THE USE OF GENETIC MARKERS TO REVEAL DYNAMIC PROCESSES IN A COMMON TOAD (BUFO BUFO) POPULATION

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DECLARATION

I hereby declare that the work that is presented in this thesis is my own work unless otherwise stated. Details are given in each chapter where any of the results are produced in collaboration with other members of the research group.

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Robert Coles

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ABSTRACT

In contrast to birds and mammals for example, amphibian population studies only rarely capture information based on genealogical relationships among individuals. As a consequence, we only have very limited knowledge about individual fitness measures such as lifetime reproductive success and the consequences of such variation on the linkage between generations of amphibians in the wild. The present thesis makes use of an existing long-term study on the common toad (*Bufo bufo*) in southern England (Dorset) to genetically identify parent-offspring relationships among approximately 850 individual toads, representing two successive generations (2004/2005/2006 and 2008/2009). The dataset enabled the comparison of measures of effective population size as well as effective breeding size, revealing ratios between 0.07 and 0.26. These data also showed an increasing trend with time and were (by some estimators) confirmed by the cross-generational parentage analysis which revealed a high reproductive skew among individuals. Forty-five percent of offspring could be assigned to a least one parent; in total, 6% of male parents and 30% of female parents were inferred. The pedigree information was also used to identify a possible hereditary basis for an observed decrease in female body condition and fecundity correlated to increased environmental temperatures. There was no indication for heritability of body size, body weight and body condition, suggesting that the documented decrease is based on phenotypic plasticity rather than evolutionary adaptation. However, kinship data that shows the population is less inbred with time coupled with the effective breeding number estimates showing an increasing trend with time suggest that despite the absence of evolutionary change, this population may still be able to circumvent the adverse effects associated with decreased body condition.

CHAPTER 1:

Introduction

1.1. Long-term individual-based population studies

Some of the most valuable insights into animal ecology and evolutionary biology have come through the employment of long-term, individual-based population studies (Clutton-Brock & Sheldon, 2010). They are able to observe some of the most significant processes that affect demographic and evolutionary responses over multiple generations. Whereas population studies solely based on count data are restricted to revealing, for example, population size fluctuations without the potential to elucidate the underlying adaptive forces. In order to predict underlying mechanisms that alter population numbers and investigate environmental effects on particular life history stages, individual-based data spanning at least two generations are required to estimate parameters such as lifetime reproductive success. Seminal studies of long-term individual-based research include the ones on passerine birds (tits, *Paridae*) in Holland (Kluijver, 1951) and Britain (Lack, 1964). During research spanning more than a decade, Lack (1964) studied fluctuations in numbers of breeding pairs in a population of great tits. The ground breaking study revealed the relationship between the most commonly observed clutch sizes and the optimum brood size for reproductive success when considering survival rates of juveniles. It also showed that clutch-size and the production rate of fledglings was reduced when breeding densities were higher and that this did not affect fluctuations in the size of the breeding population. Many similar studies of birds (Harris, 1970; Dunnet *et al.*, 1975; Newton, 1985) ensued from the research by Kluijver and Lack, although the majority were restricted because individuals were seldom habituated to close observation. Subsequent research on mammals also for example began to habituate individuals to close observation (Douglas-Hamilton, 1973; Festa-Bianchet, 1989), whereas follow-up studies on birds focused on the costs and benefits of different phenotypic traits, behavioural strategies, and social groups. Clutton-Brock & Sheldon (2010) have identified six characteristics of individual-based studies of ecology and evolution which encompass the reliable provision of recording age-related changes in life history parameters, the ability to study the causes of variation in growth, breeding success and survival, social structure and kinship, the differences in breeding success between individuals and their offspring, measurements of the strength and direction of selection, and the study of quantitative genetics. Breeding success, selection, and quantitative genetics have direct relevance to the current study.

Cross-generational studies on breeding success reveal the impact that specific mating strategies have on the structure of subsequent stages of the lifespan or individual survival (Clutton-Brock, 1988). They also determine the costs and benefits of specific mating patterns that have led to the wide range of animal mating systems. Measures of the strength and direction of selection are very important when investigating the ecology and evolutionary dynamics of wild populations since selection is a central process of evolution. Changes in the temporal variation, strength, direction and form of selection have been studied (Moorcroft *et al*, 1996; Coltman *et al*, 2005; Siepielski *et al*, 2009) with varying environmental conditions frequently shown to be significant (Wilson *et al*, 2006; Robinson *et al*, 2008). For example, a study of the relationship between a secondary sexual trait (male horn length) and fitness, in Soay Sheep, showed that the association can change from positive to negative with changing environmental conditions. Individuals within this population experience a very heterogeneous environment that causes changes to the strength of selection for associations between reproductive success and male horn length generating fluctuating selection. This fluctuation of selection has been suggested as a mechanism by which genetic variance can be maintained for secondary selected traits. Furthermore, studying the temporal dynamics of, and responses to, selection can reveal information about the mechanisms maintaining variation within populations (Sasaki &

Ellner, 1997) and the potential adaptive rate which can parallel changing environmental conditions (Siepielski *et al*., 2009; Phillimore *et al*., 2010).

Quantitative genetics is concerned with the genetic basis of traits governed by multiple genes and their interactions with the environment. Since many traits in natural populations may be quantitative and the mechanisms controlling genetic variation within these traits are not fully understood (Kruuk *et al*., 2008), insights into quantitative genetics are therefore crucial for our understanding of evolution (Barton & Keightley, 2002). Moreover, the study of quantitative genetics is fundamental to our understanding of the response of phenotypic traits to selection and thus how populations will respond to global environmental changes (Ellegren & Sheldon, 2008). More specifically, studies of quantitative genetics have provided insights into inbreeding (Collevatti *et al*., 2007; Szulkin & Sheldon, 2008), herita bility (Charmantier *et al*., 2006; Kruuk *et al*., 2008), the covariance between traits (Robinson *et al*., 2008), and gene flow (Zeyl *et al*., 2009). Understanding these genetic forces requires information about the genetic composition and genealogical relationships within populations that can be generated via genetic markers and can in turn provide tools for studies into animal conservation.

1.2. Biodiversity and conservation

The natural ecosystems and habitats of the world continue to be destructed and disturbed which is causing the widespread decimation of species. Efforts to reduce such destruction and conserve current biodiversity and genetic diversity, especially since many species remain undescribed, are therefore imperative (Bickord *et al*., 2006). Biological diversity can be defined as the variation in both phenotypes and underlying genotypes of all plants and animals and of the ecosystems in which they exist. There are three currently recognisable units of diversity, the genetic diversity, species richness, and ecosystem diversity (variation in communities and their environment) (Ramanatha & Hodgkin, 2002). Many areas of conservation interest have focused on the maintenance and investigation of levels of genetic diversity within populations. Due to the adaptive ability of species with high levels of genetic diversity, it is those that are more able to undergo evolutionary change and genetically adapt to changing environments that may be adverse. Genetic variation therefore plays an important role in conservation of many species as studies seek to understand losses of variation, disentangle the effects of environmental and evolutionary responses, and unravel phylogenetic or genealogical relationships.

In order to investigate such processes, means by which individuals can be identified within populations are required. These can be achieved via the genetic identification of individuals by using molecular markers such DNA barcodes or DNA fingerprints. DNA fingerprinting can for example, unambiguously identify individuals within populations and as a result enable the reconstruction of genealogical relationships and the placement of individuals into discreet familial relationships.

These inferences of genealogical relationships of individuals (pedigrees) in wild animal populations can address many questions of evolution, ecology, and conservation (Blouin, 2003). Before the genetic inferences of such relationships could be achieved, however, the field underwent a significant developmental process. Initially, this began with the introduction of chromosomal polymorphism studies (Levine *et al.*, 1980) and later with allozyme electrophoresis (Hanken & Sherman, 1981). However it was not until DNA fingerprinting (Jeffreys, 1985a,b) emerged, allowing the unambiguous identification of individuals, that there was genuine scope for genetic parentage analysis. Although there was a subsequent surge in the number of studies (Jones & Ardren, 2003), it was the technical and statistical constraints of DNA fingerprinting applications that restricted applications to mostly mammals and birds (Gibbs *et al.,* 1990; Westneat, 1990). However, several years after the utilisation of minisatellites, microsatellites were discovered (Tautz, 1989) and soon became the molecular marker of choice for inferring parentage (Jones *et al.,* 2010). Microsatellites became the preferred markers because they were the first single-locus, co-dominant, hypervariable markers (Avise, 2004), for which much of the statistical framework had already been formulated (Jones *et al.,* 2010). Microsatellites have become one of the most useful tools in molecular ecology and are key to providing insights into the ecology and evolution of wild animal populations and, therefore, for conservation efforts.

1.3. Amphibians and conservation

The literature is replete with studies assessing, reviewing, and detailing the causes and interacting forces of amphibian declines (Blaustein & Wake, 1990; Berger *et al*., 1998; Lips, 1999; Alford & Richards, 1999; Houlahan *et al*., 2000; Blaustein *et al*., 2001; 2011; Stuart *et al*., 2004; Beebee & Griffiths, 2005; Pounds *et al*., 1999; 2006; Halliday, 2008; Allentoft & O'Brien, 2010). This is because, within the vertebrates, amphibians are the group that are most severely affected by the current biodiversity crises, with 32% of the currently known species under threat (Stuart *et al*., 2004). Conservation of amphibians is important because their current threat indicates the extinction of a diverse taxonomic group with many unique characteristics such as their life-history traits. This loss will not only significantly affect global biodiversity and genetic diversity, but will also result in a loss of benefits to humans. For example, amphibians have contributed to the study of antibiotic and anti-tumour properties, analgesics, anti-inflammatory compounds, and natural adhesives. Moreover, 10% of Nobel prizes for research in physiology and medicine have been awarded for the study of frogs (Tyler *et al*., 2007). Furthermore, critical and deleterious ecological effects could emerge signifying a collapse of the global ecosystem (Halliday, 2008).

Due to their environmental sensitivity, amphibians are generally considered as indicator species, and can therefore provide insights into subtle environmental problems (Hopkins, 2007). This sensitivity can be caused by their central place in the food chain, their utilisation of both aquatic and terrestrial environments, and their unique feeding ecologies at each different life-cycle stage (Allentoft & O'Brien, 2010). It is because of this environmental sensitivity that they are more susceptible than other vertebrates to the threats associated with a changing environment. The threats faced by amphibians range from the molecular to the community level (Blaustein *et al*., 2011) and include habitat destruction and fragmentation, increased UV-radiation due to ozone depletion, predation or competition by non-native species, sensitivity to pollutants or toxins, road-kill, overexploitation, diseases such as chytridiomycosis, and climate change (Allentoft & O'Brien, 2010; Blaustein *et al*., 2011).

As well as the detrimental effects from anthropogenic activities such as the destruction of terrestrial and aquatic habitats, environmental pollution due to fertilizers and industrial waste, recreation and general urbanisation (Kuzmin, 1999), amphibians are also suffering from anthropogenically-induced climate change (Blaustein *et al*., 2011). For example, alterations to the levels of precipitation as a result of recent climate change have been reported to increase susceptibilities to the pathogen *Saprolegnia ferax* (Blaustein *et al*., 2011). Similarly, the widespread decline of amphibian populations due to *Batrachochytrium dendrobatidis* is made worse as climate change appears to afford optimal conditions for the spread of the disease (Pounds *et al*., 2006). Amphibians also face threats, associated with climate change, to their breeding and reproductive success. For example, due to higher cloud coverage over the mountains of Costa Rica, forests can become drier and less suitable for successful reproduction (Pounds *et al*., 1997). Furthermore, as a result of early spring temperatures, many amphibian species have had their breeding phenology disrupted and breed earlier than usual (Beebee, 1995; Blaustein *et al*., 2001; Tryjanowski *et al*., 2003).

1.4. The common toad (Bufo bufo)

The common toad is the most populous amphibian in the UK and widespread throughout Europe (Figure 1.1), and debatably one of the most successful vertebrates on the globe with distributions also in central Asia and North Africa (Beebee, 1996).

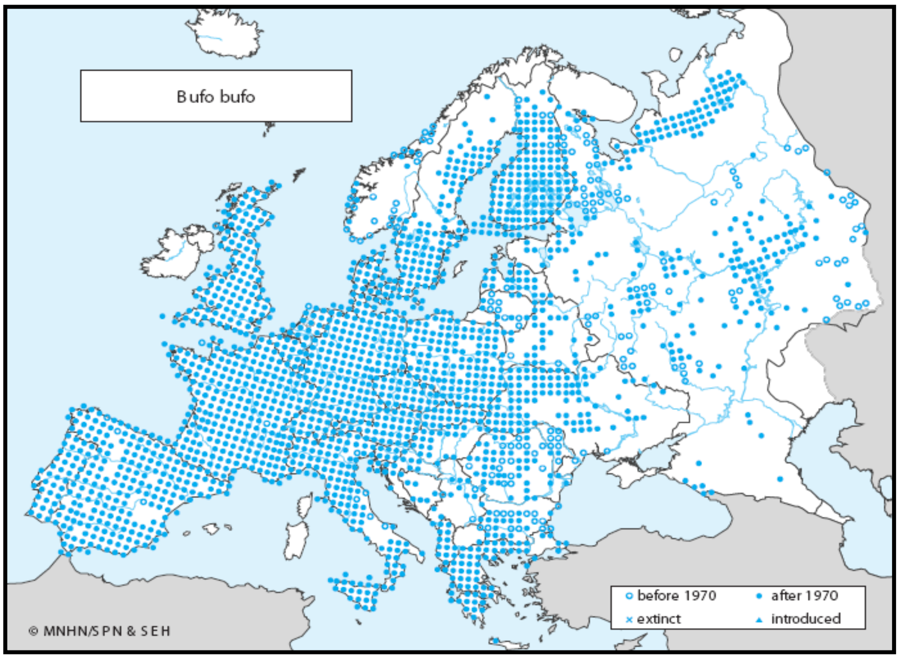


Figure 1.1. Distribution of the common toad, *Bufo bufo*, throughout Europe (Kuzmin, 1999).

The taxonomy of the genus *Bufo* is complex. Until 2006, the genus contained over 280 species before being divided into several genera (Frost *et al*., 2006). *Bufo bufo* has been recently acknowledged as to have a distinct western and eastern European species with some eastern European species now formally recognised, such as *B. gargarizans* and *B. japonicas* (Recuero *et al*., 2011; Garcia-Porta *et al*., 2012).

Recent evidence based on molecular markers now also suggests that *B. bufo* in western Europe can be divided into two separate species due to a zone of sharp mitochondrial DNA divide running through central France; Britain would remain inhabited by *B. bufo*, whereas populations in South-Western France and the Iberian peninsula would need to become recognised as *B. spinosus* (Recuero *et al*., 2011). However, despite a further study confirming the patterns of genetic divergence using different mitochondrial regions (Garcia-Porta *et al*., 2012), the taxonomy of *B. bufo* in Europe still remains undefined.

Individuals of *Bufo bufo* have warty skin, distinct bulges located at the back of the head known as the parotoid glands, and a yellow/golden brown iris with a horizontal pupil. Although colour variation exists, with some individuals observed with red brick spots, individuals tend to be a brown/greenish grey to a dirty speckled beige colour, from their dorsum to ventrum respectively. Unlike other British anurans such as the common frog (*Rana temporaria*) and the natterjack toad (*B. calamita*), individuals tend to walk not jump (Herpetofauna, 2010). As with other toads, *B. bufo* is active primarily during twilight. Individuals hibernate singularly or as a group and usually on land between September/November to March/June, depending on latitude and altitude, before migrating to their breeding pond. Hundreds or even thousands of toads arrive at their breeding ponds every spring to enter explosive periods of reproduction that last over several days (Beebee, 1996). Males amplex females with the aid of nuptial pads on their forearms (Figure 1.2) and do so for up to a few days until the female releases her spawn. Breeding may take place in lakes, ponds, ditches, large puddles and streams (Kuzmin, 1999).

Males reach sexual maturity around one year before females (average, around 3 years), and also enter the breeding ponds earlier and remain there longer (Davies & Halliday, 1979). Also because females do not breed annually, males outnumber females at breeding sites to cause male-biased operational sex ratios (OSR) typical for toad species (Arak, 1983). This leads to intense scramble competition between males and results in situations of pronounced sexual conflict, including the occasional drowning of females by competing males.

Figure 1.2. Male (attached dorsally) and female common toads in amplexus at the study site.

Females tend to be larger than males, reaching up to 13 cm and 8 cm respectively, with female fecundity being proportional to body mass. That body size is a measure of female fitness creates the possibility that female body size will play a role in male mate selection. Larger males might be at an advantage during situations when, dorsally attached in amplexus, they are forced to defend female mates from mating attempts by other males.

Due to the male biased OSR and scramble competition, male common toads have often been considered almost unlimited in their reproductive potential because they do not contribute anything to the offspring other than sperm. In an experimental investigation of sperm stores, fertilisation success, and sexual motivation, of *Bufo bufo* over the course of repeated matings, Hettyey *et al*. (2009), however, demonstrated the existence of sperm depletion after multiple matings related to body size. However, while other studies have reported body size to be important in mating success for both males and females (Davies & Halliday, 1977; Reading & Clarke, 1983), others have found no evidence (Hoglund & Robertson, 1987).

Despite still being a rather abundant amphibian, the common toad has been shown to suffer from adverse environmental effects and declines (Hitchings & Beebee, 1998; Beebee & Griffiths, 2000; Carrier & Beebee, 2003; Cooke & Sparks, 2004; Wilkinson *et al*., 2007) and is now on the Joint Nature Conservation Committee's (JNNC) UK Biodiversity Action Plan (UKBAP) priority species list (JNNC, 2007). It has been estimated that toad populations in rural areas of south-east and central England have declined by about 50% (Carrier & Beebee, 2003).

Examples of studies on adverse effects of environmental change to *B. bufo* populations include Hitchings & Beebee (1998) who used allozymes and minisatellite genetic markers to demonstrate a marked difference in genetic diversity between rural and urban populations in Britain. These authors found low levels of observed heterozygosity for both genetic markers, and high levels of genetic differentiation (F_{ST}) for populations associated with urban development linked to a loss of fitness as measured by tadpole survival rates. In a study of *B. bufo* population declines in Jersey, Wilkinson *et al*. (2007) reported measures of genetic diversity which were not be at critically low levels, but also found high levels of population structure which suggested that further urban development pressures might cause further declines. In fact, populations of Jersey common toads have been in decline for the past 40 years (Le Sueur, 1968; Beebee & Griffiths, 2000). Despite the finding that anthropogenic land use can cause reductions to heterozygosity and fitness, increased population differentiation, and general population declines, other studies have found no apparent causative agent for common toad declines. Carrier & Beebee (2003) conducted a nation-wide survey of *B. bufo* populations and found population reductions of at least 50% for south-east and central England. The study also showed that in comparison to the common frog, the common toad was faring worse, and in the absence of significant alterations to the land surrounding these populations the decline had an inexplicable cause.

1.5. Rationale

A continuous 30-year study of common toads by Dr Chris Reading (Reading, 1983; Reading, 1986; Reading, 1998; Reading & Clarke, 1995; Reading & Clarke, 1999; Reading, 2001; Reading, 2003; Reading, 2006; Reading, 2007; Reading, 2009 a,b; Reading & Clarke, 2009) based at the NERC Centre for Ecology and Hydrology, Oxford, has indicated a link between climate change and a reduction in body condition, survival, and female fecundity (Reading, 2007, Figure 1.3).

The study encompasses an extensive dataset with yearly data collection, and known individual parameters such as the sizes and weights of toads and the knowledge of which individual pairs were found mating (i.e., in amplexus). However, while long-term population studies of this kind do exist for amphibians (Pechmann *et al.,* 1991; Reading, 2007) very few currently exist that are pedigree-based and focus on a single population spanning several generations (Kruuk & Hill, 2008; Clutton-Brock & Sheldom, 2010).

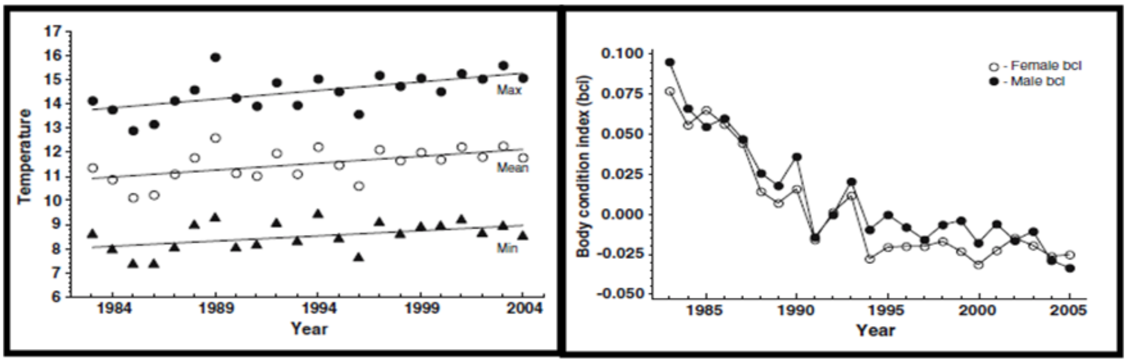


Figure 1.3. Left: Change in the mean maximum, mean and mean minimum temperatures (^{o}C) between the 1st of April each year, and the beginning of breeding season the following year for *Bufo bufo* (1982-2004). Right**:** Change in mean female and male body condition index (BCI). Reading, (2007).

1.6. Aims

Therefore, the aim of the current study is to make use of this information and create one of the first long-term pedigree-based datasets for an amphibian species. This is to be accomplished by inferring genealogical relationships via genetic data derived from tissue samples from individuals spanning two consecutive generations. Moreover, by combining the genetic data with the recorded demographic data, the aim is to quantify the heritability of fitness in the form of body condition. This is particularly important because understanding the interplay between genes and the environment and disentangling evolutionary and plastic responses is crucial for our efforts to conserve wild animal populations faced with the threat of climate change.

1.7. Objectives

- To extract DNA from *Bufo bufo* toe clippings from individuals collected in 2004/2005/2006 and 2008/2009 (forming two successive generations).
- To optimise PCR conditions for specific primers (characterised in Brede e*t al*, 2001).
- To perform PCRs on extracted DNA.
- To genotype all products that underwent PCR amplification on the Applied Biosystems ABI3130 genetic analyser.
- To score alleles from the genotyping data using the software Peakscanner.
- To convert the allele sizes (bps) from 2 decimal places to usable integers using the software Tandem.
- To perform analysis to check for errors in the data using the software Genepop, Microchecker & Tandem.
- To perform parentage analysis using the software Colony.
- To compare parentage inferences with parental relationships observed in the field.
- To calculate pairwise relatedness and inbreeding coefficients using the program KINGROUP.
- To estimate the effective population size using different methods: linkage disequilibrium, heterozygote excess, and sibship assignment.
- To regress the BCI data of the parents against the BCI data (BCI data available from Fig. 1.3) of the offspring, as per the relationships inferred by Colony, to obtain an estimate of heritability for body condition.
- To regress the N_e/N data with BCI data/inbreeding coefficients to test for patterns in the data.

- To discuss the results of chapters 3-5 independently to interpret the parentage and N_e data and to assess the evolutionary responses of this wild common toad population.
- To form a general discussion, compiling interpreted results from all chapters.

CHAPTER 2:

Materials and Methods

2.1. Study site

The study site is a pond, formed from a flooded clay pit, located to the north of the Purbeck Hills in South Dorset, southern England (Figure 2.1). It spans approximately 0.34 hectares and is flanked by dense rhododendron wood, mature deciduous woodland, wet scrub woodland dominated by birch, mature Scots pine, pasture and heathland dominated by *Calluna vulgaris* and *Ulex europaeus*.

Figure 2.1. The breeding pond, and study site. Dorset, UK.

2.2. Recording and selection of individuals

Annually, since 1980, the daily number of sexually mature male and female toads was recorded by Dr Chris Reading (e.g., Reading, 1983; Reading, 2007). Toads arriving at the pond did so from a period between January and April (Reading, 2007). The toads were also captured and marked to denote year of capture by a single toe-clipping. The size (snout-vent length, SVL, in mm) and weight (body mass, in gms) of each individual arriving at the pond was also recorded and these data were used to calculate the body condition index (BCI). For full descriptions and calculations of BCI see the methods section in Chapter 5.

Data from all individual toads (census size, *N*) required for the sampling years used in the study were obtained from Dr Chris Reading (pers. comm. 2010). The individuals were selected from the population based on known life-history traits of common toads and factors that would optimise statistical power when using computer software programs. For example, it is well known that male common toads reach sexual maturity before females and partly for this reason the operational sex ratio (OSR) at breeding sites is male biased. In the current study, the OSR is male biased by approximately 3:1 and for this reason toads were selected if they were found in amplexus. This was done to try and circumvent the problem associated with excess males in the population. By selecting male and female toads found breeding we therefore assumed that these paired individuals had a higher chance of being a mating pair and thus more chance of producing offspring. Therefore, many male toads from each parental cohort (2004, 2005, and 2006) were not sampled. Furthermore, based on the known ages at which males (3 – 5 years) and females (4 – 6 years) reach sexual maturity, individuals from the years 2008 and 2009 were selected to form the offspring cohort. Thus, individuals from the years 2004 – 2006 were used as the first generation and individuals from 2008 and 2009 as the second generation. Individuals from 2007 were not included in the study since that year had a very high number of adult individuals present for that year (census, N = 900). This would have resulted in many more potentially breeding individuals and would in turn have generated results that were statistically less reliable.

2.3. Tissue samples

In total, 898 toe-clippings (Table 2.1.) have been used for the current study. The number of samples, including single toads and pairs, varies between the years due to population size fluctuation. Individuals were selected based on the premise that pairs (males and females in amplexus) of toads used from 2004, 2005 and 2006, are the parents of toads in the later years of 2006, 2008 and 2009 (common toads reach sexual maturity at around 3-4 years).

Table 2.1. Toe-clippings as per sampling year and sex of toad.

	Sex		
Year	♂	♀	Total
2004	95	96	191
2005	58	59	117
2006	52	52	104
2008	99	99	198
2009	188	100	288
			898

2.4. Tissue digestion and DNA extraction

All toe clippings from 2004 to 2009 were dissected in preparation for digestion, using approximately 2/3 of the toe. The remaining third was stored in ethanol to be used in the future if required. Tissue samples were transferred to a digestion solution of 500µl of 1xTNE, 50µl of 1M Tris HCI pH 8.0, & 24µl of 25% SDS, along with 5µl of 20mg/ml proteinase K (Kramel Biotech, UK) and left overnight at 37^{o}C to digest. A total of 898 samples were prepared for digestion and were ready for extraction when the solution was homogenous in texture and colour.

