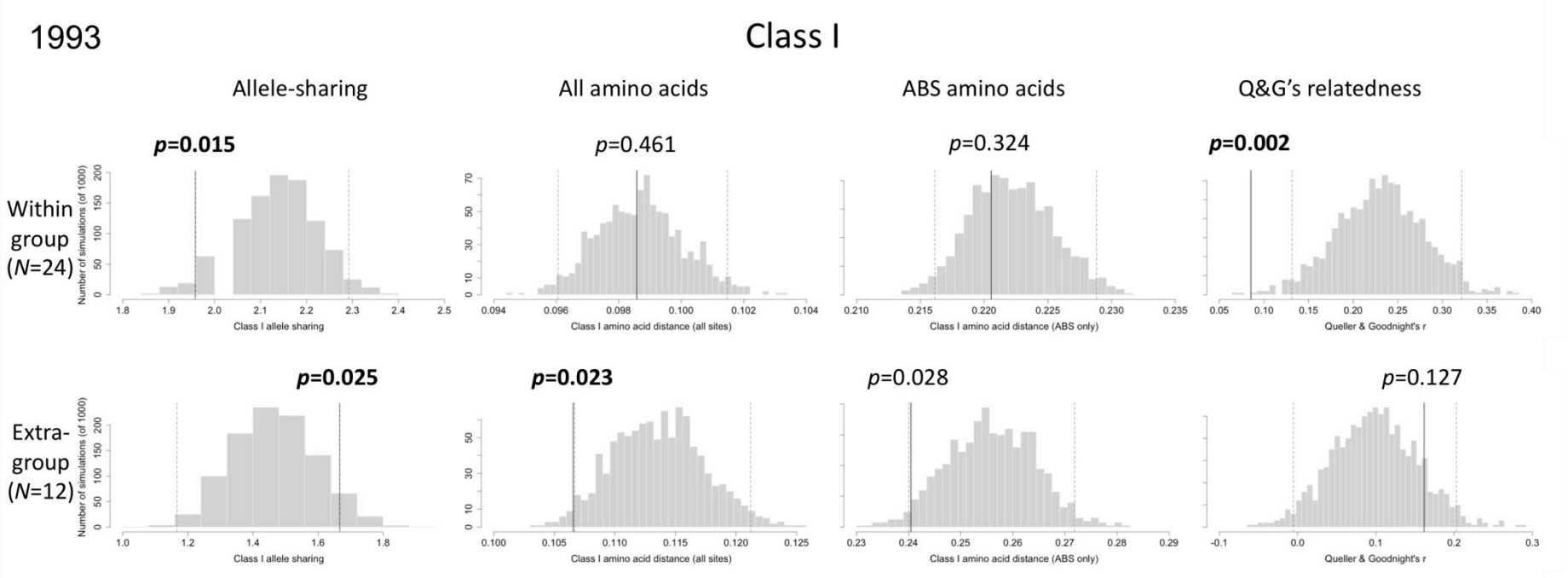


**Fig. 2a**

**Fig. 2** Mean MHC class I allele sharing, amino acid distance (all amino acid sites and ABS only) and Queller & Goodnight's index of pairwise relatedness (*R*) of assigned parent-pairs (filled line) compared with the frequency distribution of mean values generated from 1000 simulations of random pairings between breeding females and all of their within-group or neighbouring-group candidate fathers (grey bars), with two-tailed 95% CI (dashed lines) indicating cut-offs for significant departures from random mating. The data were analyzed separately for each year: (a) 1993, number of candidate fathers ($N_C$)=84, (b) 2004, $N_C$=102, (c) 2005, $N_C$=101, (d) 2008, $N_C$=136, (e) 2009, $N_C$=110, and (f) 2010, $N_C$=130. *N* indicates number of assigned parent pairs. Significant *p*-values are shown in bold.

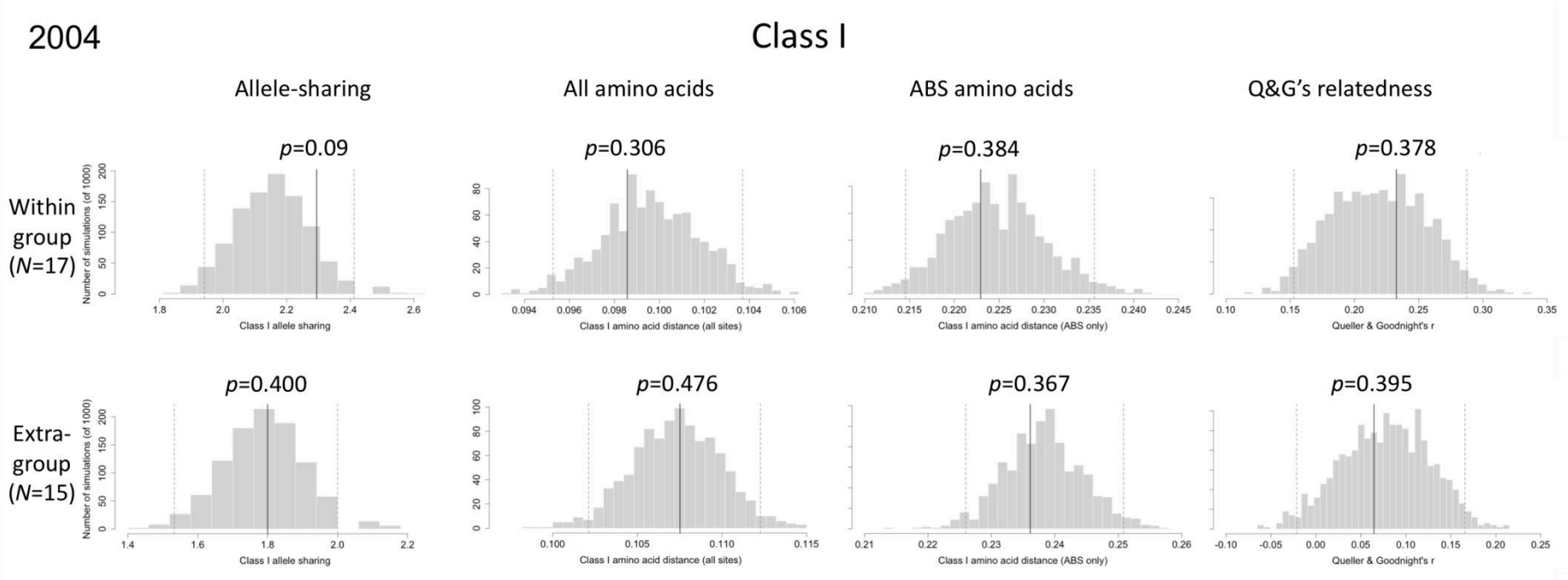


**Fig. 2b**

**Fig. 2** Mean MHC class I allele sharing, amino acid distance (all amino acid sites and ABS only) and Queller & Goodnight's index of pairwise relatedness (*R*) of assigned parent-pairs (filled line) compared with the frequency distribution of mean values generated from 1000 simulations of random pairings between breeding females and all of their within-group or neighbouring-group candidate fathers (grey bars), with two-tailed 95% CI (dashed lines) indicating cut-offs for significant departures from random mating. The data were analyzed separately for each year: (a) 1993, number of candidate fathers ($N_C$)=84, (b) 2004, $N_C$=102, (c) 2005, $N_C$=101, (d) 2008, $N_C$=136, (e) 2009, $N_C$=110, and (f) 2010, $N_C$=130. *N* indicates number of assigned parent pairs. Significant *p*-values are shown in bold.

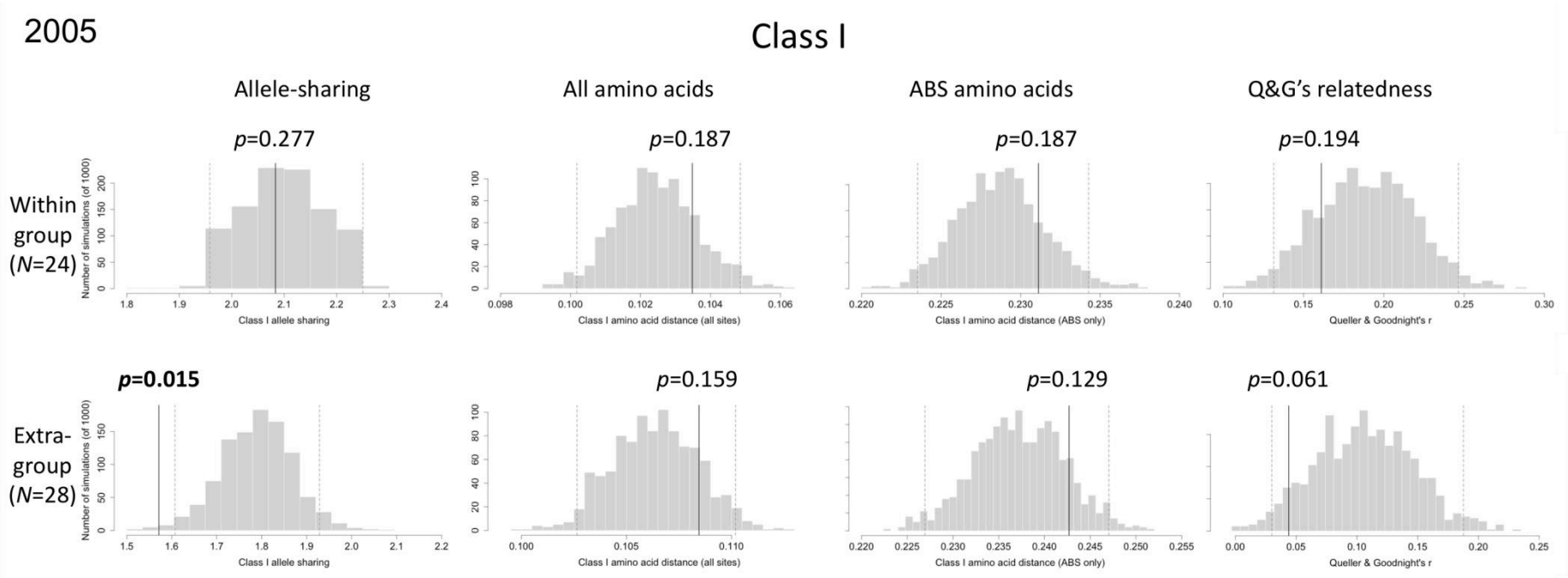


**Fig. 2c**

**Fig. 2** Mean MHC class I allele sharing, amino acid distance (all amino acid sites and ABS only) and Queller & Goodnight's index of pairwise relatedness (*R*) of assigned parent-pairs (filled line) compared with the frequency distribution of mean values generated from 1000 simulations of random pairings between breeding females and all of their within-group or neighbouring-group candidate fathers (grey bars), with two-tailed 95% CI (dashed lines) indicating cut-offs for significant departures from random mating. The data were analyzed separately for each year: (a) 1993, number of candidate fathers ($N_C$)=84, (b) 2004, $N_C$=102, (c) 2005, $N_C$=101, (d) 2008, $N_C$=136, (e) 2009, $N_C$=110, and (f) 2010, $N_C$=130. *N* indicates number of assigned parent pairs. Significant *p*-values are shown in bold.