The DNA extractions were performed by initially adding 300µl of phenol/chloroform/iso-amyl alcohol to the digested samples (Sambrook *et al*, 1989). Each sample was then mixed vigorously until forming a milky emulsion and centrifuged for 5 minutes at 13,000rpm. After centrifugation the supernatant was transferred to a labelled 1.5ml Eppendorf. This procedure was repeated with 300µl of chloroform/Iso-amyl alcohol. The DNA was then precipitated by adding 1ml of 100% ethanol to the supernatant and inverting the tube several times. After the samples were centrifuged for 10 minutes at 13,000 rpm, the ethanol was discarded. This procedure was repeated with 500µl of 70% ethanol. With the DNA pellet remaining at the bottom of each Eppendorf, the samples were left horizontally with the tube lids open overnight at 21^{o}C. This step was to ensure that any ethanol residue had completely evaporated since this can inhibit the PCR reaction. When the DNA pellet was dry it was suspended in 50µl of Tris-EDTA buffer (10mM Tris, 1mM EDTA, pH 8.0) and, with occasional gentle agitation, was dissolved at 37^{o}C for 30 minutes. After the DNA pellets had fully dissolved, they were subject to spectrophotometric quantification to reveal the DNA yield for each extraction.

DNA extractions were quantified using the Beckman Coulter nanoVette for use with the Jenway 6305 UV/visible range spectrophotometre. By pipetting 2µl of template DNA onto the nanoVette and placing it into the spectrophotometre, the concentrations of DNA, in µl/ml were recorded. This figure was then corrected for by the factor of the pathlength lid of the nanovette, and thus multiplied by 10 to give the DNA concentration in ng/ml.

Quantified DNA was diluted with specific amounts of H_20 accordingly to adjust the concentration to around 10ng/ml. For example, if a particular DNA extraction was quantified at 50ng/ml, then 50 (quantified concentration) /10 (desired concentration) x50 (the volume of extracted DNA)-50 (1 x the volume of extracted DNA) would equal 200µl of H_20 to be added to the DNA extraction.

2.5. Polymerase Chain Reaction (PCR)

Approximately 840 template DNA extractions derived from the tissue samples were prepared for PCR amplification. Initially, standard PCR reactions were set-up as follows: 30 seconds each at 94^{o}C, 55^{o}C & 72^{o}C for 35 cycles, and 10 minutes at 72^{o}C for 1 cycle. However, since these conditions resulted in weak amplifications and many failures, the touchdown program as published by Brede *et al*. (2001) was used to see if this would increase amplification success. This program was successful with many more DNA extractions amplifying, and producing brighter electrophoretic bands. The touchdown PCR program works by the elimination of nonspecific PCR products. This is achieved via 2^{o}C incremental steps applied to the annealing temperatures of the PCR primers. Since the earliest phase of the program has the highest annealing temperature, and since annealing temperature is related to primer specificity, this earliest amplified sequence (the sequence of interest) is then further amplified during the next incremental phases and out-competes the other non-specific sequences in the process. The last phase can then amplify the sequence of interest via further cycles at a final annealing temperature (Don *et al*., 1991).

Table 2.2. Microsatellite primers selected for the current study from Brede *et al*. (2001).

Locus	Repeat unit	Primer sequence (5'–3')
*Bbufμ*11	$(CA)_{19}$	GTCACATGGATAATAAATGAGACC TCTAATATTGATGACCAGACAACC
*Bbufμ*15	$(CA)_{16}$	TCAATATAGGAGTCCCAGAATGTC AATCCCCTAGCGTACACAAGATAC
*Bbufμ*24	$(CA)_{13}$	TTTGGAGAGGGGAAAACTTCACAC CGGATTCTGTTGGGGGTGCTC
*Bbufμ*46	$(TG)_{15}$	GATTTCCTGCCGTGAGCCCAGTG CGCCCGCCAAACCTTCCTGAAC
*Bbufμ*49	$(GT)_{29}$	GATCTGGGCAGTGTTGGATTG ATTCCGTCTGCTAAATGTCTCTTG
*Bbufμ*54	$(CA)_{17}$	CATTGCGCTGCTGTCAGATTACAC TTAGGGATTGCCGTCCAGTTGTC
*Bbufμ*62	$(GT)_{18}$	GCACATTCCTGTGTCCGTGTATAG ATTCCGAAAACGAAAAGAAAAGAG
*Bbufμ*65	$(GT)_{29}$	GGATCTAAGCGCTGTGAGAGTGA CGGTCCGTGTTACCACTGATGC

The choice of microsatellite markers (Table 2.2) was defined based on the fifteen dinucleotide primers characterised for *Bufo bufo* by Brede *et al*. (2001). Table 2.2 outlines the set of loci used, along with the repeat unit and repeat sequence. All PCR runs were prepared on 96-well PCR plates, compatible with the Applied Biosystems 2720 thermal cycler PCR machine, each with an adhesive sheet attached over the top to cover the reactions and prevent evaporation. Locus specific PCR profiles are given in Table 2.3.

Table 2.3 PCR profiles for the touch-down program employed per microsatellite locus.

Locus	Denaturation temp (°C)	Incremental annealing temp (°C)				Final annealing temp (°C)	Extension temp (°C)
	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 7
Bbufµ11	94	52	50	48	46	44	60 (2)
Bbufµ24	94	64	62	60	58	56	60 (2)
Bbufµ46	94	71	69	67	65	63	70 (2)
Bbufµ54	94	61	59	57	55	53	70 (2)
Bbufµ49 & 65	94	60	58	56	54	52	70 (1)
Bbufµ15 & 62	94	58	56	54	52	50	70 (1)

For all loci the PCR reaction volume was 10µl and contained 4.3µl of H_20, 1µl of template DNA, 1µl of 10x reaction buffer (Bioline Ltd, UK, 160 mM $(NH_4)2SO_4$, 670mM Tris-HCl (pH 8.8 at 25° C), 0.1 % stabilizer), 1µl of 25mM of each dNTP, 0.6µl of 25mM $MgCl_2$, 1µl of 10pmol/µl of each primer, and 0.1µl of Taq (5 units/µl).

Before genotyping the PCR products, gel electrophoresis was performed to visualise the PCR products to assess the quality and success of reactions by preparing a 1% agarose Tris Borate EDTA (TBE) gel. This was achieved by adding 0.3g of agarose (Bioline Ltd, UK) to 30ml of 1x TBE (89mM Tris-borate, 2mM EDTA, pH 8.3, Severn Biotech, UK) in a conical flask and heating on full power in a 700W microwave for about 1minute. After leaving the agarose to cool to around 50˚C, 30µl of GelRed™ (Biotium, Hayward, CA, USA) was added and mixed into the conical flask. GelRed™ is used to help visualise the DNA since it works as an intercalating agent, binding the DNA and fluorescing under UV light. The agarose was then poured into a gel tray containing a 1.5mm comb within a gel electrophoresis unit. After around 30 minutes the gel was set, the comb was removed and approximately 200 ml of 1x TBE was added to the unit immersing the gel within the buffer. Preparation of the PCR products to be run on the gel involved pipetting out 5µl of the contents of several randomly selected wells as a sample of each 96-well PCR plate. Each one of these, along with 5µl of the negative control were added to individual 0.2ml PCR tubes in addition to 5µl of loading buffer (30% glycerol containing Orange G dye). After mixing the dye with the products, the contents of each PCR tube, along with 3µl of 1Kb plus DNA marker (Invitrogen Ltd, UK) were then transferred to individual wells of the agarose gel. The unit was then connected to the power supply and run at 70V until the DNA had migrated approximately 2/3 through the gel. The PCR products were then visualised under UV light on an Alpha imager ™ 1220 (Alpha Innotech corporation, USA).

2.6. Genotyping

PCR products to be genotyped had their DNA concentrations altered by diluting them with distilled H_20. This is due to the sensitivity of the genetic analyser and was calculated by observing the DNA band intensity on the gel images from tested PCR products to estimate DNA quantity. The dilutions involved transferring 5µl of each PCR product into separate wells of a PCR 96-well plate. Since PCR was performed using the 96-well plates, the products were transferred into new PCR plates correspondingly. Thus, PCR plates with products arranged in a specific order were ordered in exactly the same way when genotyped. This was done to restrict confusion or misidentification of the products on the plates when scoring them after genotyping. In order to be more efficient with resources and time, each individual well of each plate contained three individual PCR products with different fluorescent labels. These labels were used in order for the genetic analyser to detect which specific loci were to be analysed. For example, for the locus *Bbufu11* to be modified either the forward or reverse primer becomes fluorescently labelled with a specific dye and given a code. Thus, in this case the forward primer for *Bbufu11* is labelled with a dye named 'HEX' which when detected by the genotyper fluoresces green when visualised.

Table 2.4. Microsatellite names and the 5' modification, along with the colour of fluorescence when genotyped.

Locus	Forward or Reverse	Modification	Colour of fluorescence
*Bbufµ*11	Forward	5' - HEX	Green
*Bbufµ*15	Reverse	5' - AT550	Black
*Bbufµ*24	Forward	5' - HEX	Green
*Bbufµ*46	Reverse	5' - HEX	Green
*Bbufµ*49	Reverse	5' - HEX	Green
*Bbufµ*54	Reverse	5' - AT550	Black
*Bbufµ*62	Forward	5' - FAM	Blue
*Bbufµ*65	Forward	5' - FAM	Blue

All primer modifications can be seen in Table 2.4. The PCR products were then further diluted by transferring 1µl of the PCR product mixture (three individuals combined) to a 9µl master mix of H_2O, formamide, and Liz standard. Thus, 10µl reactions were prepared and loaded onto the ABI3130 96-well genetic analyser. The data from the genetic analyser was then analysed using the software Peak Scanner^{TM} to determine allele sizes and zygosities of each successful PCR reaction.

2.7. Screening of genotypic data

After all the data were acquired from Peakscanner, they were further processed using several software programs. This is performed to check for errors associated with genotyping data that include the non-amplification of alleles (null alleles), and scoring errors caused by stutter bands. Firstly, the software Tandem v1.08 (Matschiner and Salzburger, 2009) was used to convert the alleles scored by visual inspection, which contained non-integer values, to workable integers in a process known as ‘allele binning’. Allele binning in Tandem is an automated process that sorts allele sizes into discrete classes and is more accurate than manual binning that can result in errors due to the miscalling of some allele sizes. Upon completion of the analysis from Tandem, an output file is generated containing all of the data points converted to integers and ready for all other software programs.

The software Microchecker (Oosterhout *et al.*, 2004) was used after Tandem to detect errors due to alleles being incorrectly scored in Peakscanner and the presence of null alleles indicated by homozygote excess. Once the data were checked for such errors they were processed in the program Genepop On The Web v4.0 (Raymond and Rousset, 1995) for the estimation of Hardy-Weinberg proportions. The ‘probability test’ was used with the null hypothesis that the data was in Hardy-Weinberg Equilibrium (HWE), to calculate deviations from HWE, data not in HWE reflected all P values of < 0.05. The data were also analysed in Genepop v 4.0 for basic data for each locus in each population, which comprised allele and genotype frequency data, the observed and expected heterozygosities and homozygosities and allele size ranges.

CHAPTER 3:

Measuring the effective population size over two generations in a wild common toad population

3.1. Introduction

The effective population size (N_e) is the number of breeding individuals in an idealised population exhibiting the same characteristics as the census population (the actual number of animals present, *N*, (Frankham, 2002). The concept was introduced by the geneticist Sewall Wright who stated that based on the assumptions of an idealised population, N_e would show the same distribution of alleles under genetic drift and the same levels of inbreeding as the actual population under observation. In the idealised population, there are equal numbers of both sexes and all individuals are in panmixia with equal chances of successfully reproducing. However, since wild animal populations do not meet such criteria, deviations from the idealised population will usually cause the effective population size to decrease relative to the census size. Such considerations are important, because only the effective population size determines the amount of genetic drift and inbreeding, and the rate of loss of genetic diversity per generation (Frankham, 2002). Therefore, the effective population size is important for conservation considerations because a loss of genetic diversity will limit the adaptability of a population to changing environmental conditions (Soule, 1986). It is for these reasons that the effective population size is often regarded as the most important genetic parameter in conservation genetics (Ovenden *et al.*, 2007).

The effective population size is often considered in relation to the census size (N_e/N) since it is the deviation from the ideal ratio of 1:1 from which we can measure change. The major variables affecting N_e/N ratios are unequal sex-ratio (SR), variance in family size (VFS), mating system, and fluctuations in population size (FPS) (Frankham, 2002). Factors that may cause changes to such variables include different life history aspects such as polygamy, fecundity, or mating success. Species exhibiting high fecundity for example, due to high variance in family size, and possibly increased fluctuations in population size over generations, may have reduced N_e/N ratios. While polygamous species, due to high variance of paternal gametic contributions, would also be expected to have reduced N_e/N ratios than monogamous species (Frankham, 2002).

In order to test the hypotheses that SR, FPS, VFS and life history characteristics affect N_e ratios and that taxonomic groups differ in ratios, Frankham (1995) reviewed 192 published ratios from 102 species. The review concluded very wide ranging estimates of the effective population size/actual population size ratio with comprehensive estimates averaging between 0.10 and 0.11. The lowest (0.0009) and highest (1.07) estimates of N_e were for insects exhibiting high fecundity (Butlin & Day, 1989; Nozawa, 1970). Highly fecund amphibians, with the possible exception of one study (Berven & Grudzien, 1990) all showed expected low N_e ratios. Despite some anomalies, the analysis revealed the effect of fecundity on N_e is less important than that of fluctuating population size (Frankham, 2002).

Early studies reported predictions of N_e ratios based on demographic models with values expected to be usually greater than 0.25 (Nunney & Campbell, 1993), but special circumstances required for values of much less than 0.5 (Nunney, 1993) and values of less than 0.1 expected for small organisms (Nei & Tajima, 1981). These values were contrasted further with empirical estimates of 0.5 – 0.8 (Falconer, 1989), 0.2 – 0.4 (Denniston, 1978), and, 0.25 – 1.0 (Nunney & Campbell, 1993). Furthermore, more recent estimates have been reported of 0.11 (Frankham, 1995) for demographic estimates and 0.14 for genetic estimates (Palstra & Ruzzante, 2012) with these values further still be incongruent with more contemporary findings. In a meta-analysis of 233 studies of N_e/N ratios, only 33 could be considered corrected linked ratios. Many estimates have been incorrectly linked in previous studies and the median value of N_e/N ratio from the correct ones was 0.231. Therefore, despite the recent findings that N_e/N ratios can be correctly linked, many ratios are not and demographic expectations are often dissimilar to genetic estimates. Hence, there exists a lot of inconsistency and conflict between reports of N_e/N ratios meaning significant improvements are required (Palstra & Ruzzante, 2008).

Calculation of the effective population size depends upon which of the three approaches is taken: inbreeding (N_e(*inb*)), variance (N_e(*var*)) or eigenvalue (N_e(*het*)). Other forms of *N*e have been developed but N_e(*inb*), N_e(*var*) and N_e(*het*) are the most evaluated and widely used (Luikart *et al*., 2010; Crow & Denniston, 1988). The eigenvalue *Ne* expresses the loss of heterozygosity to that of the ideal population. Similarly, N_e(*inb*) and N_e(*var*) express the increase in inbreeding and the increase in variance of allele frequency to that of the ideal population respectively. However, when a single isolated population is not changing in size, N_e(*inb*) and N_e(*var*) can be regarded as either very similar or identical (Hedrick, 2011; Luikart *et al*, 2010). Different time frames are also considered since, depending on the specific questions asked, N_e estimators maybe be used for historical, ancient or contemporary temporal scales. However, it is the contemporary time scale estimates most commonly used since these are the most viable and accurate and are the most important ones in the context of conservation science (Luikart *et al*., 2010).

A parameter related to the effective population size is N_b, the effective number of breeders. Whereas N_e is the effective number of breeders within a population that requires the breeding parental generation and the sired offspring generation to be sampled, N_b requires only a single sample of the population to be analysed. This results in the effective number of breeding adults that sired the single sample of individuals in a given breeding season, as opposed to over two season for N_e. Therefore, genetic estimation of effective population size can be broadly separated into either one-sample or two-sample estimators yielding estimates of either N_b or N_e respectively. Two sample estimators include the temporal method, a powerful approach that measures changes in allele frequencies (Luikart *et al*, 2010) over time and is based on the premise that genetic drift increases as N_e decreases. Samples of at least two, but ideally several, consecutive generations are required (Frankham, 2002) for estimation and it also requires highly polymorphic co-dominant molecular markers such as microsatellites. The temporal method, along with others such as gametic disequilibrium and heterozygote excess, is known as a moment estimator (Leberg, 2005; Pudokvin *et al.*, 1996; Bartley *et al.*, 1992; Waples, 1989).

Due to the limitation for the two-sample estimators of obtaining two samples (generations), that for many species may be somewhat spaced apart, the requirement for estimators based on one sample of the population was apparent. One sample estimators measure the effective breeding size and methods include the linkage disequilibrium (LD) approach, the heterozygote excess method, the sibship assignment method and Bayesian methods. The linkage disequilibrium method is based on the expected increase in LD due to genetic drift producing non-random associations between unlinked loci, with this being more pronounced in small than large populations (Beebee, 2009). The heterozygote excess method is based on the chance deviations of genotype frequencies over generations. Due to genetic drift, the frequencies of genotypes differ and deviate from Hardy-Weinberg expectations and this causes an excess of heterozygotes in the offspring generation. This is due to sampling error of the male and female parents in the population causing stochastic differences in genotype frequencies (Wang, 2005). The sibship assignment method works by estimating N_b from the relatedness of individual offspring in the sample. The concept is based around the number of associations of full or half siblings and the more frequent occurrences of such relationships in populations with smaller N_b.

The temporal method has been widely used to infer N_e and N_e/N ratios (Palstra & Ruzzante, 2008; Fraser *et al*., 2007; Ovenden *et al*., 2007; Palstra & Fraser, 2012).

However, a single statistical estimator which can provide a comprehensive measure of estimation does not exist (Araki *et al.*, 2007). This is primarily due to an incomplete understanding of the usefulness of different approaches when using different numbers of samples and loci in populations with varying effective sizes (Aspi *et al.*, 2006; Palstra & Fraser, 2012). Moreover, due to parametric assumptions that are commonly violated, such as non-overlapping generations, panmixia, or the absence of gene flow, some estimators can be inappropriate for particular studies and required statistical refinement (Waples & Yokota, 2007). To test the efficiency and consistency of the different statistical estimators, Aspi *et al*. (2006) employed several approaches to perform temporal analysis on a Finnish wolf population. To determine $N_e(var)$ of the population, analysis was performed using Moment based, Coalescence MCMC (Monte-Carlo Markov-Chain), MC likelihood and Pseudo-Likelihood approaches. The analyses estimated N_e to be 39.5, 40.0, 43.0 and 37.8 respectively, averaging in an effective population size of approximately 40 individuals. The study also concluded that the population was in decline despite past increases in *N*. Thus, the findings from the study have implications for the prevention of further decline or extinction of the population (Aspi *et al*, 2006), and highlight the potential for comprehensive estimates of N_e.

The precision and accuracy of N_e, for the temporal approach, depends on the number of alleles examined across all loci, the overall sample size, and the number of generations between temporal samples. Obtaining more than two sets of temporal samples also increases the precision of $N_e(var)$. However, sampling more than twice in a temporal series or increasing time frames will often prove difficult since many wildlife species have long generation times. In many cases obtaining samples spanning more than one generation will be not be feasible unless the use of a long-term population study is employed (Leberg, 2005). For precision and accuracy of single sample estimators, N_b should correlate with N, the number of polymorphic loci should be increased, and N_b should correlate nonlinearly but positively with genetic diversity (Beebee, 2009).

In conclusion, the effective population size (N_e), is the idealised population exhibiting the same genetic characteristics as the actual population under study. While the effective breeding size, N_b is the number of breeding adults in a given breeding season. There is a well-developed and refined history of statistical background for N_e (N_b) estimates and many studies have reported success using various methods. Estimates of effective population size (or N_b size) provide crucial insights into the ecology and evolution of wild animal populations and have important applications in biodiversity management and conservation (Crandall *et al*, 1999).

3.2. Aims

The current research makes use of an on-going study of a common toad population in Dorset that has indicated a link between a reduction in body condition, female fecundity, and survival of the toads and increased environmental temperatures. By using genetic data derived from individual tissue samples, the aim of the current study was to investigate the effects of the observed reduction in body condition on the effective population size, and effective breeding size of this common toad population. Moreover, given that the study population is a good model to investigate the effective population size due to availability of several hundred samples encompassing data both within and between generations, the aim was to estimate and compare measures of two distinct means estimating the total number of breeding individuals in the population.

3.3. Methods

Tissue samples of *Bufo bufo* (Table 3.1.) were obtained from the ongoing study of the common toad population in Dorset (see Chapters 1 and 2). DNA was extracted from tissue samples using a standard phenol/chloroform procedure and PCR conditions were performed as per the touchdown program described in Brede *et al*. (2001). Genotyping was performed on the ABI3130 genetic analyser and errors in the data checked for by using various software programs. These techniques are detailed in full in Chapter 2.

Table 3.1. Total number of toe-clippings as per sampling year and sex of toad.

	Sex		
Year	♂	♀	Total
2004	95	96	191
2005	58	59	117
2006	52	52	104
2008	99	99	198
2009	188	100	288
			898

Single sample effective population size estimates were calculated using the programs Colony (Wang, 2009) and NeEstimator (Peel *et al*., 2004). Colony uses a unique approach of estimating N_b by inferring sib-ships from a single sample of offspring. It is based on the premise that N_e is directly related with the number of half and full sibs found in a population. An important assumption is that the sample of individuals is randomly drawn from the same cohort. If several cohorts have been sampled simultaneously then the sample may contain parent-offspring relationships. This can lead to false sib-ship assignment given that both parents-offspring arrays and full sibs share half of their genome with each other. However, since the sampling in the current study is well defined by each year, there is no risk that any two cohorts will be mixed and thus that this assumption will be violated. Confidence intervals of 95% are calculated by bootstrapping.

The program NeEstimator employs the commonly used linkage disequilibrium method, also with a single sample of the population. It is based on the idea that N_e determines the degree of non-random associations at independent loci. Low N_e increases genetic drift which in turn increases linkage disequilibrium in the population. Confidence levels are calculated at 95% using bootstrapping and jackknifing. This same program also estimates effective breeding size via the heterozygote excess method. This method is based on the chance differences, due to genetic drift, of the genotypes between male and female parents causing an excess of heterozygotes in the offspring generation.

The temporally based effective population size estimate was calculated using the program NeEstimator. The temporal method works by calculating the change in allele frequencies caused by genetic drift over at least two generations. The calculation of the temporal approach for this study was based on the equations of Waples (2007).

Therefore, the software program Colony was used to employ the sibship assignment method and the program NeEstimator for the linkage disequilibrium method, heterozygote excess method and the temporal method.

Precision of the N_b estimators was calculated as the variance (V) defined as the difference between the confidence limits, obtained with each estimate, as a percentage of the N_b estimate. Variance was calculated as follows:

$$V = \frac{100\text{x}(C2 - C1)}{E}$$

Where, C2 equals the upper 95% confidence limit and C1 equals the lower 95% confidence limit, and *E* equal the N_b estimate (Beebee, 2009).

3.4. Results

The results from the single sample effective breeding size estimates are displayed in Table 3.2. Estimates of N_b were calculated via three different methods: sibship assignment (SA), linkage disequilibrium (LD) and the heterozygote excess (HE) methods. Table 3.2 shows the estimates of N_b as calculated via each method along with the lower and upper confidence limits of N_b at 95%. Results of Pearson product moment correlations between the sex-ratio and N_b and N and N_b/N are also displayed. Table 3.2 also shows the estimate of effective population size calculated via the temporal method.

Table 3.2 Effective breeding size estimates and census size, and N_b/N ratios.

		Sex Ratio	Effective breeding number/N_b and N ratios		
	N	♀:♂	SA	LD	HE
2004	593	0.35	69 (51–99)/0.116	∞ (1004.3–∞)	1.4/0.002
2005	473	0.18	73 (52–102)/0.154	35.6 (29.7–43.7)/0.075	1.5/0.003
2006	538	0.14	85 (63–119)/0.158	162.3 (118.9–247.5)/0.302	5.5/0.010
2008	785	0.36	116 (89–149)/0.148	320.2 (229.8–509.3)/0.408	5/0.006
2009	572	0.26	149 (117–187)/0.260	282.4 (229.4–361.5)/0.494	6.9/0.012
Mean N_b			98.4	200.13	4.06
TM = 99.7					
SR vs. N_b			-0.14	0.59	0.028
N vs. N_b/N			0.39	0.83	0.3

N = population census size, numbers in parentheses = 95% confidence limits, SA = sibship assignment, LD = linkage disequilibrium, HE = heterozygote excess, TM = temporal method, SR = Sex Ratio.

Table 3.3 Pearson product moment correlations between the three single sample estimates of *Nb*.

Nb	*r*	*P*
SA vs. HE	0.85	>0.05
SA vs. LD	0.83	>0.05
HE vs. LD	0.8	>0.05

All but one estimate (N_b from 2004 via the LD method) yielded N_b values encompassed within the lower and upper 95% confidence limits of the corresponding method. The correlation between any two of the methods yields in a correlation coefficient greater than 0.8, at however non-significant p values largely due to the low sample size (Table 3.3).

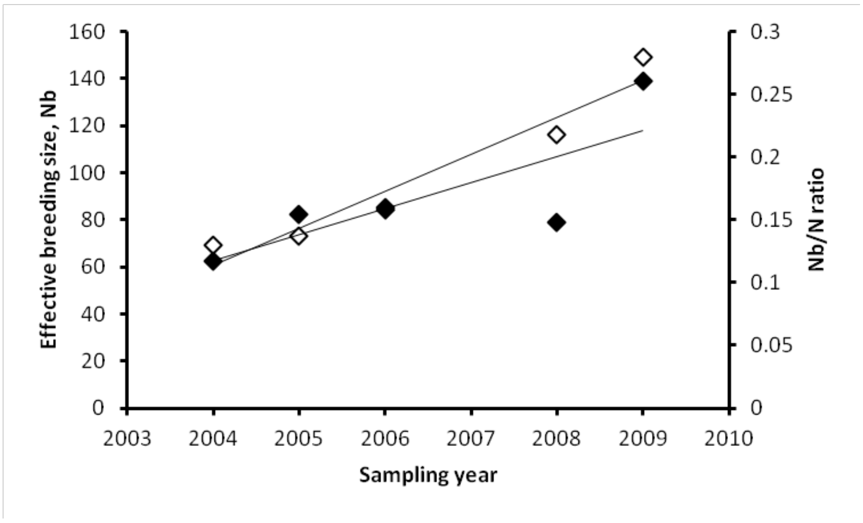


Figure 3.1 Effective breeding size, and effective breeding size and census size ratio against time for SA estimates. Left axis = N_b, right axis = N_b/N. Open symbols = N_b, closed symbols = N_b/N.