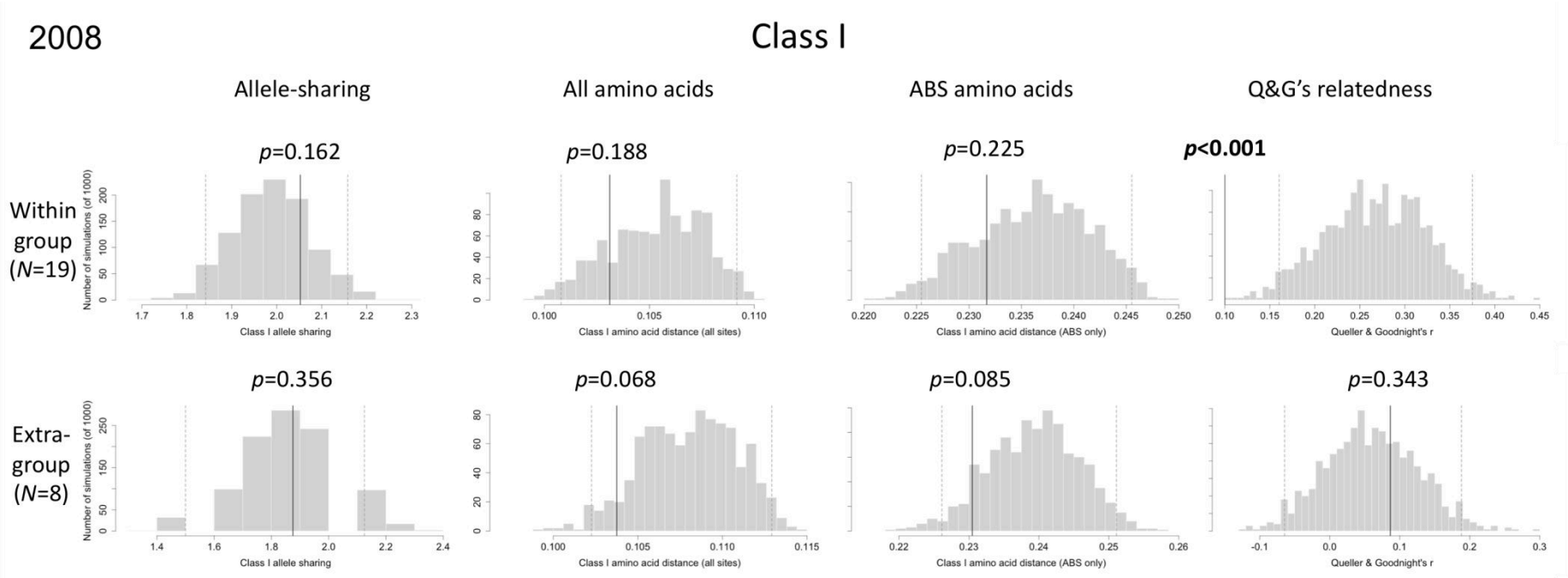


**Fig. 2d**

**Fig. 2** Mean MHC class I allele sharing, amino acid distance (all amino acid sites and ABS only) and Queller & Goodnight's index of pairwise relatedness (*R*) of assigned parent-pairs (filled line) compared with the frequency distribution of mean values generated from 1000 simulations of random pairings between breeding females and all of their within-group or neighbouring-group candidate fathers (grey bars), with two-tailed 95% CI (dashed lines) indicating cut-offs for significant departures from random mating. The data were analyzed separately for each year: (a) 1993, number of candidate fathers ($N_C$)=84, (b) 2004, $N_C$=102, (c) 2005, $N_C$=101, (d) 2008, $N_C$=136, (e) 2009, $N_C$=110, and (f) 2010, $N_C$=130. *N* indicates number of assigned parent pairs. Significant *p*-values are shown in bold.

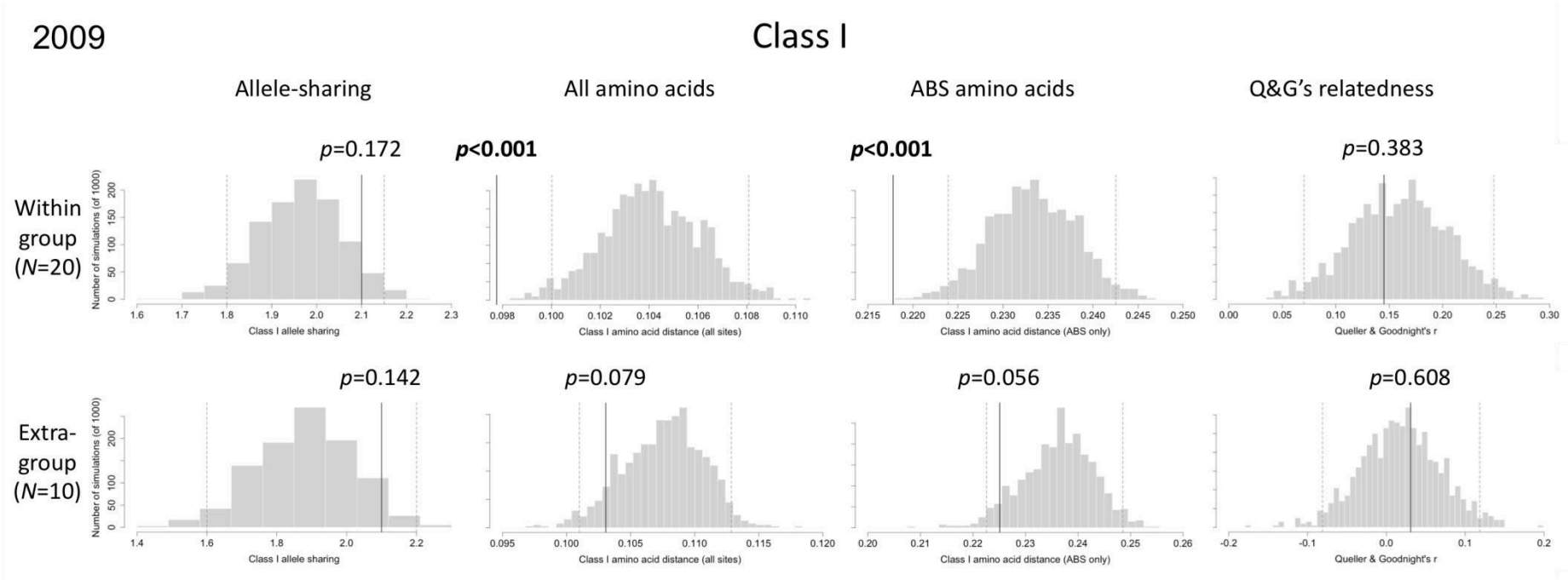


**Fig. 2e**

**Fig. 2** Mean MHC class I allele sharing, amino acid distance (all amino acid sites and ABS only) and Queller & Goodnight's index of pairwise relatedness (*R*) of assigned parent-pairs (filled line) compared with the frequency distribution of mean values generated from 1000 simulations of random pairings between breeding females and all of their within-group or neighbouring-group candidate fathers (grey bars), with two-tailed 95% CI (dashed lines) indicating cut-offs for significant departures from random mating. The data were analyzed separately for each year: (a) 1993, number of candidate fathers ($N_C$)=84, (b) 2004, $N_C$=102, (c) 2005, $N_C$=101, (d) 2008, $N_C$=136, (e) 2009, $N_C$=110, and (f) 2010, $N_C$=130. *N* indicates number of assigned parent pairs. Significant *p*-values are shown in bold.

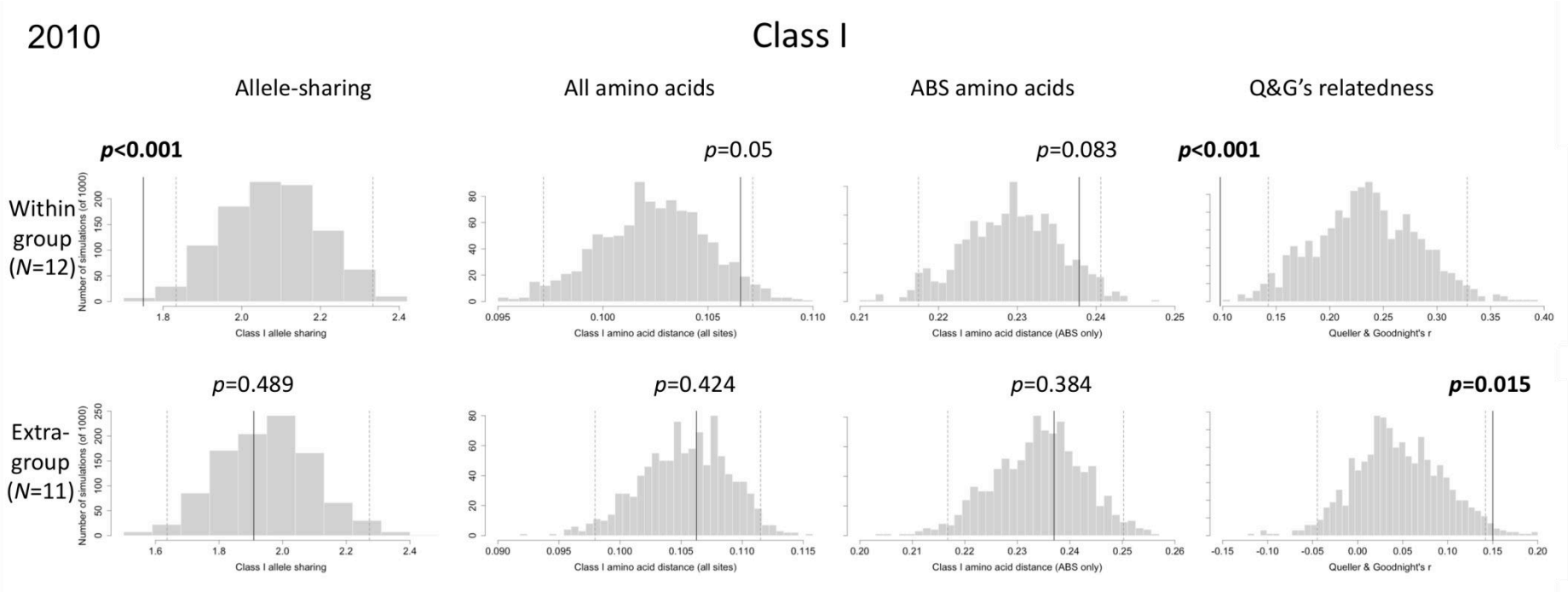


**Fig. 2f**

**Fig. 2** Mean MHC class I allele sharing, amino acid distance (all amino acid sites and ABS only) and Queller & Goodnight's index of pairwise relatedness (*R*) of assigned parent-pairs (filled line) compared with the frequency distribution of mean values generated from 1000 simulations of random pairings between breeding females and all of their within-group or neighbouring-group candidate fathers (grey bars), with two-tailed 95% CI (dashed lines) indicating cut-offs for significant departures from random mating. The data were analyzed separately for each year: (a) 1993, number of candidate fathers ($N_C$)=84, (b) 2004, $N_C$=102, (c) 2005, $N_C$=101, (d) 2008, $N_C$=136, (e) 2009, $N_C$=110, and (f) 2010, $N_C$=130. *N* indicates number of assigned parent pairs. Significant *p*-values are shown in bold.

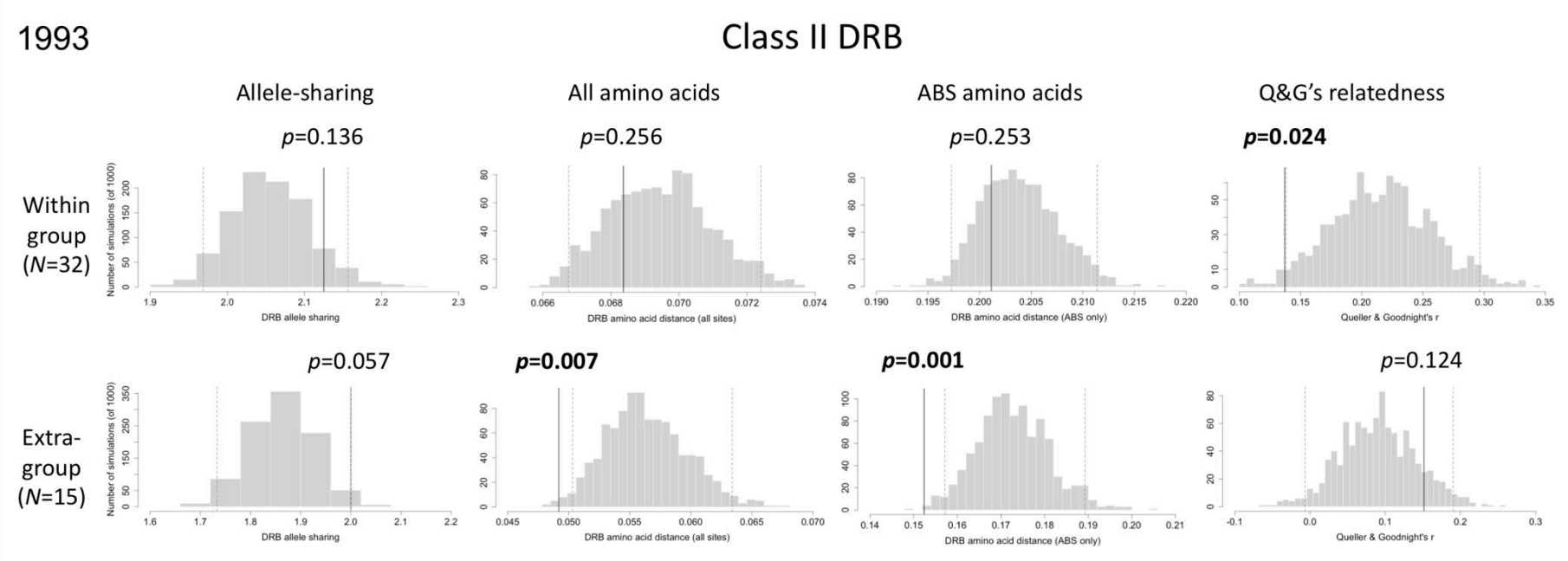


**Fig. 3a**

**Fig. 3** Mean MHC class II DRB allele sharing, amino acid distance (all amino acid sites and ABS only) and Queller & Goodnight's index of pairwise relatedness (*R*) of assigned parent-pairs (filled line) compared with the frequency distribution of mean values generated from 1000 simulations of random pairings between breeding females and all of their within-group or neighbouring-group candidate fathers (grey bars), with two-tailed 95% CI (dashed lines) indicating cut-offs for significant departures from random mating. The data were analyzed separately for each year: (a) 1993, number of candidate fathers ($N_C$)=87, (b) 2004, $N_C$=101, (c) 2005, $N_C$=100, (d) 2008, $N_C$=136, (e) 2009, $N_C$=109, and (f) 2010, $N_C$=128. *N* indicates number of assigned parent pairs. Significant *p*-values are shown in bold.