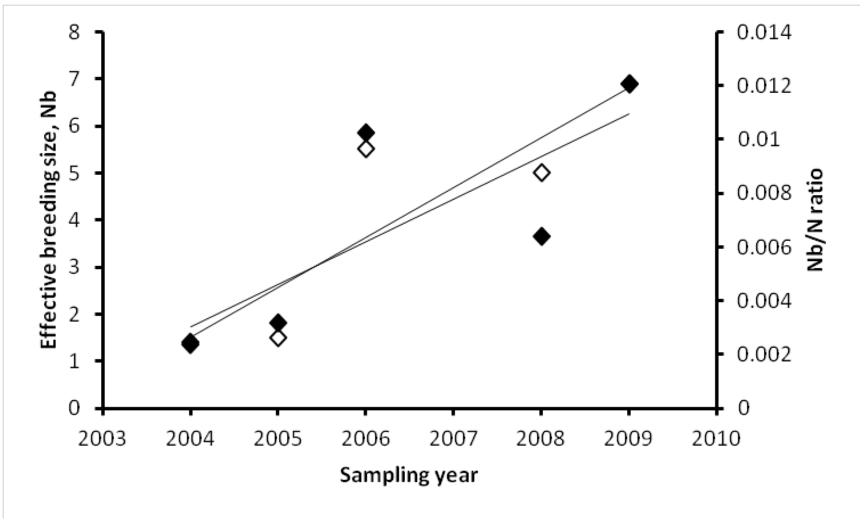


Figure 3.2 Effective breeding size, and effective breeding size and census size ratio against time for LD estimates. Left axis = N_b, right axis = N_b/N. Open symbols = N_b, closed symbols = N_b/N.

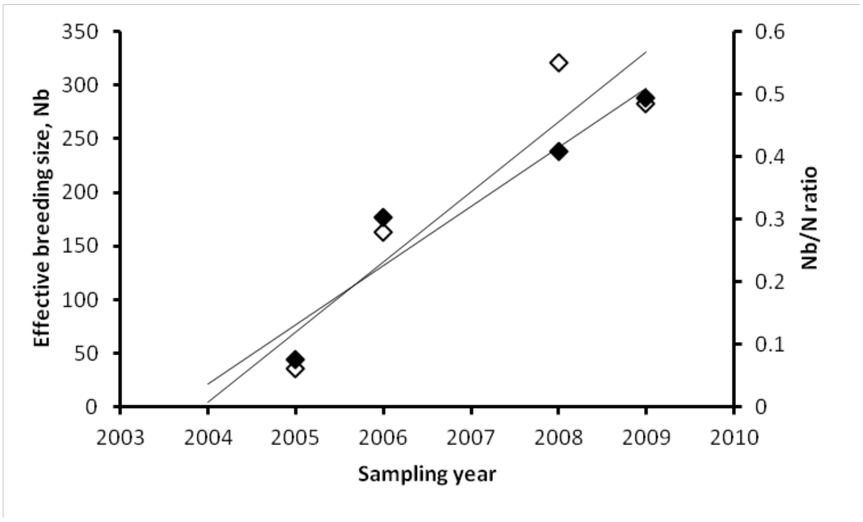


Figure 3.3 Effective breeding size, and effective breeding size and census size ratio against time for HE estimates. Left axis = N_b, right axis = N_b/N. Open symbols = N_b, closed symbols = N_b/N.

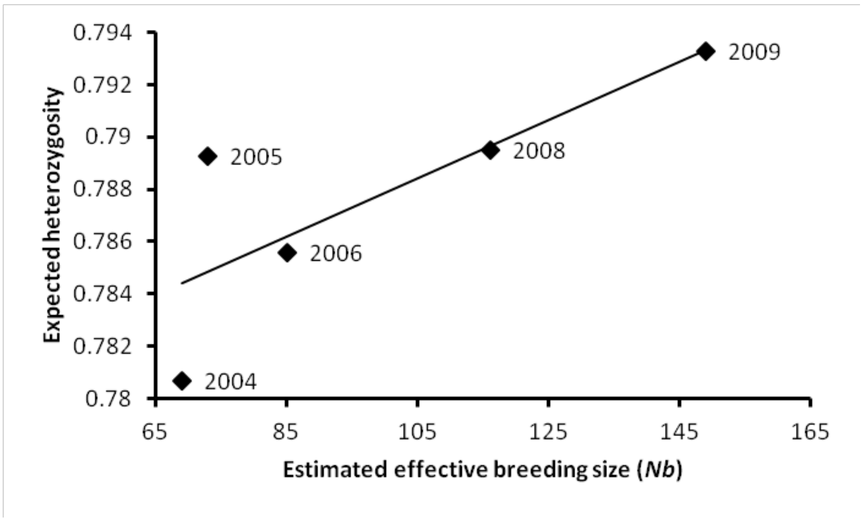


Figure 3.4 Expected heterozygosity and N_b estimates of the SA method

N_b estimates further increase from 2004 to 2009, Fig. 3.1 – 3.3). However, none of these were significant. Figures 3.1, 3.2, and 3.3 show N_b as a function of time for the estimates calculated via the SA, LD and HE method, respectively.

The figures also show the relationships between the effective breeding population size and census size ratios over the sampling period. Pearson product moment correlations were significant for sibship assignment method against time (r = 0.97, P = 0.0067), linkage disequilibrium method/census size against time (r = 0.95, P = 0.048) and the heterozygote excess method and time (r = 0.89, P = 0.04). Levels of expected heterozygosity were also related to N_b estimates for each method (Figures 3.4, 3.5, and 3.6).

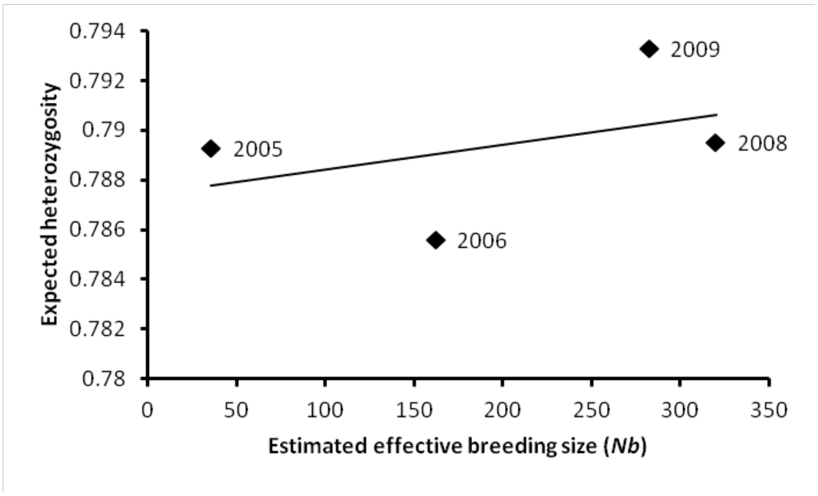


Figure 3.5 Expected heterozygosity and N_b estimates of the LD method.

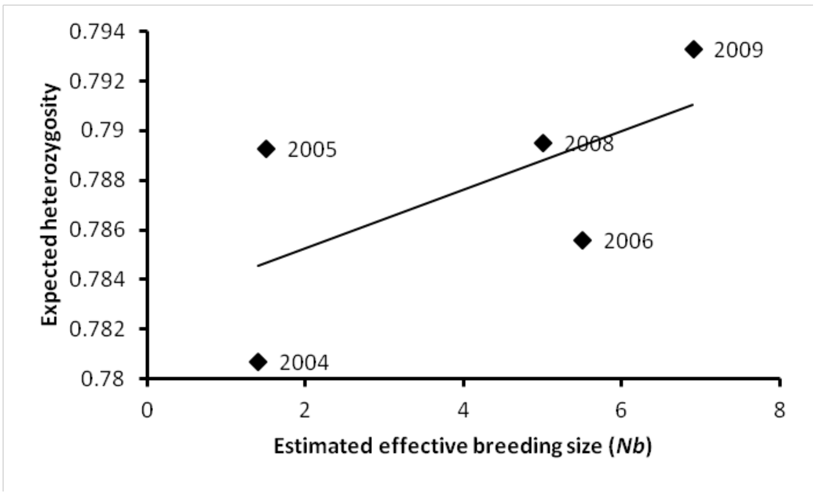


Figure 3.6 Expected heterozygosity and N_b estimates of the HE method.

The relationships between the three different *Nb* estimates and expected heterozygosity show positive but nonlinear relationships ((a) r = 0.79, (b) r = 0.62, & (c) r = 0.41), at however non-significant (P >0.05) associations.

Table 3.4 Precision of N_b estimates for the SA and LD methods.

Sampling year	*N*	Precision (*V*) SA	LD
2004	593	69.56522	–
2005	473	68.49315	39.32584
2006	538	65.88235	79.23598
2008	785	51.72414	87.28919
2009	572	46.97987	46.77762
Nb vs. *V*		-0.54	0.72

Precision increases (i.e. variance decreases) over time and is negatively correlated with *N* for the SA method, whereas precision of the LD method shows a positive correlation with *N* (Table 3.4., neither show a significant relationship at P >0.05).

Regressions of the relationship between population census size and effective breeding size showing non-significant positive correlations (SA method, r = 0.38, HE method, r = 0.30, LD method, r = 0.83).

3.5. Discussion

The effective population size (N_e) is that of an idealised population that exhibits the same characteristics as the population under observation (Wright, 1931). While the effective breeding size, N_b is the number of breeding adults in a given breeding season (Phillipsen *et al*, 2008). Estimation of N_e is particularly important because, unlike adult census size (N), it provides measures of key population genetic parameters such as, genetic drift and inbreeding which determine heterozygosity and genetic diversity (Frankham *et al.*, 2002). The different methods of genetic estimation of N_b using the single sample estimators used in the current study vary in their underlying theoretical approaches. The underlying theories are based on life histories and different population aspects and assumptions. One such assumption for the heterozygote excess method that may cause questionable values of N_b, for example, is the requirement of random mating. All N_b estimators (and $\mathrm{N_e}$ estimators) require random mating but the HE method may be a particularly incorrect or an exaggerated assumption of this method (Beebee, 2009) when applied to most empirical scenarios. It has been suggested that due to this requirement, this method may be better applied to 'broadcast spawners' such as coral (Schwartz *et al*., 1998). This is to say that due to the nature of spawning for coral, the random mating may be sufficient to fulfil the assumption of the HE method. Due to this possible violation for one of the principles of this method, results using this approach are often inconsistent or incongruous with other single sample estimators. Beebee (2009) found that this method was in fact the least satisfactory in terms of congruency with other methods and was also unable to produce confidence intervals on many occasions. This lack of confidence limits precludes the calculation of variance estimates and therefore the comparison of estimators based on precision. This method also occasionally produces very wild estimates many orders of magnitude different, or even ‘infinity’, from the other methods for the same set of data. For example, Beebee (2009) found that, while there were a few populations of British *B. calamita* that showed N_b estimates similar to other methods for the HE method, an N_b estimate of 17,000 was generated, compared to N_b = 18, 16, and 16 for the LD, Bayesian, and SA methods respectively. These data are similar to estimates from the current study for N_b values using the same method. For example, like Beebee (2009), the HE method was problematic at yielding confidence limits. In fact, in all sampling years, no confidence limits were produced. Similarly, values were wildly different between estimators. For instance, estimates from the HE method produced values in the order of approximately twenty times lower than other methods. Despite this method being the least satisfactory in terms of precision and comparisons with other methods and producing very low values, unlike Beebee (2009) it did not produce excessively high N_b estimates.

Estimates of N_b from the other single sample estimators are varied across, but relatively consistent within methods. These results are similar to those of other studies of N_b estimates of anuran species (Beebee, 2009; Phillipsen *et al.*, 2011), however somewhat differing between individual methods. For example, Phillipsen *et al*. (2011) yielded results that varied 3 or 4 fold between the SA and Bayesian methods compared to an approximate twofold difference between the SA and LD methods in the current study. However, despite large discrepancies between the LD, HE, SA and Bayesian estimates, those generated from Bayesian and SA estimation were very congruent for Beebee (2009). Other studies that have estimated N_b or N_e in *Bufonidae* have shown similar values of effective size for single sample estimation and the temporal method of estimation respectively. In a study of British populations of *B. calamita* (Beebee, 2006) using the LD method, N_b sizes of 110 and 170 were found for populations in Holme and Sandy respectively. These compare to the current study of N_b values for the sampling years of 2008 and 2009 (respectively) using the same method of estimation. Using the temporal method of estimation, Brede & Beebee, (2006) revealed N_b measures of 34 and 49 for two different populations that are similar to certain estimates obtained from current analyses (Table 3.2). Other results from the temporal method of estimation are somewhat different, such as the results obtained by Scribner *et al.* (1997) that was based on adult-tadpole arrays for generational times. Their results from several *B. bufo* populations revealed a range of N_b values from 16 to 60 across 3 populations, compared to a temporal method N_b value of 99 for the current study.

When analysed alongside the values of census size, the above studies show some differences when comparing N_b/N ratios to the current study. Scribner *et al*. (1997) showed effective breeding size and census size ratio to range from 0.007 to 0.012 using the temporal method. This is congruent with data obtained in the current study albeit for data derived from the HE method. The HE method yielded a range of values from 0.003 to 0.012 with an average of all sampling years of 0.007, exactly that of the range minimum for Scribner *et al*. (1997). However, Brede & Beebee (2006) revealed N_b/N ratios of 0.040; despite this value being close to the estimates from the HE method it is far lower than estimates obtained from the LD and SA methods in the current study.

For wildlife species in general, the 'universal' N_e/N ratio of between 0.11 (Frankham *et al.*, 2002) and 0.14 (Palstra & Ruzzante, 2008) is a resemblance to the data obtained for at least one N_e estimator from the current study, the SA method. The mean N_b/N ratio from the sibship assignment method is 0.16 and returned the greater precision over the heterozygote excess method (as calculated as variance, see methods). These data, therefore, are in accordance with expectations as stipulated by Frankham *et al.* (2002). Furthermore, even values at the higher end of the scope of N_b values for the current study can be paralleled by more recent findings of N_b/N values. These findings come from a meta-analysis of nearly 100 studies into N_e/N or N_b/N that found empirical data to be in the order of 0.22 (Palstra & Fraser, 2012).

These estimates of effective breeding size do, therefore, show some agreement with other data from empirical studies for *Bufonidae* species (*B. bufo*, and *B. calamita).* Furthermore, congruency can also be seen between the temporally based estimates and the single sample estimates and that this the first time that such a comparison has been made for *B. bufo*. Owing to the system of the ongoing study by Reading (e.g. 2003; 2007) the sampling range and number of samples per year were sufficient to encompass both the temporal estimates and one-sample estimates of N_e or N_b respectively. These data (Table 3.2) show that the temporal method estimation of N_e is 99.7 which is very close to the average N_e from the sibship assignment method mean which = 98.4. When compared against the LD and HE methods, however, the data is somewhat dissimilar between the temporal and single sample estimates. However, mean N_b values from both the SA and LD methods can be encompassed within the range of the confidence limits for temporal method (mean SA = 98.4, mean LD = 200.13, temporal method CI at 95% = 55.5 – 216.8). Moreover, the temporal method of estimation, N_e = 99.7 fits into each CI obtained from the N_b estimates of the SA method (minimum = 51, maximum = 187).

The findings from the correlations of N_b and sampling period, and N_b/N and sampling period (Figures 3.1 – 3.3) indicate that there is a temporal trend to the data. Such a trend is visible for all the N_b estimators and denotes that over the sampling period from 2004 to 2009 the effective number of breeders has been, in general, increasing over time. This finding, on a temporal scale, cannot be seen elsewhere in the literature but spatial differences and increases to effective sizes have been observed (Phillipsen *et al.*, 2011).

What could cause an increase in the effective number of breeders in this population of common toads? The fundamental contributing forces that affect N_e and N_e/N ratios in order of importance are fluctuations in population size, variation in reproductive success, and unequal sex ratio (Crow & Kimura, 1970). These impacts reduce N_e below N by increasing the variance of the number of gametes contributed per individual to the next generation. This is because the idealised population assumes a Fisherian sex-ratio (1:1) and a Poisson distribution of offspring numbers. However, this is never the case in wild populations. Indeed, the sex-ratio of the current study population is male-biased by approximately 3:1 and therefore it would seem intuitive to suggest that such biases have some degree of a relationship between the estimates of N_b. However, as it can be seen from Table 3.2, sex ratio changes are not related to the changes in effective breeding number, and only the LD method yielded a relatively strong correlation of 0.59. Correlations between N and N_b/N (Table 3.2) for the SA and HE methods are very weak negative and positive correlations respectively and all methods yielded non-significant relationships. Therefore, given these weak and nonsignificant correlations, there is no indication that a fluctuation in population size has affected N_b/N in this population. However, this is probably not too unexpected given that fluctuations in population sizes are usually much more drastic between years (than observed in the current study) (Frankham, 1995).

The results from the correlations of genetic diversity and N_b show the data conforms to that of other another study of the common toad that assessed genetic diversity and N_b (Beebee, 2009). The neutral theory of evolution predicts that genetic diversity (measured as heterozygosity/allelic richness) should correlate positively, albeit nonlinearly, with effective population size (Soule, 1979). Such positive correlations would also provide evidence for the accuracy of N_b estimators (Beebee, 2009), but to the best of my knowledge have not yet been revealed in previous studies. Figures 3.4 – 3.6 shows the positive trend indicating that the three different measures of N_b estimation (SA, LD and the heterozygote excess) are rather congruent. Despite these data showing such a trend, in all cases, the correlation did not yield statistical significance. However, this is most likely due to the small sample size of the five considered years (5 each for Figures 3.4 & 3.5 and 4 for Figure 3.6). A dataset showing statistical significance with an *n* of at least 10 can be seen in Beebee (2009) and when compared to the current data it shows a very similar pattern for two of the N_b estimators used (LD & SA). However, this was a spatial analysis of approximately 20 populations and not, like the current study, a temporal one.

Other evidence for reliability of effective breeding size estimation is provided by the correlations between the different estimators. If data between estimators are similar, then the estimates for each sampling year should show a positive correlation. Table 3.3 shows that all correlation coefficients are above 0.8, albeit they were all non-significant. Philpsen *et al*. (2011) also showed that estimates from the LD and SA methods were positively correlated for four anuran species with strong positive correlations and statistical significance found for two of these species. Similarly, Beebee (2009) found statistically significant positive correlations for the same estimation methods (LD and SA) for 16 British natterjack toad populations.

The N_b estimates from the sibship assignment method are the most precise. This is seen by the lower degree of variance for estimates in every sampling year compared to those of the linkage disequilibrium method. When the data are regressed with census size, the negative relationship for the SA data shows that this precision increases (i.e. variance decreases) with increasing *N*. However, contrary to that finding is the precision estimate data for the LD method which shows a positive relationship of variance and *N*. However, despite neither correlation being statistically significant, the low variance associated with the SA estimates is congruent with findings from other studies. In several anuran species, precision of the SA method was shown to be greater than the LD method (Phillipsen *et al.*, 2011; Beebee, 2009), and like the current study the SA method was negatively correlated with *N* for *B. calamita* (Beebee, 2009).

In summary, for all three methods of effective breeding size estimation there is evidence that N_b follows an increasing temporal trend. This is particularly interesting since it provides evidence that this population might be well equipped to circumvent the observed adverse effects to fitness, or future perturbations to the population, caused by recent climate change (See Chapters 5, and 6 for further discussion).

CHAPTER 4:

Parentage inference of a wild common toad population from multilocus genotype data

4.1. Introduction

The inference of genealogical relationships of individuals (pedigrees) in wild animal populations can address many questions of evolution, ecology, and conservation (Blouin, 2003). However, field observations of such relationships alone are often not sufficient and can in many cases be difficult to obtain (Wang & Santure, 2009). This problem was overcome with the development of studies and the subsequent discovery of microsatellites (Jeffreys, 1985b) which allowed the unambiguous identification of individuals within populations.

Many studies have used parentage analyses covering a number of animal groups (comprehensive list given in Harrison *et al.*, 2012) via many different computer software programs that include: CERVUS (Kalinowski *et al*., 2007), COLONY (Jones & Wang, 2009), GERUD (Jones, 2005), PARENTE (Cercueil *et al*., 2002), PAPA (Duchesne *et al*., 2002), PEDIGREE (Herbinger *et al*., 2006), PROBMAX (Danzmann, 1997), and MASTERBAYES (Hadfield *et al*., 2006), to employ the various methods and approaches available. These methods of parentage analysis can be classified into six categories which encompass exclusion, categorical allocation, fractional allocation, parental reconstruction, full-probability parentage analysis and sibship reconstruction (Jones & Ardren, 2003; Jones *et al.*, 2010).

Exclusion analysis is based on the fact that in sexually reproducing diploid organisms, given the rules of Mendelian inheritance, putative parents and offspring will have at least one allele in common per locus for a co-dominant marker (Chakraborty *et al.*, 1974). A pool of candidate parental genotypes is compared with that of the pool of offspring genotypes and true parents can be excluded if they do not share an allele with a given offspring. However, certain markers can cause problems with the simple underlying logic to this approach. Mutations, null-alleles (i.e. non-amplifying alleles), and scoring errors cause markers to appear non-Mendelian in inheritance. For example, null-alleles can make the true parent and offspring of a dyad appear homozygous for different alleles at the same locus. Similarly, germ line mutations can result in an allele present in an offspring to be absent in the parent. Thus, along with scoring errors, null-alleles and mutations cause mismatches between genetic data of parents and offspring, and thereby result in incorrect exclusions in the analysis. Despite these inherent problems of the method, full exclusion parentage is the current paragon of parentage studies. However, when experimental conditions do not favour exclusion, other approaches are used to infer parentage such as the most commonly used approach, categorical allocation (Meagher & Thompson, 1986; Jones *et al.,* 2010).

Categorical allocation was developed to circumvent the problems associated with exclusion approaches that resulted in some candidate parents not being fully excluded. If for instance there were many candidate parents and low levels of polymorphism within microsatellite loci, the power of a given statistical approach to achieve complete exclusion for a given individual putative parent will be low. As a result, the analysis will yield more than one non-excluded candidate parent and thus no certainty can be assigned to any one individual parent (Jones *et al.,* 2010). Since different parental genotypes will differ in their probability of having produced the focal offspring genotype (Meagher & Thompson, 1986), the determination of the single most likely putative parent from the pool of non-excluded candidate parents is required (Jones *et al.,* 20120). Categorical allocation achieves just that by using a likelihood or Bayesian approach (Neff *et al.,* 2001), based on the Mendelian-transition probabilities (Marshall *et al.,* 1998), which is the probability of acquiring a particular offspring genotype given specified parental genotypes (Jones *et al.,* 2010).

Other methods of parentage analysis have been developed for different empirical scenarios. The fractional allocation approach allows different statistical properties to accommodate for different population-level variables such as variance in reproductive success. Similarly, the full-probability approach also incorporates population-level variables of interest that can be simultaneously calculated with parentage. Or, in the case where parental genotypes are not known but the genotypes of offspring are, parental genotypes may be reconstructed from the known genotypes of offspring in full or half-sib families (Jones, 2001). And, finally, if neither candidate parents nor sib-ship families are known then the sib-ship reconstruction approach (Wang, 2004; Ashley *et al.*, 2009) can be used to infer parentage. Parentage is inferred when sib-ships are identified before the reconstruction of parental genotypes (Jones *et al.*, 2010). This particular method is often considered to be based on one of the most powerful approaches of parentage inference. It is the nature of many approaches that do not account for information that is lost from genetic marker data and uninferred relationships that renders them not as powerful. The sibship method, however, takes full advantage of this by employing a simultaneous assignment approach by basing the inferences on information from full/half sibships and parental assignments.

Examples of parentage studies of amphibians employing one, or a combination, of these six methods to investigate aspects of life-history (mentioned further on) include the study by Tennesen & Zamudio (2003). This research used the strict exclusion approach and assumed no mutation or genotyping errors and only paired individuals if their genotypes matched 100%. Similarly, Byrne & Keogh (2008), using approximately 100 individuals, performed exclusion using the program CERVUS. They deduced maternal genotypes by subtracting paternal alleles from offspring genotypes and also would only assign parentage to individuals who matched genotypic data perfectly. These approaches are rarely performed due to stringent nature in which individuals are assigned parentage. However, for these studies, relatively few individuals were sampled (around 100 each) and were subject to controlled mating experiments.

However, using the more commonly chosen method of the categorical allocation approach, Adams *et al.* (2009) sampled 27 females each with egg clutches and reconstructed paternal genotypes from known maternal and offspring genotypes using the program GERUD. In another study by Richards-Zawacki *et al.* (2012) a multi-faceted approach was employed whereby they conducted likelihood based allocation approaches in CERVUS, Bayesian approaches in MASTERBAYES and sibship assignment methods in COLONY. The sibship assignment method has also been used, to assign paternity to egg clutches in the frog *Kurixalus eiffengeri* (Cheng *et al.*, 2013), and to infer parentage for *Allobates femoralis* (Ursprung *et al.*, 2011).