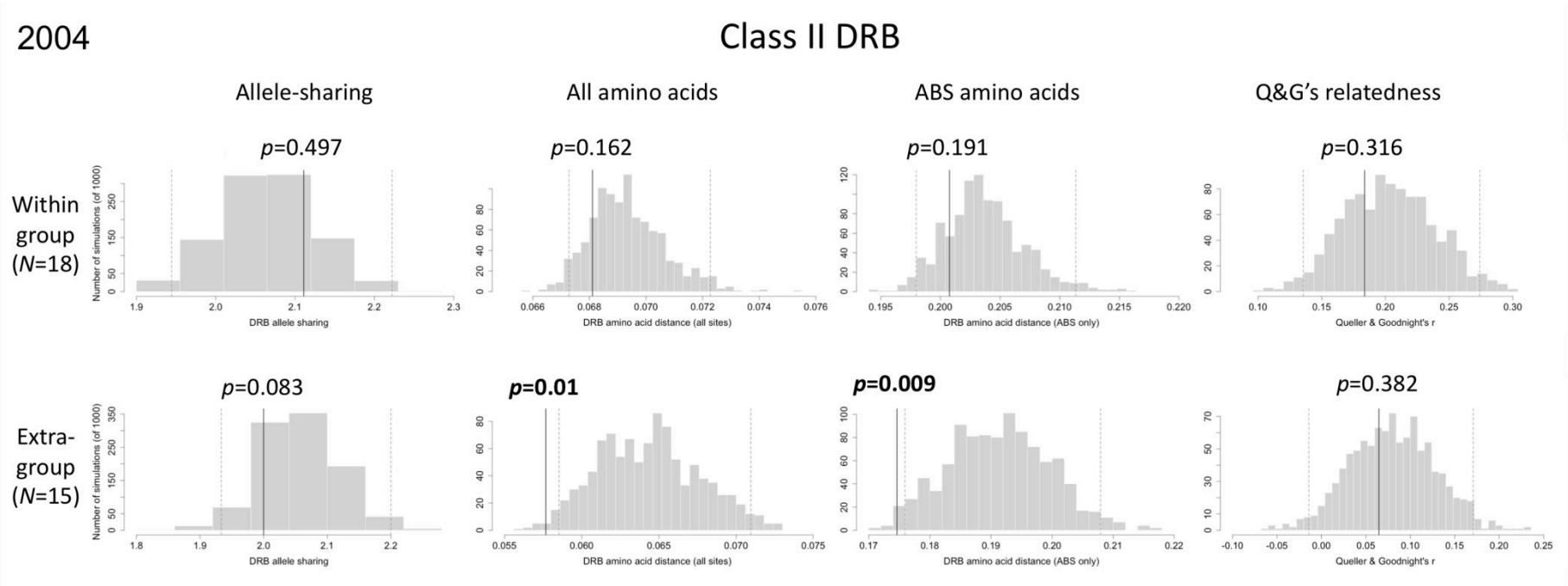


**Fig. 3b**

**Fig. 3** Mean MHC class II DRB allele sharing, amino acid distance (all amino acid sites and ABS only) and Queller & Goodnight's index of pairwise relatedness (*R*) of assigned parent-pairs (filled line) compared with the frequency distribution of mean values generated from 1000 simulations of random pairings between breeding females and all of their within-group or neighbouring-group candidate fathers (grey bars), with two-tailed 95% CI (dashed lines) indicating cut-offs for significant departures from random mating. The data were analyzed separately for each year: (a) 1993, number of candidate fathers ($N_C$)=87, (b) 2004, $N_C$=101, (c) 2005, $N_C$=100, (d) 2008, $N_C$=136, (e) 2009, $N_C$=109, and (f) 2010, $N_C$=128. *N* indicates number of assigned parent pairs. Significant *p*-values are shown in bold.