Table 4.1. Parentage publications of amphibians in the literature and the computer programs used to employ the various methods

Amphibian group	Computer software	Method	Authors
Anurans			
	CERVUS, Manually	Allocation, Exclusion	Byrne & Keogh, 2008
	COLONY, PROBMAX	Exclusion, Sibship	Cheng *et al*, 2013
	Manually	Exclusion, Kinship	Laurila & Seppa, 1998
	Manually	Exclusion	Lodé, & Lesbarrères, 2004
	CERVUS, COLONY, MASTERBAYES	Bayesian, ML, Sibship	Richards-Zawacki *et al, 2012*
	COLONY	Sibship	Ringler *et al*, 2012
	Manually	Exclusion	Roberts *et al*, 1999
	CERVUS, GERUD, Manually	Allocation, Exclusion, Reconstruction	Sztatecsny *et al*, 2006
	COLONY	Sibship	Ursprung *et al*, 2011
Salamanders & Newts			
	GERUD, Manually	Allocation, Reconstruction	Adams *et al*, 2005
	PEDIGREE, Manually	Allocation, Reconstruction	Gopurenko *et al*, 2007
	Manually	Exclusion	Jehle *et al*, 2007
	CERVUS, GERUD, Manually	Allocation, Exclusion, Reconstruction	Jones *et al*, 2002
	GERUD, Manually	Allocation, Reconstruction	Liebgold *et al*, 2006
	GERUD, Manually	Allocation, Reconstruction	Steinfartz *et al*, 2005
	Manually	Exclusion	Tennessen & Zamudio, 2003
	CERVUS, PAPA	Allocation, Exclusion	Williams & DeWoody, 2009
Caecilians			
	Manually	Exclusion	Kupfer *et al*, 2008

However, these studies of parentage/pedigree inferences in amphibian species are rather limited within this field when compared to mammals and birds. This is because amphibians exhibit certain life-history traits such as high fecundity, lifelong growth, and high variance in reproductive success, making it difficult to obtain tissue samples and reliable demographic data. Nevertheless, they have revealed important insights into amphibian genetic mating systems and life history.

Insights into the behaviour, reproductive strategies, and general life history of amphibians for anurans (Lodé, & Lesbarrères, 2004; Byrne & Keogh, 2008; Ursprung *et al.*, 2011; Cheng *et al.*, 2013), salamanders and newts (Tennessen & Zamudio, 2003; Adams *et al.*, 2005; Steinfartz *et al.*, 2005; Liebgold *et al.*, 2006; Jehle *et al.*, 2007), and caecilians (Kupfer *et al.*, 2008) have been obtained through parentage/pedigree based analyses (Table 4.1). These insights into life-history include, for example, the occurrence of multiple paternities (polyandry). Adams *et al.* (2005) showed that the need for sperm competition to be accounted for by females mating with multiple males was fulfilled. Moreover, evidence exists to suggest that within this natural population of salamander *Desmognathus ocoee*, as females mate on multiple occasions they may actually manipulate insemination and mating frequency by rejecting males. They also found that for the females that engaged in polyandry, there was one male that had a tendency to sire the majority of offspring per clutch from that female. Furthermore, these males were largely the first to inseminate the female suggesting that sperm precedence is operating. This could impact male reproductive strategies and create pressures for the play off between being the first male to mate and having sperm held in storage for longer periods. In a study by Tennessen & Zamudio, (2003) the spotted salamander *Ambystoma maculatum* showed evidence of multiple paternities due to the storage of sperm. Although this was based on experimental data, this is a potential occurrence of natural mating aggregations. Moreover, they found that the success of the mating males was dependent upon their early arrival to the pond. Thus, providing the risks of mortality associated with freezing in early spring temperature fluctuations are exceeded, this could help to explain the early migration of males to the breeding site. In summary, the study provided several insights into the reproductive strategies of the spotted salamander and male reproductive fitness by showing that, the earliest arriving males, males that encounter females first, and males having sperm stored from the previous breeding season (or mating site) are at an advantage. In extreme cases, females are promiscuous to the extent that every female within the population mates with multiple males. In fact, Byrne & Keogh (2008) showed that sequential polyandry, whereby females mate sequentially with multiple males through the duration of one breeding season, was operating as females partitioned their eggs between two and eight males. This strategy may have evolved as a mechanism of reducing variance in reproductive success and enhancing fitness. The variance in reproductive success is reduce as more males get to successfully mate while at the same time females get to receive genetic benefits from being polygamous. A number of hypotheses (albeit they were not formulated for amphibians) have been suggested to explain these benefits, such as safeguarding against mating with: infertile males (the fertility insurance hypothesis), poor fathers (paternal care hypothesis), genetically inferior males (intrinsic male quality hypothesis), or genetically incompatible males (genetic incompatibility hypothesis) (Byrne & Keogh, 2008). Since terrestrial breeding in this species carries huge risks causing nest failure, these proposed hypotheses help to ameliorate the costs associated with such failures. However, these costs account for only around 10% of all egg losses compared to the 90% of failures that occur due to desiccation caused by the poor location or quality of nests in which eggs are deposited. Therefore, females that engage in such polygamous behaviour are doing so, primarily, to ensure improved fitness chances of their offspring by depositing eggs into multiple nests. Besides other studies of frog species revealing the extent of polyandry (Ursprung *et al.*, 2011; Zhang *et al.*, 2012) and sequential polyandry (Blackwell & Passmore, 1990), this study has discovered the highest levels of sequential polyandry in a vertebrate species and was the first to show that it can help reduce the damaging environmental effects of nest failures. Conversely, male polygamy, polygyny has also been observed in a few studies of amphibian species (Ficetola *et al.*, 2009; Cheng *et al.*, 2013). The study by Cheng *et al.* (2013) on the tree frog *Kurixalus eiffengeri*, revealed sequential polygamy resulting in males using a form of parental care as a means to attract females with whom to mate. Females of this species deposit egg clutches in bamboo stumps or tree hollows, while the males are territorial at the opening of them and call to attract females. Females approach the males and matings occur that causes the new egg clutch to be deposited with the existing one, resulting in overlapping egg clutches in a nest. These overlapping egg clutches may be a reproductive strategy employed by the males to counterbalance the effects of limited breeding activity while guarding egg nests. The benefit of such behaviour is twofold, since males can ensure the survival of existing and future occurring egg clutches while remaining available to receptive females.

Other studies using parentage analyses as means to reconstruct pedigrees have revealed insights into different aspects of genetic mating systems. Such as, the study by Richards-Zawacki *et al.* (2012) that looked at mate choice with respect to colour variation. In the study species, the strawberry dart frog (*Dendrobates pumilio*) matings have previously been shown to be based on colour variation, that is, that females prefer males of the same colour morph. The results showed that under experimental conditions females may mate with males of the same colour morph (red colour morph) but selection was less specific for females of the yellow colour morph. Despite the preference for yellow females to mate with their own colour morphs, this less specific selection was likely due to the fact that these variants occur at different frequencies in the wild. Given the differences in these frequencies of the colour morphs in the wild, individuals of the yellow phenotype incur higher costs to mate assortatively (due to longer periods exposed to threats such as predation, competition from other females etc). This could therefore explain the disparity between the experimental data and occurrences in the field.

Insights into reproductive strategies have also been observed in the common toad. Under experimental conditions and in naturally breeding populations, polyandry was detected in 22% and 30% of cases respectively (Sztatecsny *et al.*, 2006) with these figures for polyandry similar to those of other studies on anurans (for, e.g. Lodé, & Lesbarrères, 2004). Multiple paternities arose as a result of toads forming a 'mating ball', in which multiple males mount a female (multiple amplexi) with no evidence to suggest fertilisation via free-swimming sperm. These instances of multiple paternities are most likely to arise under condition in which there are high population densities and male biased OSRs (Operational Sex Ratio). Given the nature of multiple amplexi, where females struggle to fight off males and may even drown as a result, female polyandry might arise unintentionally as a means by which they can avoid drowning. Therefore, unlike the cases where females are inclined to breed with multiple males (e.g. Byrne & Keogh, 2008), the case of the common toad indicates that polyandry is possibly a derivative of the heavily skewed sex ratio in favour of males.

The use of genetic markers to provide unambiguous identification of individuals (i.e. genetic fingerprints) can not only be employed to infer parentage within a population but the genetic data can also to be used to provide estimates of relatedness and inbreeding. Relatedness and inbreeding can simply be defined as the sharing of homologous alleles that are identical-by-descent (IBD) between and within individuals, respectively (Ritland, 1996). The idea of identity-by-descent forms the basis for the estimates of the 'coefficients of relatedness' (or kinship) to be calculated. This estimate is indicated as *r*, and is the probability of IBD when sampling homologous alleles. The coefficient, in outbred populations, increases with genetic dissimilarity, for example for r = 1/4 for parent-offspring and full-sib relationships, 1/8 for half-sibs and 1/16 for first cousins.

Examples of studies that have performed kinship analyses include that of Ringler *et al.* (2012) who estimated pairwise relatedness using the program KINGROUP. Specifically, the study examined the distribution of pairwise relatedness between parental dyads observed in the field with those of simulated data for 'full-sibs', 'half-sibs', and 'unrelated' individuals. The study showed that the parental dyads observed in the field had a mean pairwise relatedness coefficient of zero, matching that of the overall population mean of zero. Thus, the parental dyads observed were neither more nor less related than would be expected from random mating. Furthermore, the relatedness coefficients for full and half-sibs identified in the field, r = 0.41 and 0.21 were within the ranges obtained from the simulated full and half sibs, r = 0.489 and 0.236 respectively.

4.2. Aims

The current study makes use of part of an existing dataset encompassing nearly three decades of research of a common toad (*Bufo bufo*) population in Dorset (for more details see Chapter 2). By using genetic data derived from available tissue samples, the aim of the study was to infer parentage within the population of individuals spanning two generations. Furthermore, the parental relationships inferred from genetic data were compared with recorded information about parental pairs observed in the field.

4.3. Methods

Tissue samples of *Bufo bufo* were obtained from the ongoing study of the common toad population in Dorset. DNA was extracted from tissue samples using a standard phenol/chloroform procedure and PCR conditions were performed as per the touchdown program described in Brede *et al.* (2001). Genotyping was performed on the ABI3130 genetic analyser and errors in the data checked for by using various software programs. These techniques are detailed in full in Chapter 2.

The program COLONY (Jones & Wang, 2009) was used to perform parentage analysis with the multilocus genotyping data. COLONY employs a maximum likelihood method to assign parentage and sibship jointly and in doing so considers the likelihood over the whole pedigree rather than for just relationships between paired individuals. This improves the power and accuracy of the inferences, utilising the information that is normally lost with other current methods of parentage inference ((Jones & Wang, 2010). For example, in a pairwise approach to inference, a single offspring provides information for a single allele with regards to inferring and locating parental genotypes from a given dataset. However, the sibship method employed by COLONY considers multiple offspring in the sample increasing the probability that the full parental genotype (i.e. both alleles) can be inferred from the pool of offspring genotypes. Furthermore, by considering more individuals in the sample and designating them into groups (clusters) offspring that do not share ancestry can still provide information for other individual offspring. For example, if an offspring does not share the same parentage (either by full or half-sibship) with another offspring they may still provide information by their presence in the cluster because they may be linked via another individual offspring (Jones & Wang, 2010).

Initially, individual candidate parents from the same cohorts were used to establish full parentage of offspring from the 2008 and 2009 cohorts. New projects for each were created but each had the same set parameters. The mating system was set to 'male monogamy' and 'female monogamy' and set 'without inbreeding'. The 'species' options were set to 'dioecious' and 'diploid' and the length of run set to 'short'. The analysis method was set to 'full-likelihood (FL)', no 'sibship prior' and the 'run specifications' were set to 'do not update allele frequencies' with a random number seed of 1234, with the number of runs set to '1'. Allele frequencies were not updated since there was no prior expectation that family sizes would be large and since it makes the runs substantially more computationally intensive (see COLONY manual). The marker types and error rates input file required to indicate the level of type 1 and type 2 errors associated with microsatellite marker data was provided. The type of marker was set to '0' to represent co-dominant for all markers and the type 1 error rate (errors associated with allelic dropout) was set to the default of 0.05. The type 2 errors (errors associated with other forms of homozygote excess such as mutations) were set to the values given by MICROCHECKER (Oosterhout *et al*., 2004), as per the 'Brookfield 1' method of null allele estimation. The allele frequencies were not added during set up of the run and were selected to be calculated by COLONY.

Offspring genotypes were added from individuals within the 2008 and 2009 cohorts while maternal and paternal genotypes were added from individuals from the 2004 cohort. Known maternal and paternal sibs, excluded maternity and paternity and excluded maternal and paternal sibs were all set to zero. This procedure was repeated using females and males as candidate parents from 2005 and 2006 to form another two separate runs per cohort. A further 6 runs were performed to establish full parentage of the candidate offspring by combing the sexes from different cohorts to account for cases in which a father, or mother, was not sampled in the same year as its mating partner. Runs to estimate maternities and paternities for each parental cohort were also conducted and these were then compared to maternities from parental pairs to support assignments. If an offspring assigned full parentage was not assigned the same mother from the maternity analyses then these data were discarded as ‘untrue’ or ‘unreliable’ inferences. Similarly, the maternity assignments from all of the aforementioned parentage runs were also compared with assignments from the maternity runs alone from the corresponding cohort and also discounted if there was incongruence.

The program KINGROUP (Konovalov *et al.,* 2004) was used to calculate relatedness coefficients between all individuals within the sampling period (2004-2009). An input file containing all of genetic data available of all individuals was used for the analysis and allele frequencies were calculated within the program. Pairwise relatedness was estimated based on the calculations of Queller & Goodnight, (1989), and Goodnight & Queller, (1999) by selecting the ‘kinship’ pairwise estimator. The relatedness coefficients between any two dyads could then be found from a relationship matrix generated by the program.

4.4. Results

A total of 898 DNA extractions encompassing all sampling years underwent PCR amplification and genotyping. Table 4.2 shows the total number of individuals successfully genotyped per sampling year and per locus. The size ranges of microsatellite alleles, along with the number of alleles per locus are also shown. The fewest number of alleles was 7 (for Bbufµ15), while the most polymorphic locus was Bbufµ49, yielding 25 alleles. The mean number of alleles per locus was 14.

Table 4.2. Results from genotyping data

Locus			No. of individuals per sampling year					Total no. of
	Allele size range (bps)	Alleles per locus	2004 191	2005 117	2006 104	2008 198	2009 288	Individuals Genotyped
Bbuf µ11	103–131	14	103	58	60	165	223	609
Bbuf µ49	160–216	25	87	100	81	137	169	574
Bbuf µ62	163–203	13	96	94	53	98	230	571
Bbuf µ65	158–202	23	48	19	67	135	229	498
Bbuf µ24	128–158	13	136	110	99	179	172	696
Bbuf µ46	132–154	10	112	54	96	174	233	669
Bbuf µ54	166–190	10	95	107	97	168	251	718
Bbuf µ15	158–174	7	148	93	85	168	235	729

Figure 4.1 shows a visualisation of the PCR products after genotyping and subsequent analysis in the software program Peakscanner. The tall green peak represents the fluorescently labelled locus *Bbufu*24, with the singular peak denoting that this individual at this locus is a homozygote. Similarly, the two tall blue peaks indicate that this individual is heterozygous for the locus *Bbufu*65. The smaller peaks, at both loci, are the stutter bands that precede the taller peaks that are the microsatellite alleles. The RFU on the *y* axis indicates the Relative Frequency Units and shows the intensity of the microsatellite peaks as detected by the genetic analyser. The *x* axis gives the length of the

microsatellite fragments in base-pairs (bps) and therefore it can be seen that this individual has the homozygous genotype 151 bps and 151 bps for locus *Bbufu*24 and the heterozygous genotype 182 bps and 186 bps for locus *Bbufu*65.

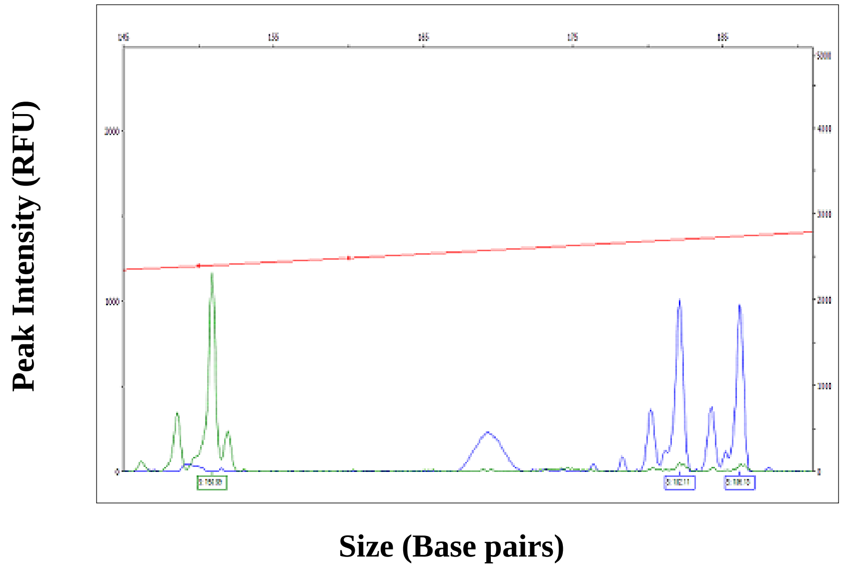


Figure 4.1. Scored alleles for *Bbuf*µ24 (green) and *Bbuf*µ65 (blue) for the same individual from 2009. RFU = Relative Fluorescence Units.

Table 4.3. Expected and observed heterozygosity, the Hardy-Weinberg test, and the number of individuals tested per locus for each sampling year.

Locus	2004				2005				2006				2008				2009			
	n	H_E	H_O	*P*	*n*	H_E	H_O	*P*	*n*	H_E	H_O	*P*	*n*	H_E	H_O	*P*	*n*	H_E	H_O	*P*
Bbufµ11	103	84	93	0.496	58	50	56	0.482	60	52	52	0.323	165	139	137	0.647	223	193	201	0.021
Bbufµ49	87	82	74	0.106	100	94	88	0.006	81	77	71	0.025	137	129	110	0	169	159	157	0.018
Bbufµ62	96	69	60	0.005	94	73	59	0.007	53	40	39	0.014	98	75	67	0.583	230	172	190	0.009
Bbufµ65	48	43	36	1E-04	19	18	18	0.007	67	62	58	0.091	135	124	115	0.04	229	212	184	6E-04
Bbufµ24	136	106	103	0.378	110	86	80	0.484	99	74	69	0.886	179	142	141	0.11	172	128	116	0.07
Bbufµ46	112	68	61	0.114	54	33	29	0.189	96	58	55	0.267	174	106	106	0.101	233	154	151	0.303
Bbufµ54	95	70	75	0.379	107	76	70	0.067	97	72	69	0.22	168	125	135	0.925	251	188	180	0.064
Bbufµ15	148	104	93	0.017	93	64	57	0.049	85	59	50	0.347	168	117	98	0.008	235	165	136	0

H_E = expected heterozygosity, H_O = observed heterozygosity, *P* = exact value estimated by the Markov Chain method (Guo & Thompson, 1992), *n* = number of individuals tested.

The results from the Hardy-Weinberg test (Table 4.3) show the estimates close to, and departures from, HWE (*P* values at 0.05 ά). Most years show estimates close to HWE for 4 or more loci while 2009 shows 5 loci deviating from HWE. Loci *Bbuf*µ24, *Bbuf*µ46 and *Bbuf*µ54 are in HWE for all sampling years. All estimates were based on an exact *P* value test (Raymond and Rousset, 1995) calculated from a Markov Chain method (Guo & Thompson, 1992).

Parentage analyses were inferred using the software COLONY (Jones & Wang, 2009) on all individuals genotyped at a minimum of six loci. Table 4.4 shows the parentage inferred where a mother and a father were assigned to at least one offspring, and where the maternal data were congruent with separate tests of maternity. Male and female parents from 2004 are displayed first and are denoted with the prefix 'E'. Individual parents from 2005 and 2006 (prefixed with 'D' & 'C' respectively) are subsequently shown, followed by the combinations of sexes from different sampling years (for example, after parents from 2006 were analysed, females from 2004 were analysed with males from 2005, and so on). Of a total of 31 parental pairs that were assigned offspring, 17 were assigned to one individual, while the highest number of offspring (6) was the inferred progeny of female *D254f* and male *E356m*.

Table 4.4. Inference of parentage as performed by COLONY (Jones & Wang, 2009) for individuals from the parental generation in 2004, 2005 & 2006 and the offspring generation in 2008 and 2009.

Mother	Father	Offspring						Probability
A363f	A298m	E537						1
A375f	A395m	E341	D471					0.99
A501F	A261m	E122						1
A102f	A376m	E293	D322	D537				
B239f	B080m	E012	E096	E299				0.97
B152f	B286m	E179	D474					0.99
C130f	C131m	E571	D385	D724				1
C136f	C314m	E400						1
C217f	C168m	D530						1
C454f	C133m	D495	D576					1
A362f	B324m	E497						0.8
A241f	B155m	E172						0.8
A466f	C067m	E040						0.97
A241f	C262m	D156	D540					0.97
A108f	C241m	D194	D317					1
A433f	C166m	D437	D632					1
A106f	C168m	D725						0.98
A150f	C275m	D015						0.91
A229f	C021m	E136						1
B059f	A221m	D665						1
B061f	A458m	D325	D710	D777				0.99
B254f	A356m	E017	E257	E511	D041	D052	D538	0.83
B059f	C330m	E332						0.99
B092f	C431m	E070						0.94
B246f	C224m	E317						1
B336f	C262m	E393	D275					
B447f	C222m	E408	D081					
C369f	A125m	D624						1
C074f	B406m	E424						1
C213f	B062m	E491						1
C327f	B324m	D294	D499					1

A = individuals from 2004, B = individuals from 2005, C = individuals from 2006, D = individuals from 2008, and E = individuals from 2009, m = males, f = females.

The probabilities of the inferred relationships are also given in Table 4.4, using 0.8 as the threshold. A total of 3 parental pairs, marked by asterisks, were inferred by comparing offspring assignments of maternity and paternity and were not inferred conjointly, as parentally paired, offspring triads. For example, when offspring assigned to female E102f were compared with offspring assigned to male E376m, 3 of those assignments (A293, B322 & B537) were paired with both individual parents. These genetically inferred parental pairs were compared with the parental pairs observed in the field resulting in only 1 case of congruence between the two sets of paired individuals. Toad numbers C130f and C131m, inferred to have sired 3 offspring, are the only two individuals to be assigned offspring that were also observed to be paired together in the field.

The complete data obtained from inferences of maternity and paternity are summarised in Figure 4.2. The number of individual offspring assigned to a maternal and paternal parent can be seen, with the majority of assignments being 1 and 2 offspring per parent while the highest number of offspring (10) was assigned to a female (C027f).

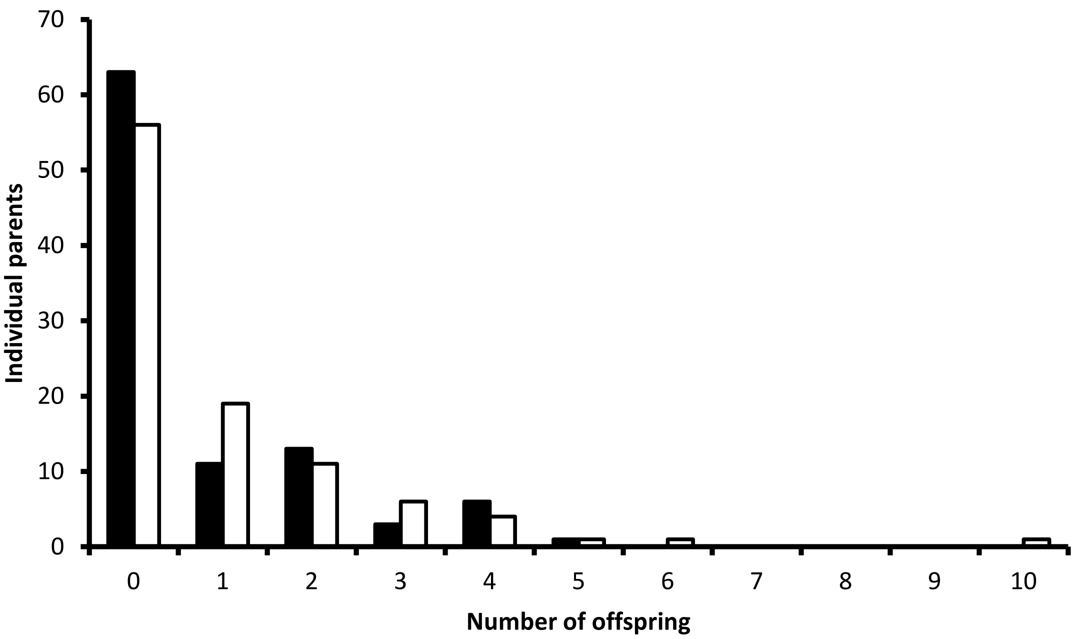


Figure 4.2. Number of progeny assigned parentage from paternity (dark bars) and maternity (light bars) analyses in COLONY.

A total of 116 and 95 offspring were assigned to 48 mothers and 40 fathers respectively. However, after comparison of these offspring assignments between sexes, 20 of which were shown to be allocated both a mother and a father. These individuals were omitted since it required categorising them as either maternally or paternally assigned or grouping them with the offspring allocated full parentage (as per Table 4.4). The new total was 96 offspring assigned to 43 mothers and 75 offspring assigned to 34 fathers and thus, the total number of offspring assigned either maternity or paternity was 171. In addition to the number of offspring allocated full parentage, which was 54, (see Table 4.4) the number of offspring assigned either maternity or paternity was 175.

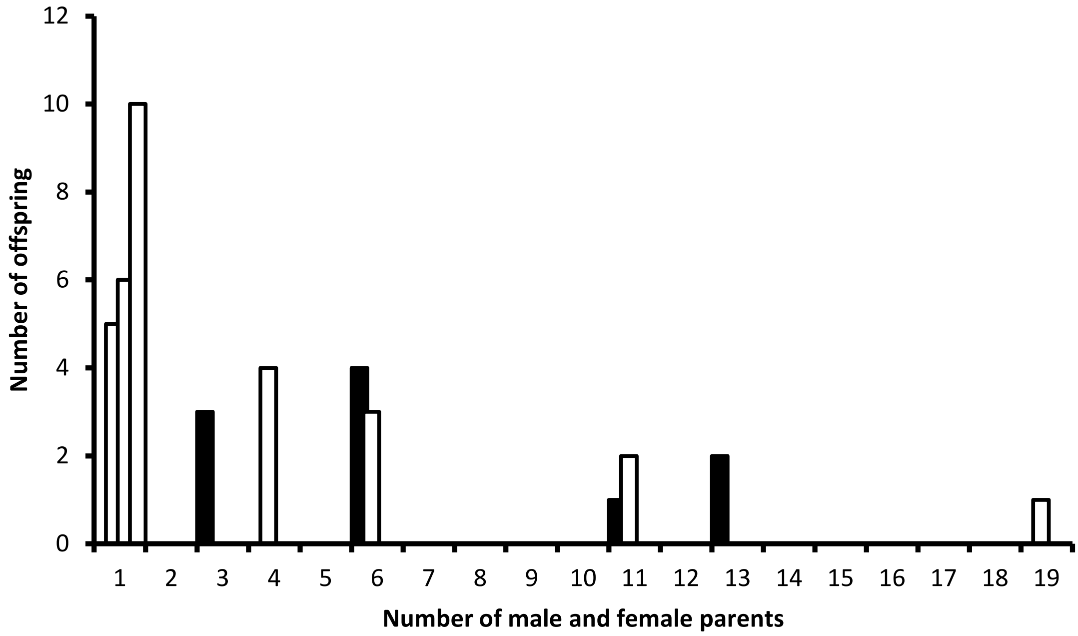


Figure 4.3. The number of male and female parents (*x* axis) assigned offspring (*y* axis), from separate analyses of paternity and maternity in COLONY. Black bars = males, Light bars = females

This therefore results in a total of 229 (47%) individuals from 2008 and 2009 used as candidate offspring assigned either full or singular parentage. Figure 4.3 shows the paternity and maternity assignments from the parental perspective, representing the number of males and females to sire offspring and size of progeny array per parent. Thus, the total number of parents per progeny array is illustrated.

For example, 1 female sired 4, 1 female sired 6, and another female sired 10 offspring each. Similarly, 3 male parents sired 3 offspring, and so on. The mode of offspring assigned parentage is 1 for paternity and maternity, with 1 offspring being assigned a single father on 11 occasions, and 19 occasions for maternity assignments. The total number of individuals inferred as parents along with the total number of offspring they sired are displayed in Table 4.5.

Table 4.5. Results from parentage analyses and the number of individuals inferred as parents, comparative to numbers of individuals sampled and population census size, per sex and per year.

	Candidate parents sampled	Parents typed at min 6 loci	Parents inferred		Offspring assigned	Proportion parentage of inds. Sampled		Population census size *N*	Proportion parentage of census size	
Parents			Males	Females		Male	Female		Male	Female
2006	105	79	20	14	87	0.36	0.29	538	0.04	0.21
2005	119	66	7	12	38	0.12	0.2	473	0.018	0.16
2004	196	59	6	16	46	0.06	0.16	593	0.0014	0.1
Total	420	204	33	42	171	0.15	0.2		0.026	0.14

The proportion of total number of individuals sampled and total number of individuals in the population (census size, *N*) that were inferred as parents are also displayed. These data are also divided between male and female toads. The highest number of parents inferred and offspring assigned are from the parental cohort of 2006 with the lowest in 2005. The proportion of individuals inferred parentage of the population census size increases from 2004 to 2006 for both sexes. The proportion parentage of individuals sampled was calculated by dividing the number of inferred parents for each sex with the total number of individuals sampled for that sex. These latter values are not present in the table and are as follows: the total number of males sampled is 213, and the total number of females sampled is 207. The data from Table 4.5 are for paternity and maternity assignments only and do not include cases of offspring assigned full parentage (see Table 4.4).

The pairwise relatedness calculated in KINGROUP (Konovalov *et al.*, 2004) generated a kinship matrix (see Appendix) giving the relationship coefficients of any two individuals. All parental pairs, assigned offspring through the parentage analyses in COLONY (see Table 4.4) were used to create a boxplot to visualise the distribution of relatedness.

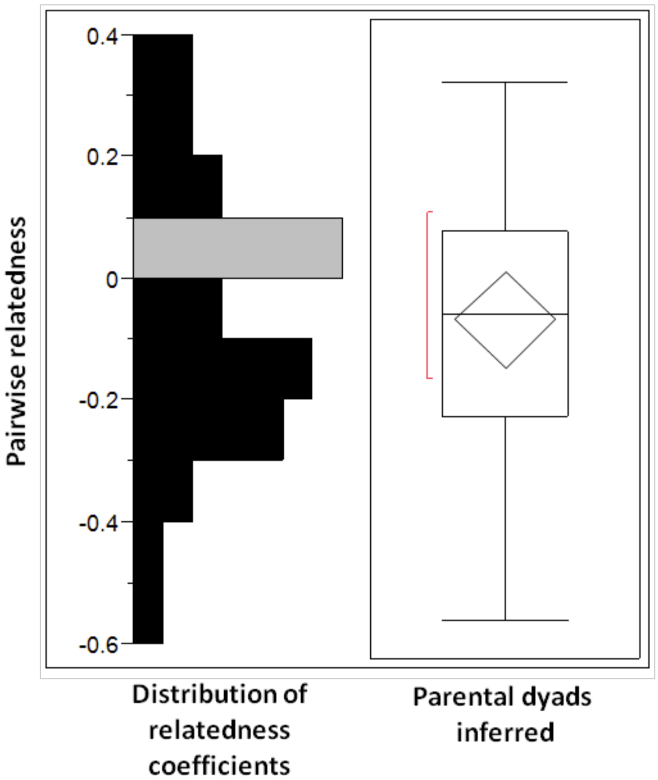


Figure 4.4 Relatedness coefficients as calculated by KINGROUP with a boxplot showing the distribution of values for all parental pairs that were assigned offspring. Grey bar = mode.

The data conforms close to a normal frequency distribution and the modal coefficients are distributed within the 0 – 0.1 quantile (Figure 4.4). Approximately 53% the coefficients are distributed in quantiles below zero, with zero being set as the default population average value of pairwise *r* in KINGROUP. The mean pairwise *r* for inferred parental dyads was r ± SD = -0.067±0.2 and therefore below the population mean of zero. Pairwise values of *r* for the upper and lower quartiles are 0.078 and -0.23 respectively. Inbreeding coefficients *F*, were calculated in the program Coancestry (Wang, 2011) giving *F* for individuals from all sampling years, totalling 898 individuals. An average of *F* was taken for each sampling year and graphically represented in Figure 4.5, along with data of the proportion of parents sampled that were inferred familial relationships (see Table 4.5). The figure shows the proportion of parents sampled that were assigned offspring increases from 2004 to 2006 (as mentioned above) and that the level of inbreeding shows a general decreasing trend at the same time.

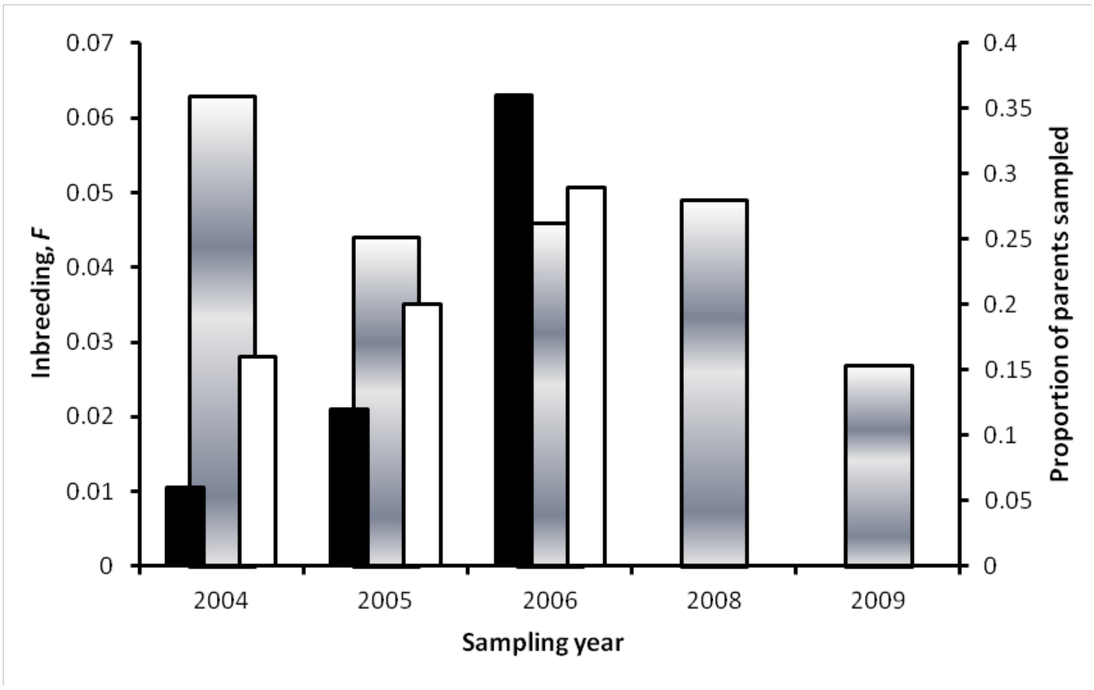


Figure 4.5 Proportion of parents sampled that were inferred as mothers (light bars) and fathers (dark bars) along with the inbreeding coefficient, *F*, (grey/light bars) for all years. Inbreeding estimates at 95% confidence.

4.5. Discussion

The allelic data derived from the current study (Table 4.2) are similar to that of other studies (Brede *et al.*, 2001; Wilkinson *et al*., 2007; Martinez-Solano & Gonzalez, 2008) whereby high levels of polymorphism for *Bufo bufo* microsatellite markers were found. Although these findings correspond to the relative levels of polymorphism between loci in Brede *et al.* (2001), I found the highest numbers of alleles compared to previously published levels. Brede *et al.* (2001) found that *Bbuf*µ49 & *Bbuf*µ65 were the most polymorphic loci with 17 alleles each, whereas the current study found 25 and 23 alleles, respectively, for these loci. However, Brede *et al.* (2001) studied a population in Sussex, as opposed to Dorset, which may explain some variation in polymorphism between the two sites. The sample size of Brede *et al.* (2001) was also smaller than the current study which could have resulted in some rare alleles not being sampled. Martinez-Solano & Gonzalez (2008) used two (at a total of five loci) of the microsatellite loci used in the current study, and found high levels of polymorphism for *Bbuf*µ49 and *Bbuf*µ11, with 21 and 24 alleles respectively, for populations in Spain. The study found that these were the most polymorphic loci as did the current study, with *Bbuf*µ49 closely matching the number of alleles found in the current study to that of Martinez-Solano & Gonzalez (2008) with 25 alleles.

The results from the Hardy-Weinberg tests (Table 4.3) reveal that, with the exception of 2009, estimates are significantly close to HWE at the 5% confidence level for most of the eight loci used. In practice, genotypes are rarely in exact HWE since natural populations are exposed to at least one of the disturbing influences proposed by the Hardy-Weinberg law. Moreover, the deviations from HWE are within the expected norms and most likely are due to the presence of null alleles and/or scoring errors within certain loci and finite population size. For example, the data are out of HWE most frequently across loci and specifically for loci *Bbuf*µ49, *Bbuf*µ62, *Bbuf*µ65, and *Bbuf*µ15. The deviation from HWE, therefore, shows this within loci pattern as opposed to being more spread across the whole population for all years. These deviations from HWE, as derived from the program GENEPOP, are congruent with null allele frequency rate as calculated by MICROCHECKER and CERVUS. However, such errors were corrected for by reassessing erroneous alleles, as indicated by the program Tandem, and accounting for the rate of null alleles and errors associated with stutter bands before using the data for parentage analyses.

Very few pedigree based studies of amphibians exist owing to certain life-history traits such as life-long growth, high variance in reproductive success and high fecundity. These factors can make it difficult to capture information based on genealogical relationships among individuals of an amphibian population. However, analyses within the current study were able to ascertain parentage for 229 offspring out of a total of 486 individuals using 8 polymorphic microsatellite loci. This is similar to studies of other anuran species that also used 7 (Ursprung *et al.*, 2011) and 10 microsatellite loci (Cheng *et al.*, 2013) with similar levels of polymorphism to conduct parentage analyses in the program COLONY. This shows, therefore, that these (similar) levels of loci used and polymorphisms yielded have been sufficient to successfully infer parentage in this program for published studies on other anurans. Parentage assignments of at least one parent could be achieved for approximately 60% of offspring in the study by Ursprung *et al.* (2011), similar to the assignment rate in the current study that was close to 50% of the sampled offspring.

The results from the parentage analyses whereby offspring were assigned a father and a mother (Table 4.4) shows some variation in reproductive success. A total of 16 parental pairs sired one offspring and 10 pairs sired two offspring, whereas four pairs sired three offspring and two pairs sired four and six offspring each, respecitvely. Moreover, the results from the singular parentage analyses also show some degree of variation among successfully reproducing individuals. For example, data from the maternity tests indicates that an individual mother (C02f) has sired 10 offspring, whereas the highest number of offspring sired by any single male is five. These data denote differences in reproductive success between the sexes and would suggest some level of polyandry was operating within the population. Polyandry in *Bufo bufo*, has been observed where 30% (in the field), and 22% (experimentally) produced egg strings were sired by more than one male (Sztatecsny *et al.*, 2006). However, various runs via COLONY to test for such a mating system by selecting the 'polygamous' option did not yield any evidence to suggest multiple paternity and hence this could be an artefact of incomplete sampling of the males.

At many amphibian breeding foci, there is a bias in the operational sex ratio (OSR) in favour of males especially for explosively breeding species where it can be as high as 10:1 (Wells, 1977). This is apparent at the breeding population of the current study as males outnumber females by approximately 3:1. Despite the difference in the individuals available to sample, members of the population were sampled based on their association with mating partners. That is to say, male and female toads that were found in amplexus together in the field were sampled as 'mating partners' and thus providing a means to circumvent the problem of having many males unsampled. However, as the results from the parentage analyses show, only one parental pair inferred by COLONY matched with the parental pairs observed in the field (numbers shown in italics in Table 4.4). Therefore, given the relative accuracy of parentage analyses, it is likely that the individual toads observed in amplexus do not represent the true mating partners. This could have resulted from the manner in which the toads actually pair up. For example, some female toads at the breeding site changed males several times and the male classified as the breeding individual was recorded as the last male with whom the female was associated (pers. comm. Chris Reading, 2009). Therefore, the last male to be associated with a given female may have been usurped by a different male following the recording due to the separation of the toads from amplexus. Male and female toads in amplexus are separated to be measured and weighed and then regrouped before being placed back in the pond. Furthermore, given the strong intrasexual competition from males (Wells, 2007) in the common toad, the act of 'scrambling' (scramble competition) for a female mate could make this situation more likely. Thus, as the toads are replaced into the pond, scramble competition results in the recorded male being supplanted by another male as many males try to gain access to a female mate.

Biases to the operational sex ratio can cause greater variance in reproductive success for the limited sex (Emlen & Oring, 1977), in this case the female. This bias in OSR could help explain differential success between the sexes, for example the additional 20 offspring that were assigned to female parents as opposed to male candidates. Because of the bias, the numbers of female parents of the total number of breeding adults sampled were close to 70% but the males were closer to 20%. This skewed sex ratio could account for the higher number of offspring assignments to maternal parents since many males from the population remain unsampled.

The results from the KINGROUP pairwise relatedness coefficients (Figure 4.4) show that mean r for inferred parental pairs (-0.06) is below the population mean of zero and that 56% of individuals are 'unrelated'. The mean r data derived from these analyses are similar to that of another study on an anuran species. Ringler *et al.* (2012) showed mean relatedness coefficients of $r \pm$ SD =0.003±0 .127 for observed parental dyads. However, 82.4% of these dyads were classed as 'unrelated individuals' and probably reflects the greater n (100) for that study. With r = -0.06, the genetically inferred parental dyads are therefore less related to one another than would be expected by random chance. However, with SD = 0.2, the variance around the mean is high representing a wide distributional spread and with n = 32, this might not be indicative of the actual mean of genetically inferred paired parents. Nevertheless, a mean r = -0.06 indicates that the highest levels of reproductive success is for parental pairs of less than intermediate genetic relatedness. This therefore means that there is, from a genetic perspective, a degree of viability for this population of common toads since inbreeding appears to not be prevalent. Explanations for this lack of inbreeding could be based around the notion of mate choice. Mate choice, as it is most commonly referred to from the female perspective, can be defined as the choice of sperm to fertilise an egg (Eberhard, 1996). Thus, for a number of reasons, females chose to mate with specific males (Halliday, 1983). However, due to scramble competition of *Bufo bufo* and the inability for most females to dislodge unwanted males, this sexual selection mechanism would be absent as females appear to be somewhat limited in their choice of males (Davies & Halliday, 1979). Even though it has been argued that males may be selected for by females by choosing those individual males that are most persistent (Kokko *et al*., 2003), it is not equivalent to the actual choosing of males from a wider subset of the male population. Thus, as inbreeding requires some level of choice of females with which males to mate, this lack of choice could explain the lack of inbreeding. Indeed, when the results of Figure 4.5 are considered, it can be seen that inbreeding (as shown through the coefficient of inbreeding measures, F) shows a decreasing trend from the years 2004 to 2005 and thus indicates that inbreeding has recently been somewhat reduced. Inbreeding has been shown to cause an increase in the number deleterious alleles through the decrease in heterozygosity, reducing fitness in a number of species (Keller & Waller, 2002). It has been indicated to be a key component of fitness and directly affect population persistence making it an integral area of research in conservation biology. However, given the evidence to suggest that inbreeding has been reduced in this population, its effects might not be as detrimental in this study. One mechanism to explain a reduced effect of inbreeding could be due to ‘purging’ (Keller & Waller, 2002). Purging is a process whereby the deleterious alleles accumulated through inbreeding are selected against, reducing the mutational load (Boakes *et al*., 2006). This could, therefore, emerge in harsh environmental conditions that cause the reduction in fitness or other life-history traits, such as the reduction in BCI and survival of both sexes and the reduction of fecundity in females as observed in the current study. If these effects begin to cause an increased rate of inbreeding then the process of purging could ameliorate these adverse effects by removing the deleterious alleles in the population. Therefore, this finding that inbreeding has been somewhat reduced on a contemporary scale, (and thereby mitigating the associated adverse effects) is promising evidence for the well-being and viability of this population. Particularly, since the adverse effects that have been reported for this population might indicate an increased risk of the deleterious effects of inbreeding and that it might be more pervasive. This is because, populations with reduced fitness and survival might be expected to become smaller and smaller populations are more susceptible to environmental and demographic stochasticity. And, this can in turn lead to the population becoming further affected by reduced survival and fecundity as well a further increased vulnerability to inbreeding (Keller & Waller, 2002).

In summary, the results show that for two parental years, females were assigned offspring more often than males and that from 2004 to 2006 there was an increase in the number of parental-offspring dyad assignments. Data from the relatedness coefficients show that the population does not appear to be suffering from inbreeding as confirmed by the inbreeding coefficients which interestingly show a temporal trend.

CHAPTER 5:

Assessing evolutionary and ecological responses to changing environmental conditions in a wild common toad population.

5.1 Introduction

Current climate change, involving the rise in temperature associated with alterations in precipitation and atmospheric CO_2 concentrations is considered to have been instrumental in the estimated global biodiversity decline of more than 25% over the last 35 years (Collen *et al.,* 2008). As a result of changing climate, species have responded by altering their physiology, phenology and distribution (Hughes, 2000). Alterations in atmospheric CO_2 levels directly affect the metabolism and development of many organisms, while life cycle events can be affected when environmental cues such as photoperiods are altered (Ellis *et al.,* 1997).

Shifts in distributional ranges have been observed in many animals, such as flying insects, birds, marine invertebrates and terrestrial mammals (Parmesan *et al.,* 1999; Beever *et al*., 2003) and involve individuals moving upwards and polewards in response to shifting isotherms. Indeed, a 3^{o}C increase in mean annual temperature equates to an approximate shift in isotherms of 300-400 km in latitude or 500 m in altitude (Hughes, 2000).

The concept of an alternative state in phenotype in response to changing environmental conditions for a given genotype has a historical basis. The ancient philosophical debate of the roles of 'nurture versus nature' is the basis for the study of the relative contributions of genes and the environment (Pigliucci, 2001). Phenotypic plasticity is the modern embodiment of the environmental aspect. The first evidence provided for the idea of phenotypic plasticity came from Woltereck (1909), who showed that a range of phenotypic outcomes can result from changed environmental stimuli for clones of *Daphnia*. Using the trait 'helmet length' the study showed that when subjected to the presence of a predator, clones of *Daphnia cucullata* expressed different helmet length sizes and ‘neck teeth’. These phenotypes, the presence of which is effective at reducing predation pressure, spanned a range of traits from low to intermediate to high and were named ‘reaction norms’. Since the seminal study of Woltereck (1909), further empirical evidence and key developments for plasticity were provided by Schmalhausen (1949), Waddington (1952), Bradshaw (1965), Via & Lande (1985), Schlichting & Smith (2002).

Phenotypic plasticity has been observed in amphibian species such as the parsley frog *Pelodytes punctatus.* In a study by Jourdan-Pineau *et al*. (2012), frogs were shown to change their breeding behaviour, and breed in the autumn in some years and in the spring in others, according to the specific environmental conditions under which they were naturally subjected. Examples of phenotypic plasticity causing changes to phenotypes as a result of climate change include causing an advancement of parturition dates in: the red squirrel *Tamiasciurus hudsonicus* (Reale *et al*., 2003), the great tit *Parus major* (Charmantier *et al*., 2008), and the collared flycatcher *Ficedula albicollis* (Przybylo *et al*., 2000).

Evolutionary adaptations can also occur in response to environmental change, whereby genetic alterations causing evolutionary change arise at the level of a species or population. For example, in Darwin’s Finches, beak shape and body size were altered in response to the effects of climate change on food resources (Grant & Grant, 2002). Similarly, pitcher plants mosquitoes (*Wyeomyia smithii*) have shifted their genetically controlled photoperiodic response toward shorter, more southern day lengths over the last 30 years in response to a longer growing season (Bradshaw & Holzapfel, 2001). Another study has revealed that whole chromosomal shifts within *Drosophila robusta* is an evolutionary response to climate change (Levitan & Etges, 2005). Microevolutionary adaptations not only occur at the level of the species or population but also in subpopulations (demes) that confer the highest fitness to a specific habitat patch of their environment. When other forces and constraints are absent each local population, usually by means of divergent selection, new traits that are beneficial within the new local environment can evolve.

These fundamental responses shown by populations due to climate change, physiological or phenological change, range shifts, or adaptive change, are all well documented (Hughes, 2000; Postma & Van Noordwijk, 2005; Visser, 2008; Phillimore *et al*., 2010). However, discerning the magnitude of each response, especially plastic versus evolutionary change (Gienapp *et al*., 2008) is essential for our understanding of how populations will respond to anticipated climate change.

The selection pressures imposed upon wild animal populations as a result of climate change are causing these responses of range shifts, plasticity, and evolution. However, distributional range shifts are likely to only provide a very short term solution for many taxa. Similarly, plastic responses will also only be a short term solution and like shifting ranges are limited in their ability to mitigate long-term effects of continued environmental change. Evolutionary responses, however, can provide the means of successful and lasting adaptation through Darwinian natural selection. This is not attainable for plastic responses because they are unable, from the plastic genotype, to produce an extreme phenotype as required in the new environmental conditions. Evolutionary responses can produce such genotypes and overcome the adverse effects on fitness that plastic responses cannot mitigate. It is, therefore, important to disentangle the responses of plasticity and evolution as many organisms face threats associated with environmental change.

One way in which this can be achieved is with the concept of heritability. Heritability is defined by the measure of the proportion of phenotypic variation within a species that is due to genetic factors. However, in order to discern between the relative contributions of additive genetic variance (V_A) and the effects of epistatic interactions (V_I) and dominance (V_D), heritability can be classified as either broad-sense (H^2) or narrow-sense heritability (h^2) (Allendorf *et al*, 2012).

Broad sense heritability is a measure of the proportional variance that is a result of the total genetic differences between individuals. For example, if genetic variance (V_G)/phenotypic variance (V_P) = H^2, then $H^2 = V_A + V_I + V_D/V_P$, allowing for the effects of epistasis and dominance to be measured. However, since only additive genetic variance is the variance upon which natural (and artificial) selection can act, measures of H^2 do not permit the response to selection to be estimated. For example, in a hypothetical scenario, species *X* has a two allele system (A_1A_2) that determines body length. The heterozygous state (A_1A_2) whereby individuals are the longest in length occur at a frequency of 0.50 (2*pq*) and both homozygous states (A_1A_1 and A_2A_2) that produce smaller individuals occur at the frequency 0.25 each (p^2 and q^2) and the allele frequencies are therefore equal. If the longest individuals were desired and to be artificially selected then this would thus result in all heterozygous individuals being chosen for breeding. However, given the laws of Mendelian segregation, the progeny sired as a result of an all heterozygous parental generation would contain the same genotype frequencies. Thus despite H^2 being 1, due to all of the phenotypic differences resulting from genetic differences, the response to selection will be 0 due to the fact that the genetic effects are caused by dominance. Narrow sense heritability, meanwhile, estimates the response of a trait to selection by measuring the proportion of phenotypic variation that is due only to additive genetic variation. Thus, narrow sense heritability is given by $h^2 = V_A/V_P$ (Allendorf *et al.*, 2012).

There are a number of methods used to estimate heritability that all rely upon the comparison of phenotypes between relatives, either from known pedigrees or genetic inferences (Allendorf *et al*., 2012). Methods include the 'animal model' (Kruuk, 2004) that evaluates the quantitative genetic variation and breeding value of parents by assessing phenotypic similarity of half, or full-siblings (Visscher *et al*., 2008) Alternatively, the additive genetic value of individual animals as opposed to related groups can be estimated by partitioning variance components (environmental and genetic) using best linear unbiased prediction models (BLUPs) (Allendorf *et al*., 2012). One of the most commonly used methods to estimate heritability is a parent-offspring regression whereby phenotypic values of a specific trait for offspring and parents are linearly regressed. Heritability in the narrow sense can be estimated by the slope of the regression of the mean progeny values on the mean of the mother and father trait values (mid-parent value). However by regression of the values of either the mother or the father alone on female or male progeny values, h^2 is given by twice the value from the slope of the regression (Frankham *et al*., 2009).

Evidence from the fossil record provides clear indications of the relationship between periods of past global warming and organism size. The Paleocene-Eocene Thermal Maximum (PETM), a period of around 10,000 to 20,000 years occurring over 55 million years ago (Bralower *et al*, 1997), was associated with rapid global warming, biotic extinction and migration, and fundamental perturbations to the carbon and hydrological cycle (Rodriguez-Tovar, 2011). Evidence for this period indicates that, during the warming phase, invertebrates such as ants, bees, beetles, spiders and wasps shrank in size by 50-75%. Similar evidence can be found, but during different periods of past warming, for diatoms, pocket gophers (Hadley, 1997), California squirrels and woodrats (Smith *et al.,* 1995, Finkel *et al.,* 2005).