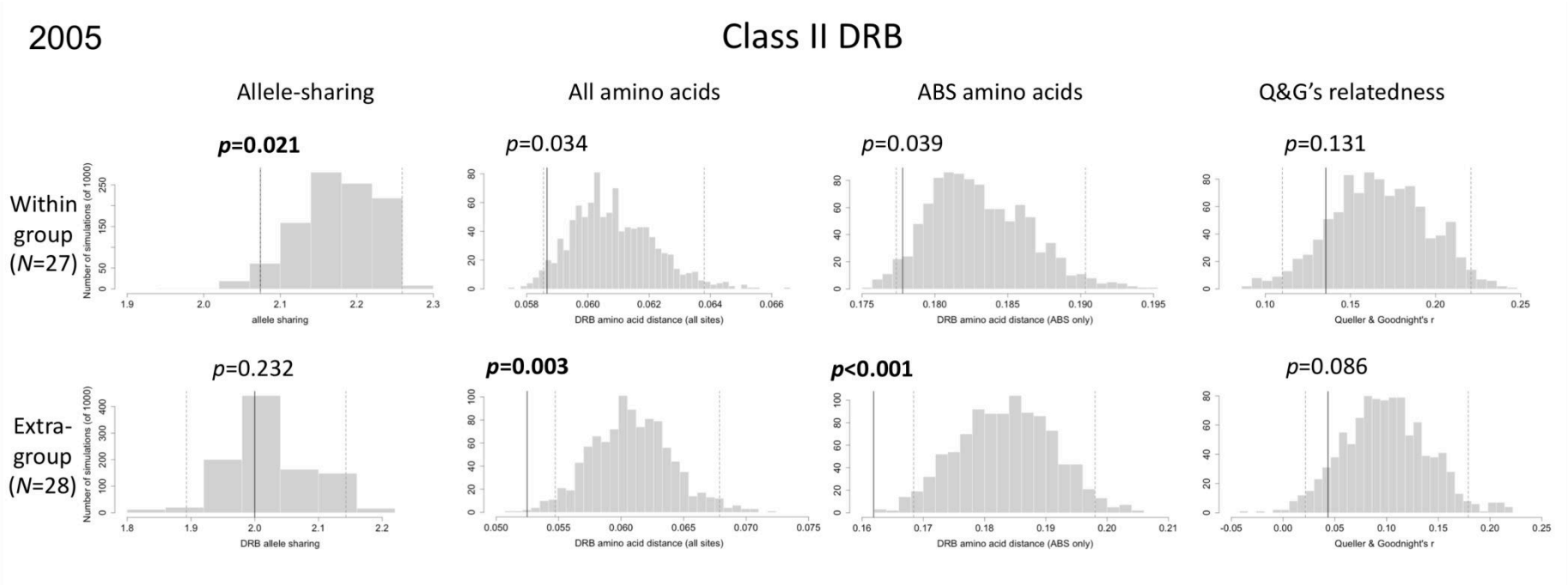


**Fig. 3c**

**Fig. 3** Mean MHC class II DRB allele sharing, amino acid distance (all amino acid sites and ABS only) and Queller & Goodnight's index of pairwise relatedness (*R*) of assigned parent-pairs (filled line) compared with the frequency distribution of mean values generated from 1000 simulations of random pairings between breeding females and all of their within-group or neighbouring-group candidate fathers (grey bars), with two-tailed 95% CI (dashed lines) indicating cut-offs for significant departures from random mating. The data were analyzed separately for each year: (a) 1993, number of candidate fathers ($N_C$)=87, (b) 2004, $N_C$=101, (c) 2005, $N_C$=100, (d) 2008, $N_C$=136, (e) 2009, $N_C$=109, and (f) 2010, $N_C$=128. *N* indicates number of assigned parent pairs. Significant *p*-values are shown in bold.

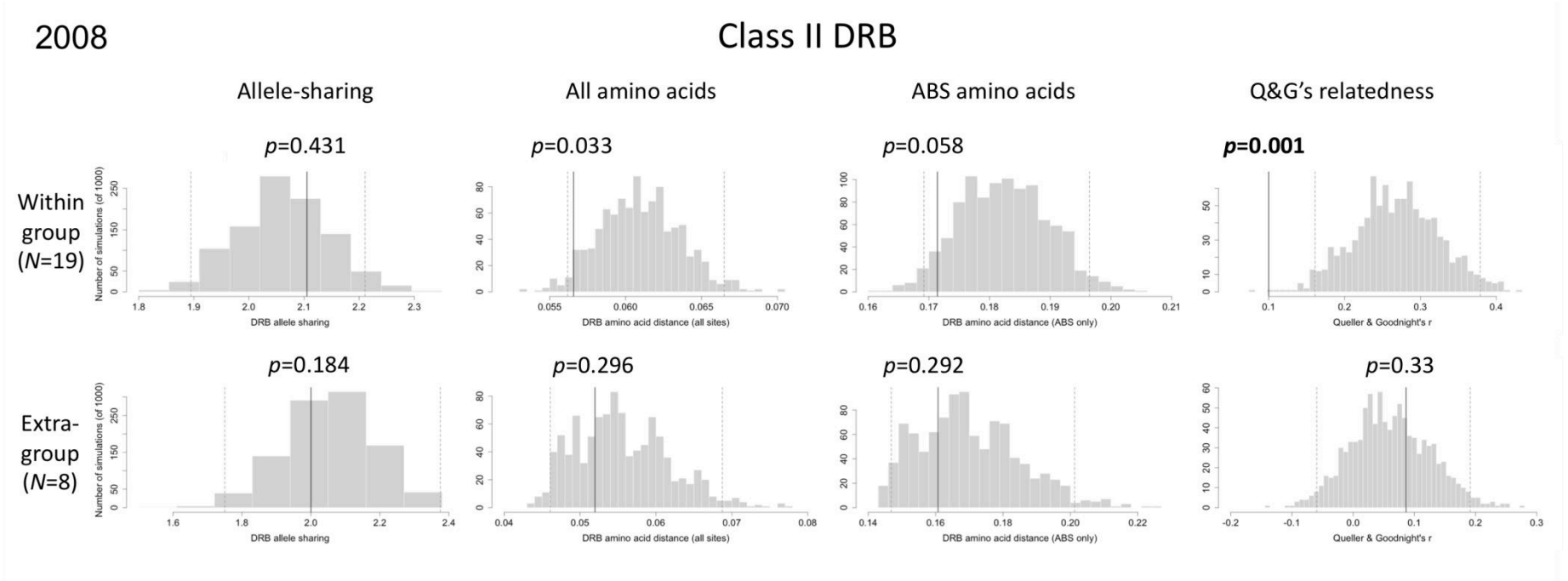


**Fig. 3d**

**Fig. 3** Mean MHC class II DRB allele sharing, amino acid distance (all amino acid sites and ABS only) and Queller & Goodnight's index of pairwise relatedness (*R*) of assigned parent-pairs (filled line) compared with the frequency distribution of mean values generated from 1000 simulations of random pairings between breeding females and all of their within-group or neighbouring-group candidate fathers (grey bars), with two-tailed 95% CI (dashed lines) indicating cut-offs for significant departures from random mating. The data were analyzed separately for each year: (a) 1993, number of candidate fathers ($N_C$)=87, (b) 2004, $N_C$=101, (c) 2005, $N_C$=100, (d) 2008, $N_C$=136, (e) 2009, $N_C$=109, and (f) 2010, $N_C$=128. *N* indicates number of assigned parent pairs. Significant *p*-values are shown in bold.

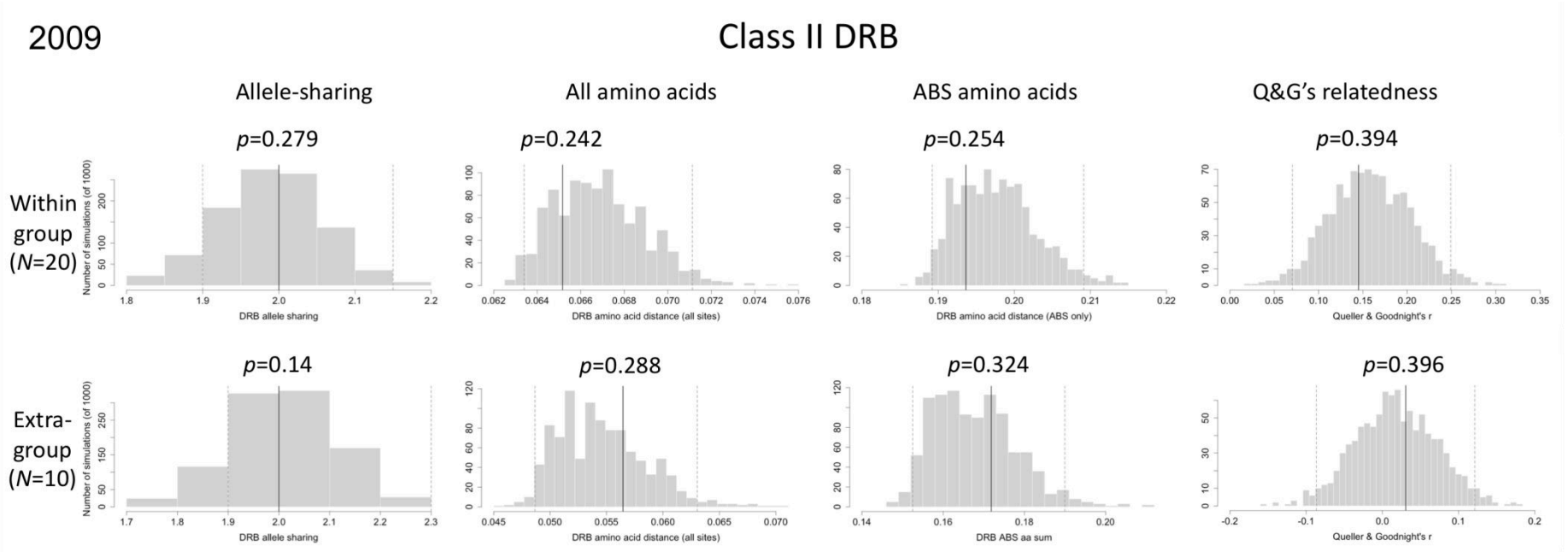


**Fig. 3e**

**Fig. 3** Mean MHC class II DRB allele sharing, amino acid distance (all amino acid sites and ABS only) and Queller & Goodnight's index of pairwise relatedness (*R*) of assigned parent-pairs (filled line) compared with the frequency distribution of mean values generated from 1000 simulations of random pairings between breeding females and all of their within-group or neighbouring-group candidate fathers (grey bars), with two-tailed 95% CI (dashed lines) indicating cut-offs for significant departures from random mating. The data were analyzed separately for each year: (a) 1993, number of candidate fathers ($N_C$)=87, (b) 2004, $N_C$=101, (c) 2005, $N_C$=100, (d) 2008, $N_C$=136, (e) 2009, $N_C$=109, and (f) 2010, $N_C$=128. *N* indicates number of assigned parent pairs. Significant *p*-values are shown in bold.

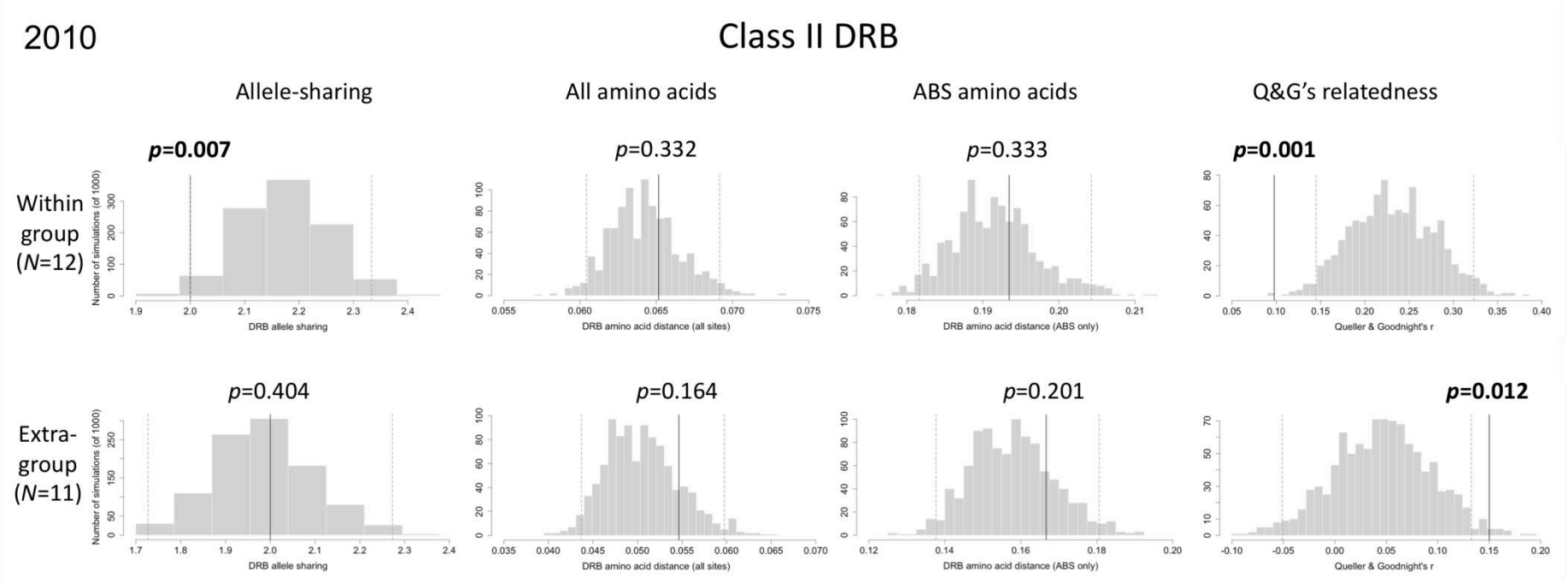


**Fig. 3f**

**Fig. 3** Mean MHC class II DRB allele sharing, amino acid distance (all amino acid sites and ABS only) and Queller & Goodnight's index of pairwise relatedness (*R*) of assigned parent-pairs (filled line) compared with the frequency distribution of mean values generated from 1000 simulations of random pairings between breeding females and all of their within-group or neighbouring-group candidate fathers (grey bars), with two-tailed 95% CI (dashed lines) indicating cut-offs for significant departures from random mating. The data were analyzed separately for each year: (a) 1993, number of candidate fathers ($N_C$)=87, (b) 2004, $N_C$=101, (c) 2005, $N_C$=100, (d) 2008, $N_C$=136, (e) 2009, $N_C$=109, and (f) 2010, $N_C$=128. *N* indicates number of assigned parent pairs. Significant *p*-values are shown in bold.

## A2.5 Discussion

Our study provides evidence for MHC-assortative mate choice. Although both class I and class II DRB allele sharing of assigned within-group pairs overall was less than expected under random mating, the class I and class II DRB amino acid distances overall were more similar in both assigned within- and neighbouring-group pairs, which indicates mate choice for MHC-similar males.