Since climatic changes during the PETM, such as temperature increases of between 3-7^{o}C and precipitation decreases of approximately 40%, are comparable to expected global climate change over the next century, such information could be valuable in attempts to estimate anticipated changes to organism size. Despite current climate change occurring much faster than previous periods of warming, contemporary reductions in growth rates and body size (Sheridan & Bickford, 2011) as well as alterations to the distribution, phenology and behaviour of many organisms (Hughes, 2000; Bradshaw & Holzapfel, 2006), have been observed due to environmental change.

It is, however, only until recently that studies have focused on the effects of climate change on development and growth, and therefore organism size (Sheridan & Bickford, 2011). Since development and growth are affected by temperature and water availability (Irie & Fischer, 2009; Parolin *et al*, 2010) climate change will affect organism size. Daufresne *et al,* (2009) were one of the first studies to suggest that, at least for aquatic taxa, the reduction of body size as an ecological response to climate change and many types of wild animals such as amphibians (Reading, 2007), reptiles (Wikelski *et al.,* 2000) mammals (Smith *et al.,* 1998; Ozgul *et al.,* 2009), birds (Gardener *et al.,* 2009), and fish (Desai *et al.,* 2009) have shown reduced growth rates and body size as a result. For example, Ozgul *et al.* (2009) showed that environmental change has resulted in a reduced growth rate of Soay sheep in St. Kilda, explaining the observed reduction in body size. Similarly, mean body mass of woodrat populations was shown to have decreased significantly over several years in correlation with increasing temperatures (Smith *et al.,* 1998). Further evidence is provided by laboratory experiments on marine molluscs (Jokiel *et al.,* 2008), and marine invertebrates (Daufresne *et al.,* 2009) that have shown similar negative effects due to alterations to temperatures and CO_2 concentrations.

There are numerous mechanisms proposed for the observed reduction in organism size for a number of different taxa, however, the most pronounced types appear to be related to increased metabolism and quicker development (Sheridan & Bickford, 2011). Particularly for ectotherms, metabolic rate is dependent primarily on temperature and body size (Gillooly *et al.,* 2001). Therefore, with an estimated global temperature increase of 1.1-6.4^{o}C by 2100 (Solomon *et al*, 2007), ectothermic metabolic rate is expected to increase 10-75% (Bickford *et al.,* 2010) if metabolic demands are not met. Alternatively, the temperature-size rule suggests that organisms that develop at higher temperatures will be small relative to individuals at lower temperatures (Angiletta *et al.*, 2004). This is due to the inverse relationship between temperature and duration of development (Jarosık, *et al.,* 2002) and has been evidenced in multiple taxa (Ray, 1960). Another empirical generalisation of temperature and body size is Bergmann's rule (Bergmann, 1847) in which it is proposed that, due to the smaller surface area to volume ratio of larger individuals, evolution favours the reduction of heat loss in colder climates (Walters & Hassal, 2006). Thus, individuals of a particular species tend to be larger in body mass in colder regions. While Bergmann's rule was initially considered primarily a generalisation for endotherms, many ectotherm groups have also shown such temperature-size trends (Ray, 1960)

Evolution will also be a fundamental force in the reduction of organism size. Historic periods of global warming that affected the body size of many mammal species have seen genetic responses for smaller body size in woodrats (Smith *et al.,* 1995) and horses (Secord *et al.,* 2012). The effects of shrinking body size are apparent due to the risks of desiccation from evaporative heat loss in amphibians, for example (Sheridan & Bickford, 2011) and can for most organisms affect their physiology, anatomy, behaviour, ecology, life history and survival (Walters & Hassall, 2006). Therefore, the need for evolutionary responses to emerge due to shrinking body size is apparent. Moreover, as evident from the fossil record, evolution is expected to play a significant role if organisms are to circumvent the adverse effects associated with a reduced body size (Hoffmann & Sgro, 2011; Sheridan & Bickford, 2011).

The measure of energy reserves is intimately related to the health of an animal and is functional to a variety of ecological observations, such as environmental stress, parasite load and reproductive investment (Blas *et al.*, 2005; Castellano *et al.*, 2000; Narayan *et al.,* 2013; Neff & Cargnelli, 2004; Whiteman and Parker, 2004). However, some measures are destructive such as estimating fat deposits which is undesirable especially in the field of conservation research. The use of the body condition index (BCI) as a management tool was proposed by Anderson and Neumann (1996), subsequently providing a non-destructive and relatively straightforward way to compare energy reserves among populations. Common BCIs used are residuals from a linear regression of body mass against body size indicator (BSI) and, ratios between body mass and linear measures of BSI. The use of BCI, however, is not without contention even though numerous ecological studies have been carried out utilising these approaches and the results have been considered highly reliable by many authors. Bancila *et al*. (2010) compared three BCI methods using body mass data from 24 populations of yellow-bellied toad *Bombina variegata*. The three BCIs used were Fulton's index, relative body condition mass index and residual index. Fulton's index (Sztatecsny and Schabetsberger, 2005) uses the Fulton's factor to compare populations based upon the assumption that those with a higher K (weight/length^3) contain more energy reserves, and thus have a better body condition, than those with a lower value of K. While the relative mass condition index (W_r) was calculated as $W_r = 100 \times W/W_S$, where W_S is the body mass predicted from the linear regression of body mass on SVL. Lastly, the residual index uses the residuals of the linear regression of SVL against weight. Many data assumptions exist when using these methods in order to gain an accurate interpretation of the results and should not be violated where possible. However some assumptions cannot be verified which is one of the reasons that their reliability have been questioned (Green, 2001). Bancila *et al*. (2010) states these assumptions as follows: body mass increases linearly with BSI (following any data transformation), BCI is independent of BSI, BSI is an accurate measure of structural size, there is no correlation between BCI and other structural components, and BSI is measured without bias. Bancila *et al*. (2012) tested the three indices for statistical independence of SVL and normality of distribution. They found that when using the Fulton's index, BCI was not independent of SVL and data using the relative body condition mass index was not normally distributed. The residual index, however, did not violate either of these assumptions and, therefore, was considered to be the most reliable method of analysis for these data and the application of this index was recommended as a tool in analysing data of amphibians. Green (2001), however, tested a residual index using the ordinary least square (OLS) linear regression of body mass against a linear measure of size in an avian morphometric data set. The purpose of the analysis was to illustrate how this method can easily lead to Type I and Type II errors by the violation of data assumptions. The paper states that significant relationships are particularly vulnerable to being spurious when the correlation coefficient and BSI is low. Although in the current study this was not the case, other caveats need to be drawn attention to, such as the presumption that BCI accurately correlates with the size of energy stores.

5.2. Aims.

The current research makes use of an existing long term study a common toad population in Dorset. By employing data derived from chapters 3 and 4, the aim is assess evolutionary responses of the population by using measures of effective population size and heritability. In doing so, the aim is to acquire an understanding into the genetic mechanisms underlying the adverse effects of climate change in a wild common toad population. Specifically, by performing regression analyses of known phenotypic values of parents and their offspring (as per the inferred relationships of Colony, see Chapter 4), the aim is to estimate heritability of a trait adversely affected by climate change: body condition index (BCI). Moreover, by combining data of effective breeding size estimates and BCI, the aim is to investigate any relationship between these two parameters.

5.3. Methods

The mean BCI data used was calculated from the data obtained from the on-going population study (Reading, 2010, pers. comm.) and by following methods performed previously for this population (residual index, Reading, 2012 pers. comm.). It was calculated by firstly transforming the size and weight data to log(10) for all individuals of the population for which both measurements were available, for all sampling years (2004, 2005, 2006, 2008 & 2009) and separated by sex. Subsequently the log(10) values of size and weight were regressed returning residuals and it was from these residuals that the average BCI was calculated by taking the mean for each sampling year. Table 5.1 shows the number of individuals used for BCI calculation and the number of individuals forming census sizes per year and per sex. The table also shows the mean BCI separated by each year and sex.

For the heritability regressions, the same method of BCI determination was performed and for the midparent BCI and mean offspring BCI regression a scaling factor was applied to the BCI calculation for male toads. The scaling factor was the difference in the average snout-vent length of female toads compared to male toads and used so that the average male sizes could be multiplied by this value. This was performed to account for the size differences between the sexes (female toads are usually much larger than males).

The effective breeding size data used for the N_b/N and BCI regressions were obtained from the estimates presented in Chapter 4.

Table 5.1. Numbers of individuals used for calculation of BCI, and mean BCI for each sampling year and sex.

	Females			Males		
Year	*n* used	*N*	BCI	*n* used	*N*	BCI
2004	150	153	0.003003	439	440	0.002068
2005	71	73	-0.00026	398	400	0.002671
2006	65	67	-0.00273	471	471	0.004066
2008	193	212	-0.0023	573	573	-0.00196
2009	113	117	-0.00601	455	455	-0.00609

N = population census size (Reading, 2006), *n* = number of individuals used (that had available size & weight data).

5.4. Results

Estimates of heritability, for those individuals of which pedigree information was obtained (see Chapter 4), were performed and are shown in Figures 5.2 to 5.4. They represent parent-offspring regression of mean BCI, BCI of female parents and mean BCI of female offspring and BCI of male parents and mean BCI of male offspring respectively.

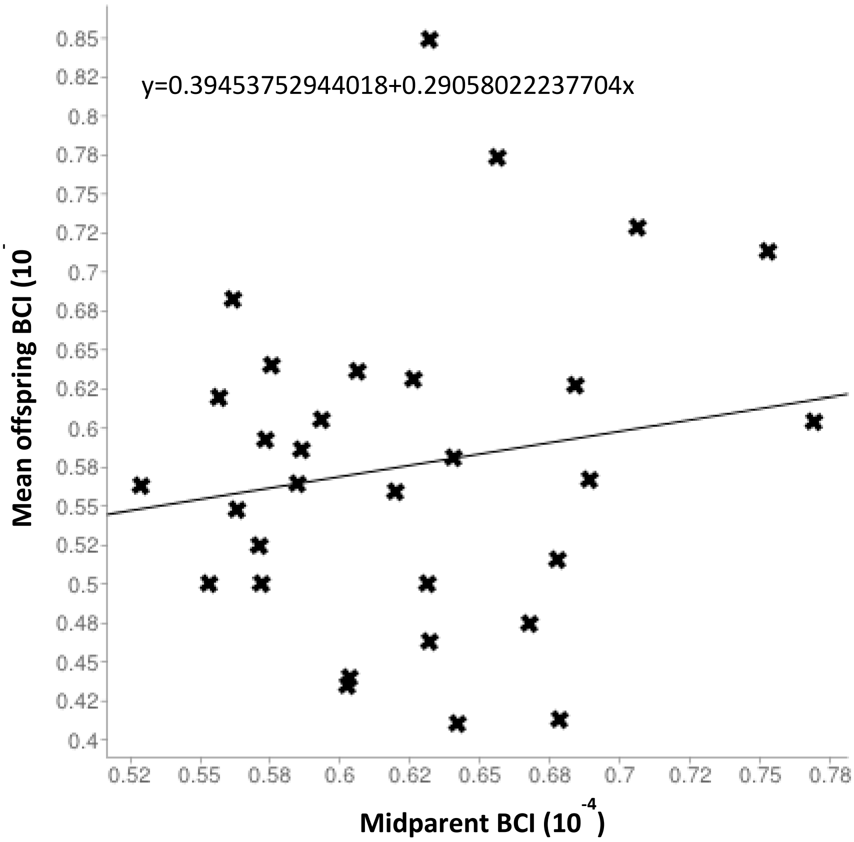


Figure 5.1. Parent-offspring regression of the mean BCI of parental pairs (midparent value) and the mean BCI of their offspring, as inferred by Colony.

The Pearson product-moment correlation was used to obtain all correlation coefficients, with the midparent and offspring regression (Figure 5.1) having r = 0.16, (P = > 0.05, df = 29) and a slope, and thus the narrow sense heritability h^2, of 0.16. The data for mothers and daughters (Figure 5.2) and fathers and sons (Figure 5.3) are both negatively correlated, with correlation coefficients of -0.17 (P = > 0.05, df = 25) and -0.033 (P = > 0.05, df = 26) respectively.

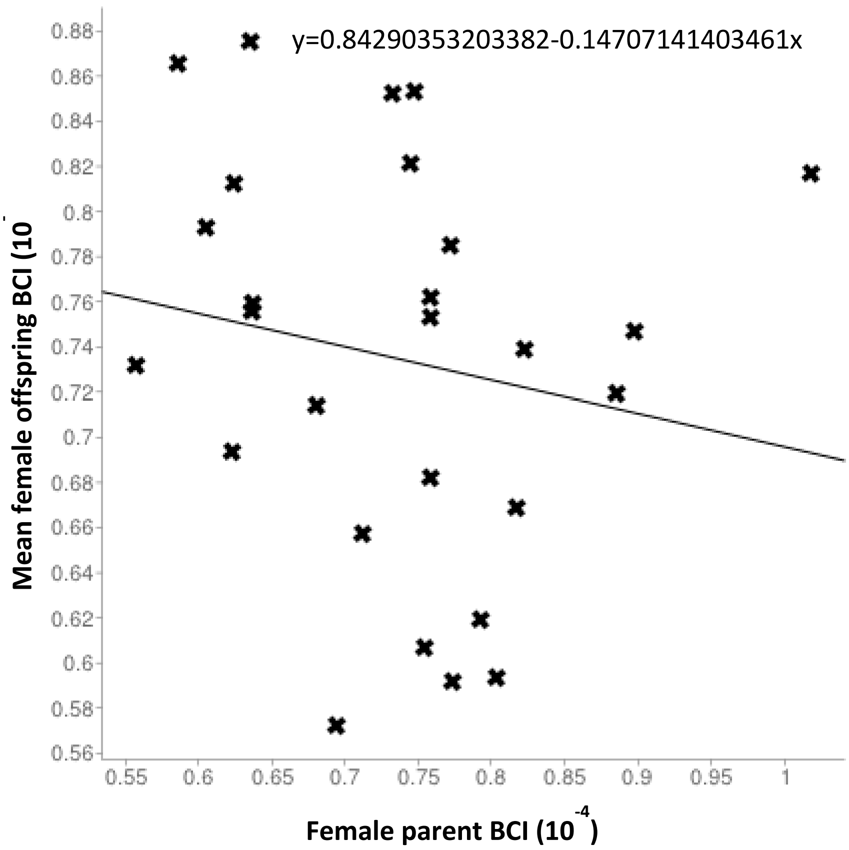


Figure 5.2. Mother-offspring regression of mean BCI values of relationships as inferred by Colony.

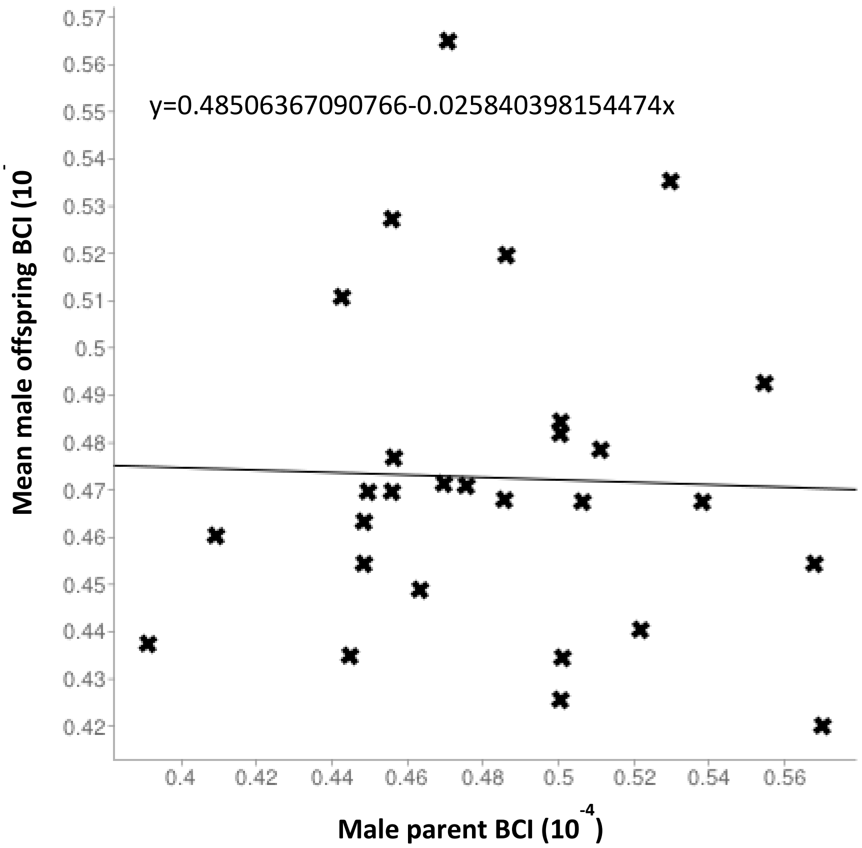
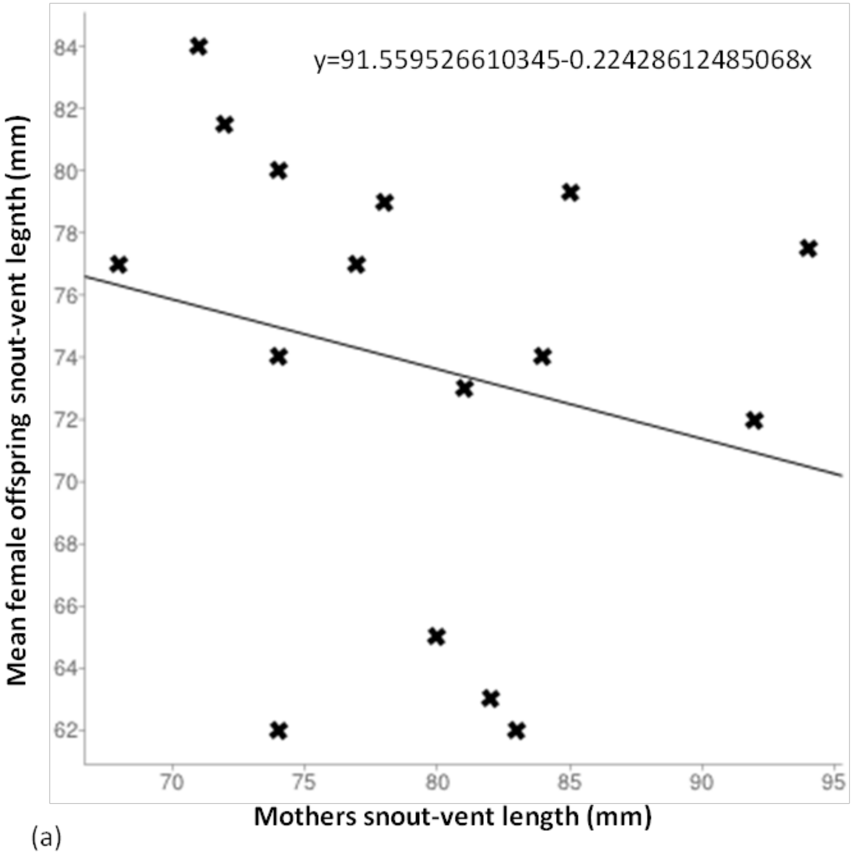


Figure 5.3. Father-offspring regression of mean BCI values of relationships as inferred by Colony.

To see if the size or weight of individual offspring and inferred parents showed heritable variation for these traits, mean female and male offspring values were regressed on either maternal or paternal parental values respectively. Figure 5.4 shows the heritability of snout-vent length in (a) mothers and daughters and (b) fathers and sons, showing weak negative and positive correlations respectively. Pearson product moment correlation coefficients were *r* = -0.22 (*P* = >0.05, df = 14) for females and *r* = 0.2 (*P* = >0.05, df = 26) for males and thus the narrow sense heritability of snout-vent length for males is h^2 = 0.4. Similarly, Figure 5.6 shows the heritability of body weight for (a) females and (b) males and *r* = -0.11 (*P* = >0.05, df = 14) and 0.10 (*P* = >0.05, df = 26) respectively (male h^2 = 0.2).



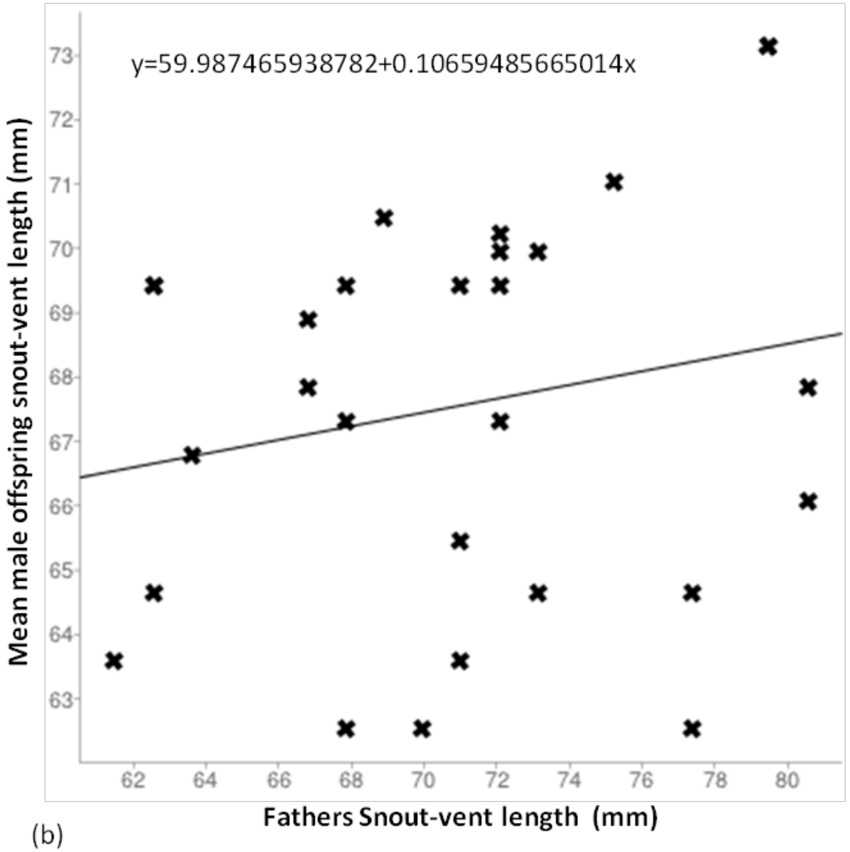


Figure 5.4. Parent-offspring regressions of inferred relationships from maternity and paternity tests in Colony for the estimation of heritability of snout-vent length in *Bufo bufo*: (a) mothers-female offspring regression; (b) father-male offspring regression.

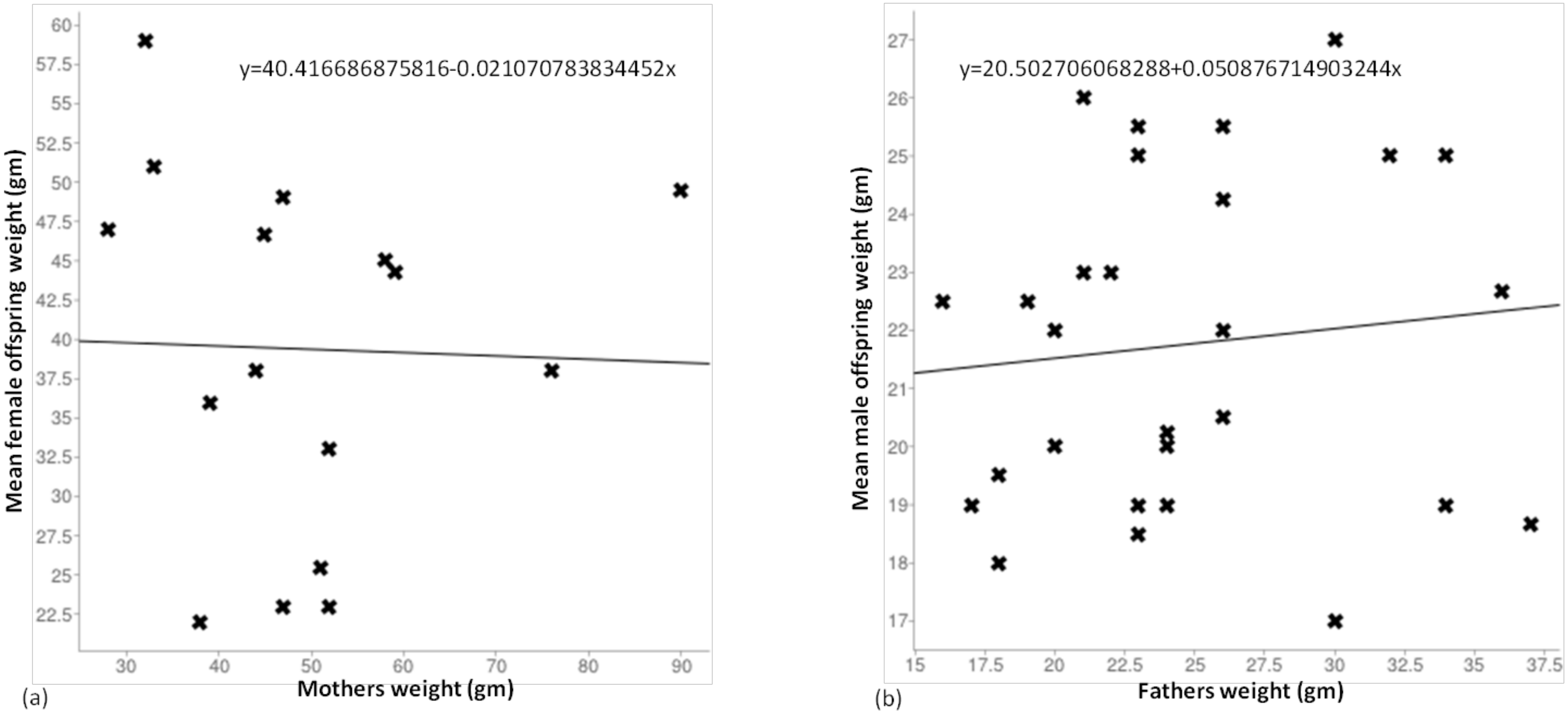


Figure 5.5. Parent-offspring regressions of inferred relationships from maternity and paternity tests in Colony for the estimation of heritability of body weight in *Bufo bufo*: (a) mothers-female offspring regression; (b) father-male offspring regression

Results from the effective breeding size estimates for sibship assignment (SA), linkage disequilibrium (LD), and heterozygote excess (HE) methods (see Chapter 3) show an increasing trend with time (sampling year). Since the data for BCI also show a similar trend (see Chapter 1 for background) the two sets of data were regressed to visualise the relationship. Significant correlations can be seen for effective population size/census size and mean female BCI regressions (Figures 5.6 – 5.8). N_b estimates calculated via the sibship assignment (SA), linkage disequilibrium (LD), and heterozygote excess (HE), methods show mean female BCI is negatively correlated with effective breeding size/census size ratio (N_b (SA) $r = -0.88$, $P = 0.048$, N_b(LD) $r = -0.92$, $P = 0.02$, & *Nb*(HE) $r = -0.78$, $P = >0.05$). Thus, when mean female body condition index is low as per relatively later sampling years (e.g. 2008/2009) the N_b/N ratio is highest.