The results based on MHC allele sharing are different from those based on MHC amino acid distance. This is not unexpected as amino acid distance incorporates functional difference between allele. The divergence between these parameters is also evident in other studies, which have shown the amino acid distance to be more informative than allele sharing in identifying MHC-based mate choice (Landry *et al.*, 2001; Miller *et al.*, 2009).

There were considerable annual fluctuations in the occurrence of MHC-based mate choice. MHC-based mate choice based on class II DRB genes was shown in more years than based on class I genes. There was mating based on both genes in some years (e.g. 1993 neighbouring-group pairs), but some years showed neither (e.g. 2010 neighbouring-group pairs). Although overall there was significant effect of MHC-based mate choice based on both genes in both within- and neighbouring-group pairs, this marked fluctuation in the level of MHC similarity between assigned parent pairs suggests that females may actively choose MHC-similar males in some years. We cannot provide a definitive answer to which conditions favoured MHC-assortative mating in those years. Fluctuation in population relatedness structure is proposed to be affected by ecological constraints such as predation and disease risk (Jaatinen *et al.*, 2012). We speculate that MHC-based mate choice may only pay for females when ecological constraints such as disease risk are severe enough, and the specific conditions needed for MHC-based mate choice to be favoured may vary over time. There was also difference between within- and neighbouring-group pairs with respect to MHC-based mate choice. Neighbouring-group pairs showed a stronger effect of MHC-assortative

mate choice than within-group pairs (Table 1), which indicates females may be able to seek MHC-similar extra-group males more actively than within-group males.

Our analyses confirmed that MHC genotype does influence mating preference. This is different to MHC-disassortative mating found in other species (Landry *et al.*, 2001; Gillingham *et al.*, 2009; Miller *et al.*, 2009; Juola & Dearborn, 2012), as badgers do not maximize MHC differences between mates. There was a lack of finding for MHC-assortative mating pattern, i.e. preference for MHC-similar individuals, which was reported in mice (Yamazaki *et al.*, 1978), humans (Roberts *et al.*, 2005) and tiger salamanders (*Ambystoma tigrinum*; Bos *et al.*, 2009). Males with MHC alleles that are more similar to a female over males exhibiting more divergent MHC alleles may have reproductive advantage (Bos *et al.*, 2009). The lack of preference for MHC-dissimilar individuals suggests that such mating do not increase female reproductive success. Possible disadvantage arise from having parents with highly dissimilar MHC include increased loss of T-cells (Lawlor *et al.*, 1990; Nowak *et al.*, 1992), increased risk of autoimmune diseases or disruption of co-adapted gene complex (Kaufman, 1999) in offspring. The choice of a genetically similar mate would, on average, minimize heterozygosity. A study in mice shows MHC heterozygotes had lower resistance to infection, survival and reproductive success compared to homozygotes (Ilmonen *et al.*, 2007), however, heterozygote advantage that MHC heterozygous individuals might benefit from the ability of binding a wider range of pathogenic antigens was reported in a number of species (Penn & Potts, 1999; Worley *et al.*, 2010).

MHC-disassortative mating has been proposed as a mechanism for inbreeding avoidance (Potts *et al.*, 1994). However, there was no preference for MHC-dissimilar males in the years in which inbreeding avoidance was suggested for within-group mating in our study, which suggests that MHC-based cues were not used as a reference for within-group inbreeding avoidance. The small MHC amino acid distance of within-group or neighbouring-group pairs was not a by-product of inbreeding, which was only found in neighbouring-group pairs in 2010 and no MHC-assortative mating was found in that year.

MHC-based mate choice could be achieved by pre-copulatory choice, through a preference to mate with MHC-similar males. MHC genotypes might be recognizable through olfactory cues (Yamazaki *et al.*, 1982; Brennan & Kendrick, 2006), as the MHC molecules and/or peptides derived from them can contribute to individual olfactory profiles. For example, MHC genes are involved in the generation and recognition of odour-types in mice (Leinders-Zufall *et al.*, 2004; Ziegler *et al.*, 2005). Badgers potentially exchange information about individual specific parameters through subcaudal gland scent (Buesching *et al.*, 2002). This secretion may include information on the MHC genotype of the badger that deposited it. Another possibility is MHC genes may influence microbiota (e.g. inside the subcaudal pouch; Sin *et al.*, 2012a) that generate secondary metabolites and contribute to the actual odour of the host (Singh *et al.*, 1990). The odour of badgers is thus likely to play an important role if pre-copulatory MHC-based mate choice happens in this species. An alternative, but not mutually exclusive, explanation is that MHC-based mate choice operates after copulation, through cryptic female choice such as selective fertilization and selective abortion (Wedekind *et al.*, 1996; Ziegler *et al.*, 2002; Ziegler *et al.*, 2005). MHC-based post-copulatory choice occurs in mammals such as grey mouse lemurs (*Microcebus murinus*; Schwensow *et al.*, 2008a). Putatively superfoetation and embryo reabsorption in badgers (Canivenc & Bonnin, 1979; Yamaguchi *et al.*, 2006) is likely to facilitate multiple paternity (Dugdale *et al.*, 2007) and post-copulatory sexual selection. However, the mechanism behind MHC-assortative mate choice is yet to be discovered.

There is growing evidence indicating a trend of mate preference towards minimal or intermediate MHC divergence (Milinski, 2006; Forsberg *et al.*, 2007; Bos *et al.*, 2009; Kalbe *et al.*, 2009; Woelfing *et al.*, 2009) in addition to maximal divergence (Landry *et al.*, 2001; Miller *et al.*, 2009; Juola & Dearborn, 2012) − i.e. optimal divergence. There is also growing evidence showing a complex and context-dependent pattern of MHC-based reproduction (Richardson *et al.*, 2005; Bos *et al.*, 2009) rather than simple MHC-disassortative mating. Mate choice based on the MHC but not on genome-wide background has been reported for a number of species (e.g. Landry *et al.*, 2001;

Juola & Dearborn, 2012), but there is a lack of study in wild mammals currently. This is the first published study of MHC-based mate choice in the order Carnivora, of evidence for MHC-assortative mating, which is contrast to most other studies that found a preference for MHC dissimilar mates (e.g. Landry *et al.*, 2001; Miller *et al.*, 2009; Juola & Dearborn, 2012) or no preference (e.g. Westerdahl, 2004). Our study thus adds to the support for the evidence-lacking MHC-assortative mating and the changing view of MHC-based mate choice.

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