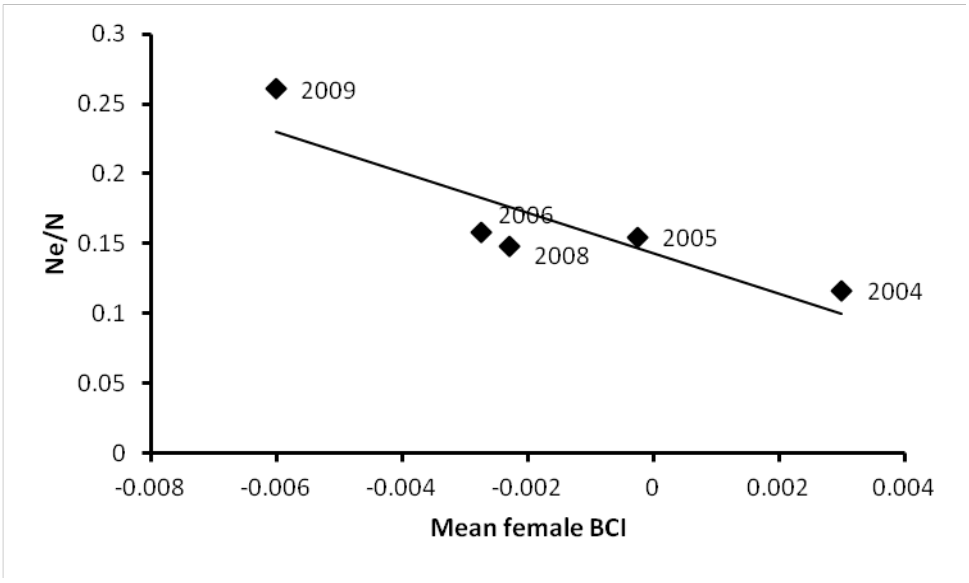


Figure 5.6. Effective breeding size/census size data regressed on mean female body condition index for N_b estimates calculated via the sibship assignment method.

Data for 2004 obtained from the linkage disequilibrium method were omitted from Figures 5.7 and 5.8 because the N_b value computed by NeEstimator was infinity (∞) and thus could not be correlated with other data.

Data for BCI were divided by sex to account for the differences in body mass since females have up to an additional 30% of weight when captured and processed due to egg masses. Mean male BCI and effective population size/census size correlations also show negative relationships, indicating a similar trend for that of females.

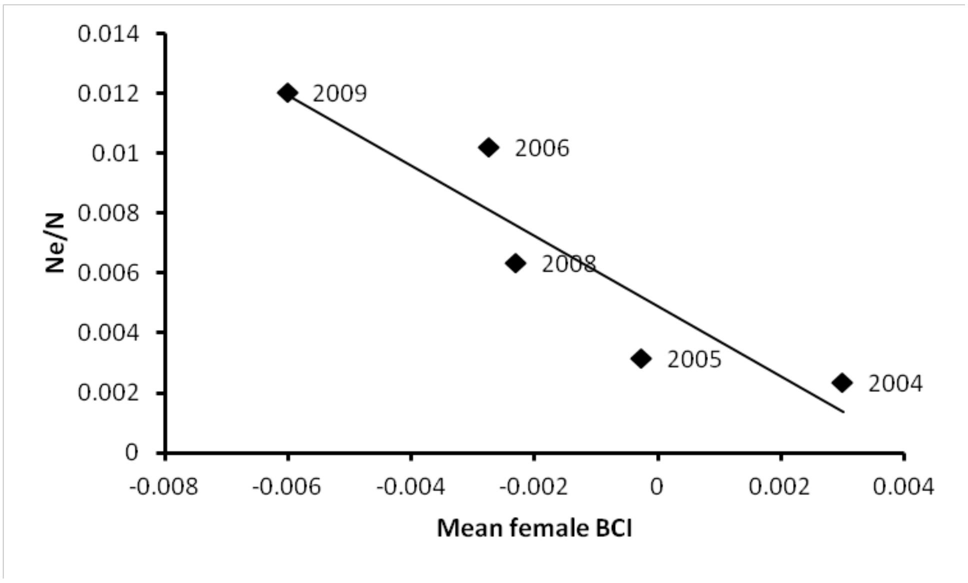


Figure 5.7. Effective breeding size/census size data regressed on mean female body condition index for N_b estimates calculated via the heterozygote excess method.

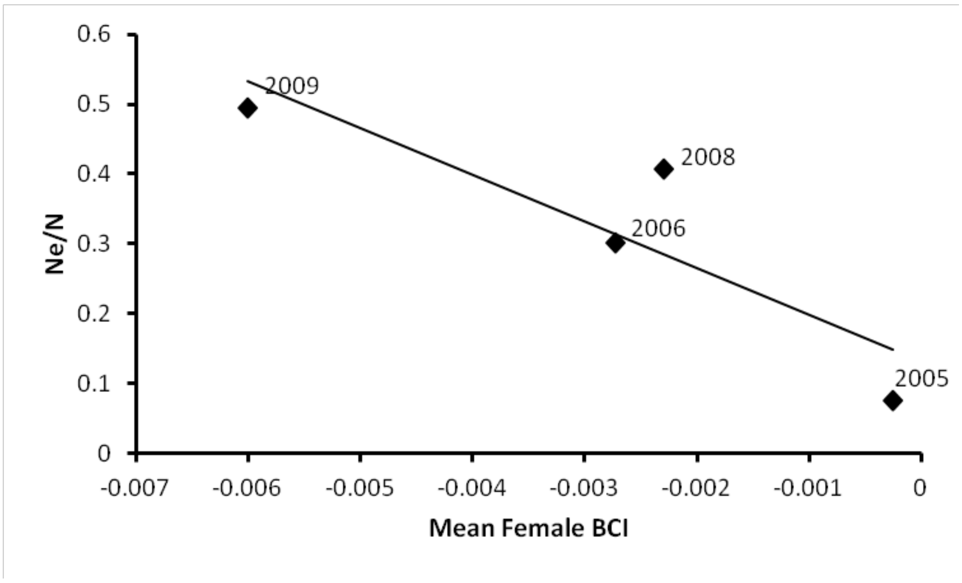


Figure 5.8. Effective breeding size/census size data regressed on mean female body condition index for N_b estimates calculated via the linkage disequilibrium method.

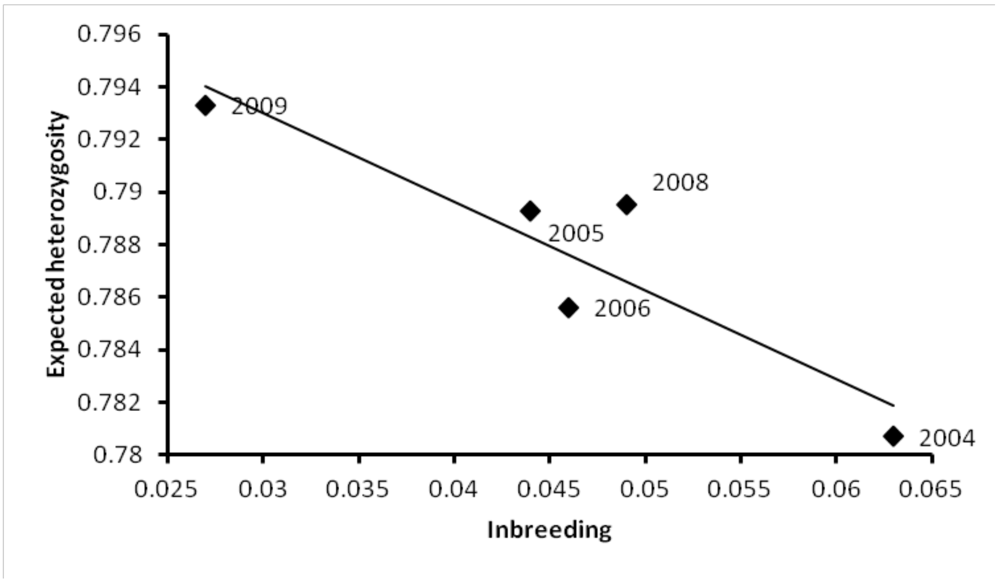


Figure 5.9 Inbreeding and expected heterozygosity as per sampling year.

All correlations were, however, insignificant with *r* = -0.78 for the sibship assignment and linkage disequilibrium methods and -0.51 for the heterozygote excess method (all 3, *P* = >0.05). Since effective population size/census size ratios increase with decreasing body condition index, and since a reduction in fitness, and fecundity and increased mortality (see Chapter 1) are associated with an increase in inbreeding, a correlation of inbreeding, *F* and N_b/N was obtained. An average for inbreeding of all individuals in each sampling year that were given inbreeding coefficients in the program Coancestry was calculated. Figure 5.9 shows the significant negative correlation (*r* = -0.91, *P* = 0.031) between average inbreeding coefficients calculated by the program Coancestry and expected heterozygosity.

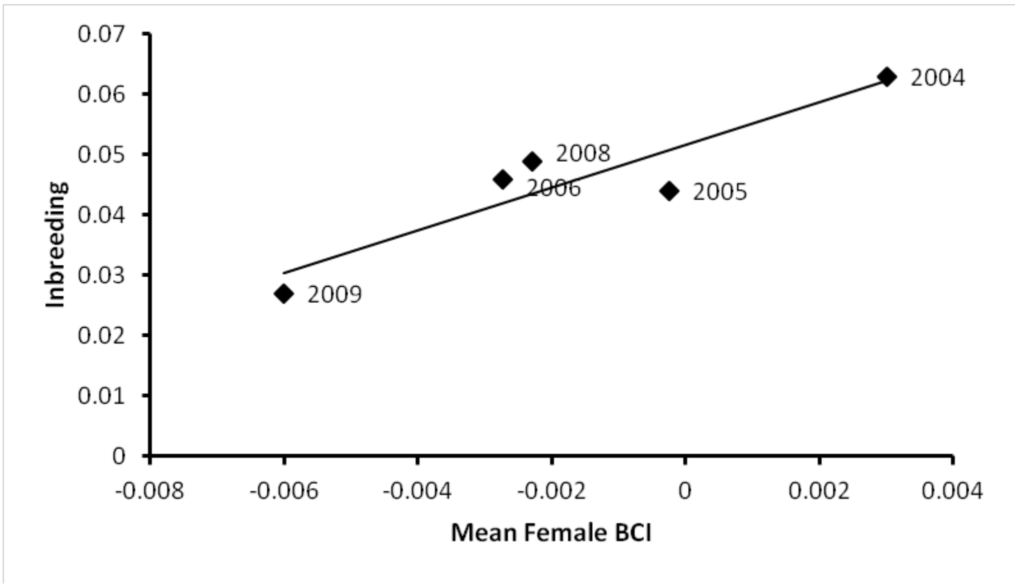


Figure 5.10. Mean female BCI and inbreeding as per sampling year.

Furthermore, mean body condition index and inbreeding should therefore conversely show a positive correlation (given the increasing effect of N_b/N with decreasing BCI). For mean male BCI and inbreeding, like that of BCI and N_b/N is not significant but is nevertheless a positive relationship (r = 0.65, P = >0.05). However, mean female BCI and inbreeding (Figure 5.10) shows a significant and positive correlation (r = 0.92, P = 0.028).

5.5. Discussion

A number of studies have investigated heritability of traits in wild animal populations but have been focused on birds such as the collared flycatcher (Merilä *et al*, 2001a, b), the great tit (Boyce & Perrins, 1987), the snow goose (Cooch *et al*, 1999), the barnacle goose (Larsson *et al*, 1998), and mammals such as the red deer (Kruuk *et al*, 2000 & 2001) and Soay sheep (Milner *et al*, 1999; 2000). At present there are no such studies that exist for amphibians due to the difficulties associated with obtaining tissue samples and reliable measures of traits such as body mass and length relative to mammals and birds. Moreover, other factors such as the ectothermic nature and lifelong growth of amphibians and their large genomes with few genetic resources do not make the study systems optimal. Therefore, since no studies currently exist that have performed heritability estimates in amphibians, there is no data to which the current study can be compared. However, within studies of birds and mammals, heritable genetic variation for body size has been found for lesser snow geese (Davies *et al.*, 1988), Soay sheep (Milner *et al.*, 1999) and humans (Maes *et al*, 1997). Heritability of body weight in Soay sheep has been shown to be as low as 0.054 (Milner *et al.*, 1999) and in humans as high as 0.93 (Maes *et al.*, 1997) compared to the narrow sense heritability of 0.16 for this population of common toads. This, r = 0.16, illustrates a small fraction of variance shared between parental and offspring BCI and that h^2 is very low for this population. Furthermore, the results from the mothers and daughters and fathers and sons regressions (Figures 5.2 & 5.3 respectively) show slopes of negative correlations which are to be interpreted as a lack of heritable variation for body size in this population. The data from Figures 5.4(a) and 5.5(a) showing the heritability estimates for mothers and daughters of snout-vent length and body mass respectively are also congruent with heritability estimates of BCI for both males and females. These data support the interpretation that heritability is very low in this population, despite the positive correlations for male length and weight (Figures 5.4(b) & 5.5(b)) since these were very weak and not significant (P = >0.05).

The results from the heritability analyses therefore show that there is no correlation between parents and offspring for BCI or traits associated with BCI. The absence of any heritable variation for body condition is an indication that the observed declined of this trait is largely, if not completely, due to environmental causes. Therefore, phenotypic plasticity has occurred within the population in response to increased temperatures as a function of the temperature-size rule (Angilletta *et al.*, 2004). These findings indicate that there is no heritable variation for body condition meaning that there is no evolutionary potential for this population of common toads. Since genetic adaptation is thought to be the most sufficient mechanism of circumventing the adverse effects on fitness associated with increased temperatures, this population therefore lacks the ability to track current climate change.

Since the results from the effective breeding size estimates (see Chapter 3) and the body condition index (see Chapters 1 and 2) both showed a trend with time, correlating the two variables seemed logical. Thus, is there evidence for a functional relationship between BCI and N_b? The data from the mean female body condition index and effective breeding size/census size ratios show a negative relationship for all three of the N_b estimates (Figures 5.6 to 5.8). Thus, at times when BCI is high the N_b/N ratio is low and vice versa. This is particularly interesting since, given the observed decline in female fecundity and BCI for both sexes as well as increased mortality (Reading, 2007), N_b might be expected to decrease. This reduction in female fitness and increased mortality would result in pressures within the population for reproduction. These pressures would be associated with aspects such as fewer female mating partners (in a population already naturally male biased) and a reduction in the number of viable eggs per strings in a system whereby egg strings are vulnerable to desiccation and predation. Therefore, with reductions in the potentially available female (and male) mating partners and available female gametes, it would be expected that fewer individuals would be available to successfully contribute to reproduction. As a result, this would cause a reduction in the effective breeding size due to further changes to the sex ratio and potential changes to family size and changes to the population size associated with increased mortality. However, despite these adverse effects (such as body size reduction) having the potential to cause a reduction in N_b, the effective population size could actually increase under this scenario. In the presence of sexual selection pressures, the effective population/breeding size can be reduced as a result of a portion (usually males) of the population being limited in their reproductive contribution (Moller & Birkhead, 1994) Thus, in systems with naturally male biased sex ratios and intense male competition (such as scramble competition), as with the current study, many males do not successfully reproduce and therefore do not contribute. However, if these pressures are reduced, sexual selection can become less important and a less instrumental force driving reproduction. For instance, in the current study, both sexes could, arguably, be subject to sexual selection for body size as for example, large females are more fecund and large males may benefit when competing with other males or forming amplexus (or both). However, the observed reduction in body size of both male and female toads (Table 5.1 & Figures 5.6 to 5.8) could make the pressures associated with, for example, male competition less intense. This could emerge as a result of female toads being less selective about body size of the male toads. Conversely, the same could occur for male toads when selecting female partners and under certain circumstances could even prevent the detrimental effects (such as death of the female due to drowning) associated with multiple males amplexed with one female. Therefore, since sexual selection can reduces N_e or N_b (Moller & Birkhead, 1994), a reduction in sexual selection could increase N_e or N_b by removing, in the case of the current study, the need for a trait such as body size to be selected for.

Other factors that could adversely affect body condition index values in amphibians include those associated with, for example, nutritional deficiencies (Krause *et al.*, 2011) or habitat change (Karraker & Welsh, 2006). For example, since nutritional intake is vital for metabolism which is directly linked to body condition, individual toads that have a poorer nutritional intake will have reduced assimilation of energy reserves and therefore a reduced body size. However, although individual toads within the study population have been shown to suffer from the reduced ability to assimilate, and increased depletion rate of, energy reserves, these factors have been associated with increased temperatures during the spring and summer months and the occurrence of more mild winters. Thus, these effects to energy reserves are more likely to be related to increased environmental temperatures as opposed to a change to the dietary intake of the population since there is no documented evidence of any reported changes to the surrounding habitat or the breeding pond itself that may have caused changes to nutritional intake.

The data from Figures 5.6 to 5.8 that shows effective breeding size increases with decreasing body condition index, therefore, indicates a mechanism by which this population can offset the effects of reduced body size and fecundity. However, although studies have shown that sexual selection can reduce N_e, no such results exist in the current literature that can show a reduction in body condition to be correlated with higher levels of N_e (or N_b) or increases in N_e/N (or N_b/N). In fact, very few show that N_e can be increased within populations. Temporal (Lage & Kornfield, 2006) and spatial (Phillipsen *et al*, 2011) studies on vertebrate species have shown alterations to N_e but these are typically reductions and associated with populations suffering from ecological perturbations such as habitat destruction or fragmentation and given obstructions to gene flow for example, would be expected to lose genetic diversity and thus have reduced N_e.

Furthermore, studies tend not to report findings that support increases in N_e in response to adverse environmental or ecological alterations. Those few studies that report such cases have noted that when the population census size is low, increases in N_e/N are apparent and this phenomenon has been termed 'genetic compensation'. Beebee (2009) describes genetic compensation as 'manifest as a nonlinear relationship between N_b/N_c ratios and N_c' and was evident in that same study. Other studies of amphibian species (Jehle *et al.*, 2005; Palstra & Ruzzante, 2008) have also shown such correlations. Jehle *et al.* (2005) showed a negative but nonlinear relationship between population census size, N and N_b/N. The study found that when effective breeding size and census size ratios were lowest, the population census size was at its highest and vice versa. For example, when N_b/N ratios were around 0.1, population census size was between 150 and 225 individuals and conversely when N_b/N ratios were between 0.5 and 0.65, census size was below 25 individuals. This therefore means that at times of very low N the majority of individuals within the population reproduce and it is this characteristic for which the term 'genetic compensation' is required. This phenomenon, albeit manifested in a different manner, may be applied in the current study to explain the findings that show increased Ne/N (or N_b/N) ratios correlated negatively with decreased BCI.

To summarise, the data from the heritability estimates show that there is no evidence for the existence of significant heritability for BCI. This is concerning for the long-term viability of this population of common toads since responses emerging from plastic genotypes are not sufficient to circumvent the adverse effects associated with climate change. However, the data for effective breeding size shows an increasing temporal trend which is negatively correlated with the body condition suggesting that the observed detrimental effects to fitness (i.e. fecundity and body size reduction) may be offset by the ability of individuals to increase the effective breeding number possibly by reducing the variance in reproductive success due to decreased sexual selection pressures.

CHAPTER 6:

General Discussion

Despite the common toad (*B. bufo*) being the most populous amphibian in the UK and widespread throughout Europe, with populations in decline (e.g., Beebee & Griffiths, 2000) it is now listed as a priority species (JNNC, 2007). A loss of genetic diversity and fitness, (Hitchings & Beebee, 1998) and surveys showing that toad populations fare worse than those of common frogs (Carrier & Beebee, 2003), have provided some insights into the decline of the common toad in the UK. Furthermore, a long-term population study has indicated that increased temperatures are linked to the reduced body condition, fitness and survival of a common toad population in Dorset, UK (Reading, 2007). While survey-based or population-level studies can contribute to the revealing of population density, distribution, size fluctuations and other demographic processes, they are limited in their ability to elucidate the underlying forces for observed declines. In order to document and predict the mechanisms that alter population numbers and investigate environmental effects on particular life history stages, individual-based data spanning at least two generations aid to estimate parameters such as lifetime reproductive success. By combining data derived from the study by Reading (e.g., Reading, 1983; 2007) with data on individually recognisable members of the population and their paternity share in successive generations, the current study could elucidate some underlying forces contributing to population dynamic processes, including the observed decline in fitness and survival of the studied population.

Data from the effective breeding size estimates in Chapter 3 reveal that there is an upward trend for the effective breeding number from 2004 to 2009. This temporal trend was also apparent when analysed as the N_b/N ratio and was produced by all three N_b estimators used. The comparison of this data with the data from the parentage analyses in Chapter 4 indicates some level of congruency. The data can only be compared for the years of 2004, 2005 and 2006, since these were the years covering the parental generations. However, the number of individuals contributing to reproduction appears to have increased both for the N_b estimates and parentage assignments. Therefore both sets of data add support to the inference that there is an increase in the effective number of breeders in this population. Furthermore, the average number of breeders relative to the adult census size (N) as inferred by the parentage assignments (see Chapter 4, Table 4.4) is 0.16 and the average number of breeders relative to census size (N_b/N) as inferred by the N_b estimate via the sibship assignment (SA) method is also 0.16. The fact that the value from the SA method matches the parentage figure of 0.16 is promising, since estimates of N_b derived from the SA method have been shown to be the most accurate when compared to HE and LD estimates (also for anuran species, Beebee, 2009; Phillipsen *et al*., 2011). Similarly, when the effective population size estimate inferred via the temporal method (using 2004 as generation 0 and 2009 as generation 1) is analysed relative to the average adult population census size, the $N_e(_{TM})$/mean N ratio is also 0.16. ($N_e(_{TM})$ = 98.4, mean N = 592.2). Thus, from different methods of inference, different statistical means, and theoretical assumptions, these data all converge on a ratio of effective population size to census size of approximately 0.16. This, therefore, shows some level of accuracy and reliability of the data and confidence that this value is likely to be the true N_e/N ratio.

The most influential forces that affect the ratio of effective population size to adult census size are fluctuations to population size, the sex-ratio, and variance in reproductive success (the former two of which are discussed in Chapter 3). Variance in reproductive success alters N_e/N ratios by affecting the number of gametes each individual contributes to the next generation. For example, an ideal Wright-Fisher population with a sex-ratio of 1:1 and a Poisson distribution of gametes would produce no variation as the average number of gametes equals 2. However, given that this is never the case for wild animal populations, deviations from an idealised population are observed allowing the effects of variance in reproductive success to be investigated. In the current study, therefore, evidence of variation in reproductive success would indicate some effects to the effective population/breeding size. The results from Chapter 4 show that apart from a single female assigned 10 offspring (Figure 4.2), the data show no significant variance in reproductive success for either sex of the toads or sampling year. These data (Chapter 4), therefore, could help explain an increase in N_b (Chapter 3, as opposed to a decrease associated with increase reproductive variance). Furthermore, although the variance in reproductive success in terms of family size does not show a decreasing trend with time (indeed, the 10 offspring assigned to one female were observed in 2009), in terms of differential success between the sexes the data is more revealing. From the results of Chapter 4 (Table 4.4) it is apparent that there is a difference between the reproductive success of males and females. In 2004, there are 10 females more than males that were assigned parentage and in 2009 the difference is only 6 additional males. Moreover, the sex-ratio data (Chapter 3, Table 3.2) is in accordance with the differential parentage assignment data (Chapter 4, Table 4.4) in that it shows a decrease in the sex-ratio resulting in a less biased ratio from 2004 to 2006. If fewer males, relative to females are present in the population then this could help explain a reduction in reproductive variance and thus an increase in N_b.

The results from the body condition index and N_b/N regression data of Chapter 5 might also help explain the low variance in reproductive success as observed from the parentage data (Chapter 4) and as implicated from the N_b data (Chapter 3). The regression analyses show that mean BCI per sampling year is negatively correlated with estimates of N_b/N per sampling year (Figures 5.8 to 5.10). Thus, when the effective breeding size is highest (i.e., in 2009) the body condition is lowest. This is statistically significant for two of the N_b estimators (SA and HE, $P = <0.05$). This means that the reductions to body size and fitness as observed (reading, 2007) appear to have not led to a decrease, but an increase, in the average contribution to reproduction by each individual in this population. If the reduction in body size reduces pressures associated with sexual selection, then this could have emerged as individuals mating less selectively. In the absence of body condition as an important determining factor of sexual selection, other individuals may achieve reproductive success and thereby increase the number of breeding individuals in the population. This would therefore explain the increase in N_b estimates (Chapter 3/5) and the increase in parentage assignments (Chapter 4). Moreover, the data from the inbreeding coefficients (F) in Chapter 3 (Figure 4.4) show that inbreeding has been reduced (see also Chapter 5, Figure 5.12), which means that F increases with increasing body condition. Thus, when the toads are of a smaller BCI they are less inbred. If this is due to an increase in the breeding number of individuals in the population then it may have emerged as those individuals whose mating chances are reduced due to increased selective pressure (due to larger toads) may then have become less choosy and thus less effective at avoiding inbreeding. However, inbreeding avoidance often leads to a loss of potential breeding opportunities (Kokko & Otts, 2006), which may as a result reduce the number of breeders in the population. Nevertheless, this increase in breeding success in spite of a reduction in body size and fitness would therefore denote that this population might be well equipped to overcome the adverse effects of increased environmental temperatures.

If body condition is an important factor in the breeding of individuals in this population then evolutionary change would be required to select for body size. The results from the heritability estimates of BCI (Chapter 5, Figures 5.2 to 5.4), however, indicate that the reduction in body size appears to be a plastic response and not a genetic one.

In conclusion, despite the lack of evidence to suggest that the observed reduction in body size of individual toads in this population is due to evolutionary change, the population has shown that it may be capable of circumventing the adverse effects associated with a reduction in body size as evident from the increase in effective breeding number. Data from the measures of genetic parameters such as a reduction in inbreeding, an increase in genetic diversity and effective breeding size, coupled with an increase in parentage assignments over time suggest that reductions in BCI, fecundity and survival have not been detrimental. However, if the observed reduction in body condition and fecundity continues then the effects of reduced reproductive competition for example, such as an increase in N_b, might not be enough to counteract the effects of an increasingly less fecund population suffering from increased mortality. For that, adaptive genetic change would be required that can be measured through estimates of heritability over longer period of time to disentangle the effects of environments from genes. The need for such analyses in future studies of conservation genetics in amphibians and all wildlife species is becoming more urgent.

CHAPTER 7:

References

7.1. References

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CHAPTER 8:

Appendix

8.1. Dilution of DNA extractions

2009	#	DNA	T.E															
Row		1			2			3			4			5			6	
A	448	59	360	341	36	258	502	32	224	407	51	410	460	48	382	462	44	338
B	496	34	235	388	67	572	417	51	408	22	68	584	320	34	239	487	41	314
C	401	82	722	538	40	300	537	45	347	475	26	157	224	115	1051	326	95	854
D	465	33	234	507	47	280	512	64	135	287	45	352	447	11	—			
E	386	20	—	273	48	377	390	69	587	428	34	240	315	103	931	330	10	—
F	346	89	715	503	26	150	550	159	1340	531	40	265	329	43	295	569	32	200
G	454	27	150	525	55	448	458	38	245	398	113	930	540	66	500	509	22	105
H		5			6			12			21			42			105	

Row		7			8			9			10			11			12	
A	452	48	383	345	41	306	321	27	175	368	101	910	325	41	310	530	36	260
B	539	37	271	522	60	503	443	45	349	317	61	511	561	31	207	384	30	200
C	339	58	484	331	80	701	488	32	219	444	29	187	335	116	1056			
D	306	52	421	380	23	80							491	77	674			
E	397	32	223	455	22	120	450	37	272	359	55	400	372	34	241			
F	348	64	490	451	31	190	456	4	—	323	35	225	311	37	266			
G	342	24	125	565	33	205	322	76	600	564	17	—	493	26	164			
H		6																

2008	#	DNA	T.E															
Row		1			2			3			4			5			6	
A	76	23	33	51	1	—	20	20	—	16	40	90	50	3	—	15	-2	—
B	218	24	41	149	9	—	151	9	—	146	18	—	111	5	—	44	0	—
C	496	-5	—	322	26	63	374	18	—	321	4	—	326	7	—	375	3	—
D	468	5	—	436	-1	—	665	26	62	588	31	85	614	-4	—	503	33	94
E	639	46	145	500	10	—	640	23	52	670	13	—	435	11	—	554	31	83
F	121	-6	—	168	14	—	458	14	—	471	10	—	485	27	152	592	-2	—
G	89	9	—	100	7	—	162	5	—	310	24	126	327	79	621	775	12	—
H		4			14			14			18			36			108	

Row		7			8			9			10			11			12	
A	25	-6	—	52	4	—	29	26	41	23	13	—	152	168	1498	664	66	508
B	24	20	—	22	32	109	49	1	—	3	27	85	215	38	251	774	44	308
C	322			333	29	96	286	6	—	275	39	146	233	102	367	309	61	481
D	721	6	—	488	7	—	707	82	360	705	19	—	381	50	360	434	37	246
E	538	56	229	442	11	—	440	30	101	472	3	—	502	16	—	525	64	436
F	593	89	397	627	14	—	710	44	170	735	44	169	540	102	829	532	44	322
G	439	14	—	539	52	211	720	38	138	395	90	399	594	64	511	537	81	671
H		2																

2008	#	DNA	T.E															
Row		1			2			3			4			5			6	
A	265	0	—	497	142	357	384	19	—	43	35	69	153	16	—	42	17	—
B	198	25	40	197	11	—	220	30	55	575	13	9	31	12	—	495	10	—
C	80	35	69	32	37	72	759	14	—	604	9	—	467	15	—	522	29	52
D	81	21	31	160	15	—	53	28	49	41	24	38	628	29	50	317	13	—
E	28	25	39	283	39	78	77	19	—	40	11	—	214	20	—	581	28	48
F	576	48	364	595	54	261	704	47	352	679	81	677	313	104	470			
G	506	31	58	387	102	248	457	41	85	461	45	95	369	15	—	449	109	268
H		2			13			19			27			47			100	

Row		7			8			9			10			11	
A	318	-2	—	175	12	—	544	18	—	269	21	29			
B	281	3	—	480	15	—	724	10	—	26	10	—			
C	392	5	—	194	18	—	164	25	40	216	18	—			
D	760	3	—	217	8	—	433	12	—	445	19	—			
E	386	19	—	387	20	—	777	16	—	204	15	—			
F															
G	310	66	151	350	48	102	495	41	84	445	84	199	355	96	232
H		-11													

Row		1			2			3			4			5			6	
A	217	75	195	27	236	677	21	10	—	452	36	77	275	4	—	132	32	66
B	287	79	207	334	37	81	89	6	—	72	66	169	498	27	52	333	68	173
C	467	19	—	224	41	92	74	49	118	314	48	113	213	89	237	286	68	174
D	133	38	85	131	47	111	266	539	1586	254	114	311	373	69	176	221	348	1015
E	572	110	301	330	5	—	135	63	159	369	67	172	372	40	90	374	49	116
F	332	5	—	338	127	352	496	7	—	272	78	203	164	8	—	425	6	—
G	453	15	—	130	13	—	28	168	473	136	47	111	377	6	—	73	62	156
H		27			-3			6			22			36			108	

Row		7			8			9			10			11			12	
A	370	37	81	325	27	50	496	27	52	273	72	185	513	21	85	268	25	115
B	368	146	407	375	44	102	222	3	—	366	50	119	495	39	220	88	47	274
C	168	46	107	262	13	—	25	81	213	40	29	57	67	24	108	166	17	—
D	86	87	232	274	91	242	167	218	623	68	264	763	422	4	—	454	-4	—
E	163	58	145	165	63	159	455	92	246	122	205	585	128	39	218	340	8	—
F	270	-8	—	327	41	92	431	-7	—	371	22	35	75	19	—	337	4	—
G	339	45	104	134	40	91	269	69	178	124	30	60	137	34	177	212	11	—
H																		

2005	#	DNA	T.E															
Row		1			2			3			4			5			6	
A	427	-8	—	292	0	—	404	6	—	320	8	—	448	-4	—	409	-4	—
B	28	6	—	359	3	—	459	18	—	141	1	—	281	25	41	152	39	78
C	400	29	52	182	-5	—	362	26	44	150	8	—	252	34	64	337	4	—
D	151	92	220	357	111	271	413	99	242	322	184	471	411	52	113	236	124	309
E	278	1	—	369	19	—	156	72	168	339	18	—	444	15	—	412	1	—
F	58	98	659	64	125	865	161	65	410	217	77	499	279	134	926	283	62	389
G	102	119	817	136	136	944	180*	474	3479	323	65	416	239	126	873	331	77	505
H					4			16			31			44			101	

Row		7			8			9			10			11			12	
A	22	0	—	249	4	—	321	2	—	365	8	—	92**	330	2397	406	75	489
B	253	15	—	237	26	44	432	15	—	129	6	—	183	126	873	254	181	514
C	256	22	33	155	41	84	243	58	130	61	132	330	230	55	334			
D	59	52	113	181	173	441	324	82	195	57	98	238	280	78	506			
E	335	19	—	458	15	—	54	189	482	368	39	78	329	70	359			
F	286	107	724	332	87	578	338	57	351	401	62	391	346	75	488			
G	364	92	612	407	60	376	410	65	414	233	55	335	363	72	469			
H		-3																

2004	#	DNA	T.E															
Row		1			2			3			4			5			6	
A	17	-9	—	230	247	1774	232	108	491	252	65	386	148	145	1009	151	33	169
B	178	68	433	343	55	337	61	22	92	154	3	—	325	244	1758	357	162	1142
C	228	38	100	176	19	—	464	91	609	497	217	1240	485	92	618	364	169	1193
D	468	189	1340	495	91	607	259	115	785	97	300	2175	86	162	1139	299	127	876
E	467	94	633	410	83	548	11	-10	—	6	41	232	206	84	556	91	39	214
F	33	-14	—	293	134	927	335	39	220	336	22	89	80	31	160	120	143	999
G	Error**			Error**			Error**			Error**			380	25	114	474	-6	—
H		13			11			-2			33			58			95	

Row		7			8			9			10			11			12	
A	400	122	838	432	130	902	440	93	621	444	103	557						
B	435	135	935	96	41	232	124	29	144	51	74	478						
C	391	122	839	499	89	591	416***			483	115	788						
D	204	22	77	465	10	—	237	901	6681	322	133	922						
E	101	-3	—	260*	220	1573	502	92	613	360	60	372						
F	127	164	1159	142	101	680	219	674	4981	456	133	923						
G	668	117	800	624	-4	—	695	83	544	776	-21	—	626	-30	—	582	64	408
H		-15																

2004	#	DNA	T.E															
Row	1			2			3			4			5			6		
A	258	50	240	474	20	60	49	10	—	363	30	170	447	20	85	292	20	85
B	239	20	85	409	30	170	229	20	85	396	10	—	469	10	—	351	10	—
C	42	20	85	291	40	255	174	10	—	142	10	—	375	10	—	133	50	340
D	496	20	60	99	20	85	482	50	340	262	10	—	245	50	340	265	70	510
E	98	10	—	290	20	85	355	0		295	10	—	92	10	—	126	50	340
F	331	10	—	361	30	100	352	20	85	54	20	85	235	20	85	5	10	—
G	157	50	340	430	10	—	94	10	—	188	20	85	438	70	510	107	20	85
H	269	10	—	487	40	180	238	20	60	263	80	420	240	120	660	327	30	120

Row	7			8			9			10			11			12		
A	203	10	—	392	10	—	433	10	—	264	10	—	317	120	935	111	20	85
B	177	10	—	104	40	255	34	10	—	247	0		412	30	170	393	50	340
C	216	10	—	102	20	85	304	30	170	242	10	—	125	130	1020	103	40	255
D	189	20	85	88	60	425	37	10	—	417	10	—	150	260	2125	87	20	60
E	156	10	—	100	30	170	321	20	85	209	30	120	356	30	170	413	10	—
F	218	40	180	90	10	—	127	50	340	300	10	—	236	30	170	466	20	60
G	335	10	—	376	10	—	197	30	170	501	10	—	219	40	255	9	60	300
H	55	10	—	457	10	—	484	10	—	340	10	—	58	30	170	323	50	240

2004	#	DNA	T.E															
Row	1			2			3			4			5			6		
A	394	20	60	105	20	60	39	10	—	431	20	60	362	20	60	231	60	300
B	498	10	—	401	130	720	365	130	720	302	90	480	297	70	360	411	40	180
C	233	30	120	146	100	540	503	40	180	207	60	300	296	120	660	95	80	420
D	175	40	180	436	20	60	414	10	—	220	20	60	221	30	120	110	20	60
E	358	10	—	224	60	300	398	10	—	155	10	—	62	30	120	223	10	—
F	441	30	120	256	30	120	475	20	60	298	30	120	458	30	120	330	90	480
G	8	120	660	36	80	420	442	10	—	294	70	360	402	130	720	53	190	1080
H	187	20	60	359	20	60	13	50	240	439	20	60	38	40	180	257	20	60

Row	7			8			9			10			11			12		
A	241	20	60	128	30	120	443	30	120	271	10	—	130	70	360	303	40	180
B	63	60	300	35	60	300	60	0		399	30	120	205	120	660	147	40	180
C	261	40	180	395	150	840	324	80	420	50	70	360	191	80	420	48	100	540
D	397	20	60	106	20	60	208	70	360	29	60	300	56	60	300	416	180	1020
E	52	10	—	316	80	420	59	130	720	28	80	420	437	30	120	43	30	120
F	108	10	—	149	80	420	217	20	60	89	70	360	329	90	480	486	40	180
G	328	210	1200	7	90	480	227	100	540	366	50	240	326	110	600	202	60	300
H	32	10	—	446	10	—	333	30	120	93	10	—	123	80	420	459	40	180

8.2. Routine PCR plates configuration

Routine PCR plates											
A	1	2	3	4	5	6	7	8	9	10	11	12
A	448	341	502	407	460	462	452	345	321	368	369	449
B	496	388	417	22	320	487	539	522	443	317	310	350
C	401	538	537	475	224	326	339	331	488	444	495	445
D	465	507	512	287	447	325	306	380	561	335	355	
E	386	273	390	428	315	330	397	455	450	359		
F	346	503	550	531	329	569	348	451	456	323		
G	454	525	458	398	540	509	342	565	322	564		
H	387	457	461	491	372	311	493	530	384	506		
B	1	2	3	4	5	6	7	8	9	10	11	12
A	76	51	20	16	50	15	25	52	29	23	434	695
B	218	149	151	146	111	44	24	22	49	3	525	776
C	496	322	374	321	326	375	582	333	286	275	532	626
D	468	436	665	588	614	503	721	488	707	705	537	-ve
E	639	500	640	670	435	554	538	442	440	472	380	
F	121	168	458	471	485	592	593	627	710	735	474	
G	89	100	162	310	327	775	439	539	720	395	668	
H	152	215	233	381	502	540	594	664	774	309	624	
C	#											
Row	1	2	3	4	5	6	7	8	9	10	11	12
A	427	292	404	320	448	409	22	249	321	365	336	358
B	28	359	459	141	281	152	253	237	432	129	333	154
C	400	182	362	150	252	337	256	155	243	61	457	135
D	151	357	413	322	411	236	59	181	324	57	460	157
E	278	369	156	339	444	412	335	458	54	368	97	405
F	58	64	161	217	279	283	286	332	338	401	62	251
G	102	136	180	323	239	331	364	407	410	233	184	190
H	92	183	230	280	329	346	363	406	254	285	87	179
D	1	2	3	4	5	6	7	8	9	10	11	12
A	217	27	21	452	275	132	370	325	496	273	454	.
B	287	334	89	72	498	333	368	375	222	366	337	.
C	467	224	74	314	213	286	168	262	25	40	.	.
D	133	131	266	254	373	221	86	274	167	68	.	.
E	572	330	135	369	372	374	163	165	455	122	340	.
F	332	338	496	272	164	425	270	327	431	371	212	.
G	453	130	28	136	377	73	339	134	269	124	.	.
H	513	495	67	422	128	75	137	268	88	166	.	.
E	1	2	3	4	5	6	7	8	9	10	11	12
A	265	497	384	43	153	42	433	175	544	269	251	272
B	198	197	220	575	31	495	281	480	724	26	82	546
C	80	32	759	604	467	522	392	194	164	216	268	725
D	81	160	53	41	628	317	760	217	318	445	632	669
E	28	283	77	40	214	581	386	387	777	204	726	491
F	576	595	704	679	313	113	39	529	264	727	487	530
G	163	274	736	27	54	245	325	778	244	156	99	108
H	261	499	88	437	441	543	260	145	165	438	479	neg
G	1	2	3	4	5	6	7	8	9	10	11	12
A	258	474	49	363	447	292	203	392	433	264	317	111
B	239	409	229	396	469	351	177	104	34	247	412	393
C	42	291	174	142	375	133	216	102	304	242	125	103
D	496	99	482	262	245	265	189	88	37	417	150	87
E	98	290	355	295	92	126	156	100	321	209	356	413
F	331	361	352	54	235	5	218	90	127	300	236	466
G	157	430	94	188	438	107	335	376	197	501	219	9
H	269	487	238	263	240	327	55	457	484	340	58	323

A	394	105	39	431	362	231	241	128	443	271	130	303
B	498	401	365	302	297	411	63	35	60	399	205	147
C	233	146	503	207	296	95	261	395	324	50	191	48
D	175	436	414	220	221	110	397	106	208	29	56	416
E	358	224	398	155	62	223	52	316	59	28	437	43
F	441	256	475	298	458	330	108	149	217	89	329	486
G	8	36	442	294	402	53	328	7	227	366	326	202
H	187	359	13	439	38	257	32	446	333	93	123	459
I	1	2	3	4	5	6	7	8	9	10	11	12
A	333	318	435	497	250	471	556	405	432	424	164	316
B	38	563	393	570	446	510	336	505	243	453	150	67
C	177	485	136	282	365	459	86	225	274	349	76	93
D	57	21	171	238	15	254	293	92	157	438	504	377
E	251	137	479	294	190	400	70	426	338	394	344	73
F	280	154	192	399	332	334	28	199	96	162	402	319
G	34	292	275	90	227	131	337	123	17	40	233	358
H	133	147	244	234	532	265	286	7	248	74	135	Neg
J	#											
Row	1	2	3	4	5	6	7	8	9	10	11	12
A	163	343	207	97	172	340	127	79	3	23	466	257
B	100	63	107	231	72	249	18	266	144	288	12	281
C	252	347	219	218	71	327	246	141	357	545	408	276
D	389	116	395	555	554	62	85	29	179	477	295	134
E	396	571	122	472	283	239	304	128	94	10	105	9
F	35	272	145	255	278	56	61	289	371	101	82	363
G	166	508	543	410	296	229	526	517	255	514	104	20
H	140	149	39	11	232	170	411	223	511	195	277	neg
K	1	2	3	4	5	6	7	8	9	10	11	12
A	260	96	444	178	93	80	153	284	445	428		
B	447	149	240	433	88	241	250	327	408	118		
C	255	334	403	402	246	360	194	330	331	426		
D	324	267	220	214	126	329	367	233	376	328		
E	127	424	456	123	319	226	335	336	80	120		
F	142	219	114	758	250	403	385	404	2	492		
G	157	545	706	531	132	191	109	299	513	16		
H	58	533	564	77	176	53	194	242	neg			

8.3. Unbinned genotypes

npops = 5																
nloci = 8																
pop = A																
	Bbutu11		Bbutu49		Bbutu62		Bbutu65		Bbutu24		Bbutu46		Bbutu54		Bbutu15	
PopA	116.4	125.9	180.2	183.9	196.1	198.2	170	170	151.8	151.8	131.9	144.2	166.4	188.9	166.6	166.6
PopA	118.3	127.9	?	?	183.6	197.8	163.9	177.4	146.1	157.5	132	144.2	166.6	173	166.9	169
PopA	103.4	106.2	178.1	193.5	183.8	183.8	183.1	189.8	147.2	158.4	144.2	146.4	166.5	185	166.6	166.6
PopA	116.3	118.2	210.4	212.2	183.8	196	164.1	169.7	139	147.5	131.9	144.2	176.6	184.8	171	171
PopA	110.7	127.7	166.5	178.2	198	204	169.8	183.1	146.9	151.6	141.1	144.2	184.7	184.7	167.1	169.3
PopA	?	?	?	?	183.7	196	164	164	147.2	147.2	?	?	166.3	166.3	?	?
PopA	116.2	127.7	187.4	187.4	?	?	164	167.9	154	154	135.9	146.6	188	190.2	?	?
PopA	?	?	?	?	?	?	?	?	?	?	131.8	144.1	166.3	166.3	170.3	172.3
PopA	106.4	126	?	?	?	?	184.1	186	152.3	152.3	144.4	146.5	?	?	169	171.1
PopA	106.1	125.9	185.8	187.7	183.7	198.1	157.8	184	151.7	153.9	137.8	144.2	172.5	176.5	166.7	171
PopA	106	118.2	178.3	180.1	183.6	198	157.7	163.8	138.8	151.7	144.2	146.4	166.3	186.9	168.9	168.9
PopA	118.3	125.8	174.2	189.4	183.5	197.7	186.7	199.8	150.6	150.6	144.5	144.5	184.9	184.9	158.8	171.2
PopA	103.6	109	189.8	191.6	184	198.4	160.2	169.9	146.1	150.9	132	144.1	166.3	172.5	170.8	175.5
PopA	125.9	127.9	178.4	200.9	183.8	198	164.2	177.7	138.9	151.7	135.8	146.4	186.9	190.9	170.9	170.9
PopA	?	?	193.5	204.6	183.7	198	167.7	196.4	125.9	150.9	131.9	146.5	166.2	184.6	166.9	169
PopA	102.4	125.7	200.9	202.8	197.9	197.9	177	177	?	?	146.4	146.4	166.2	184.7	166.5	166.5
PopA	108.8	125.7	197	202.4	?	?	160	163.9	143.4	145.8	132.2	138	166.5	185	167.2	171.4
PopA	118	118	187.6	195.1	183.7	183.7	179	182.8	137.5	151.6	135.9	148.5	166.2	166.2	167	167
PopA	106.2	121.8	174.6	187.9	183.6	197.9	183.9	183.9	145.9	150.7	132.1	144.5	166.5	185	?	?
PopA	103.2	129.5	166.4	189.4	196	198	163.9	163.9	151.6	153.8	132	144.2	185	189.1	166.6	168.7
PopA	118.3	127.9	201.4	203.2	183.7	198	164.3	166.3	143.7	150.9	144.3	144.3	?	?	173.1	173.1
PopA	?	?	?	?	184.4	199.9	?	?	?	?	?	?	185	189.1	?	?
PopA	110.6	121.7	187.8	210.3	198.1	202	161.7	177.7	151.7	151.7	144.2	144.2	166.6	166.6	170.9	170.9
PopA	103.4	127.9	178.4	180.2	183.7	198	169.6	171.6	146	150.7	140.2	144.7	166.6	187.1	?	?
PopA	?	?	?	?	?	?	167.2	185.6	?	?	144.2	144.2	166.2	188.8	169.3	171.3
PopA	118.1	122	158.8	178.3	200.1	204.1	159.9	183.4	151.8	151.8	144.1	144.1	184.7	186.8	169.4	171.4
PopA	116.2	127.7	164.5	174.1	197.9	204.1	157.6	185.3	138.9	151.6	144.2	146.4	?	?	158.4	170.9
PopA	106	106	179.9	198.9	183.7	200	169.3	186.9	151.6	151.6	131.9	144.2	184.6	184.6	169.4	171.5
PopA	103.7	128	186.2	186.2	183.7	200	177.8	198.2	?	?	131.9	144.2	166.7	173.3	?	?
PopA	118.2	125.9	?	?	183.8	198.1	189.8	189.8	144.8	158.5	137.8	144.3	166.6	166.6	?	?
PopA	106	116.2	185.7	187.5	198	204.1	159.7	177.6	146.9	151.6	139	139	166.3	166.3	168.7	170.9
PopA	118.1	121.8	?	?	162.8	185.9	157.7	189.6	137.4	151.7	137.8	144.2	185	189.1	169.3	169.3
PopA	?	?	174.5	188.1	183.7	187.8	178	190	?	?	?	?	?	?	169	171.1
PopA	125.9	127.9	?	?	183.6	198	?	?	?	?	144.3	144.3	166.3	166.3	169	171
PopA	?	?	193.4	200.9	?	?	164.2	181.2	?	?	?	?	?	?	166.7	170.9
PopA	?	?	?	?	183.4	197.7	163.7	163.7	?	?	146.4	146.4	166.2	184.8	?	?
PopA	122	125.8	187.7	193.4	198.2	198.2	160.1	187.2	151.8	160.4	146.3	146.3	166.3	166.3	166.6	170.9
PopA	118.2	122	178.3	187.9	196	200.1	?	?	156.3	156.3	131.8	146.4	?	?	169.2	169.2
PopA	103.3	125.7	189.5	195.2	183.6	200	178.1	194	153.9	158.3	?	?	166.5	166.5	168.8	168.8
PopA	118.2	122	158.8	191.5	?	?	160	160	152	160.6	137.9	144.4	166.4	166.4	166.7	168.9
PopA	106.2	128	164.6	174.3	183.5	197.8	169.8	177.7	146	146	144.3	144.3	166.3	185.8	166.8	168.9
PopA	118.2	125.8	170.4	197.1	183.6	193.7	162	164	?	?	144.3	146.4	184.8	184.8	168.9	170.9
PopA	106.4	111	182.2	189.9	?	?	165.1	186.9	?	?	?	?	185.1	185.1	171.5	171.5
PopA	106.2	125.8	159	182	196.1	200.2	160	164.1	147.2	156.2	137.7	137.7	166.3	184.8	166.7	166.7
PopA	122.3	126.2	?	?	183.8	196	170.2	186	146.6	146.6	?	?	166.5	188.9	166.9	169
PopA	103.3	125.8	?	?	200.1	200.1	178	198.7	137.5	151.6	135.9	144.2	166.3	184.8	166.7	171
PopA	127.6	129.6	?	?	192.1	198.2	177.6	185.8	151.8	154	131.9	144.2	185	185	168.8	172.9
PopA	125.8	127.7	185.9	187.8	196	198	164	177.6	151.9	151.9	?	?	166.3	166.3	168.9	173
PopA	121.8	125.8	189.6	195.2	183.7	183.7	189.8	189.8	147.2	147.2	?	?	?	?	169.4	169.4
PopA	125.8	127.7	176.3	178.3	183.7	183.7	186.3	194.1	151.9	151.9	131.8	144.1	?	?	168.9	168.9
PopA	123.9	125.7	164.6	187.6	198.1	204.1	158.1	190	?	?	146.4	146.4	166.2	184.7	169.3	169.3
PopA	?	?	?	?	?	?	?	?	?	?	146.4	146.4	172.5	188.9	?	?
PopA	121.9	125.7	180	187.6	188	196.1	159.7	189.5	139	151.6	131.9	131.9	166.5	185.1	169.2	171.3
PopA	106	129.6	?	?	183.7	183.7	164	193.4	147	151.7	137.8	144.2	166.2	188.7	169.3	171.3
PopA	116.3	118.2	?	?	183.6	200	184.1	186	?	?	132	144.4	166.6	188.4	171.1	171.1
PopA	121.8	125.8	164.7	191.5	183.8	183.8	164.2	196.8	139	147.5	137.7	146.4	180.6	188.8	167.1	167.1
PopA	?	?	185.8	187.8	196.1	198.1	164	189.8	151.8	154	131.9	146.4	185	185	166.7	171
PopA	116.4	125.9	176.1	198.9	?	?	164.1	193.5	151.8	151.8	144.3	146.4	166.4	166.4	?	?
PopA	?	?	?	?	197.8	197.8	171.4	185.5	?	?	144.2	144.2	166.4	188.9	167.3	169.2
PopA	118.1	125.8	?	?	183.5	197.8	?	?	?	?	132.2	144.5	166.5	185	167.3	171.6
PopA	118.1	127.7	172.4	187.5	?	?	187	196.1	151.7	151.7	?	?	166.3	184.7	166.5	170.8
PopA	103.7	103.7	?	?	?	?	162.2	186	?	?	131.9	144.2	?	?	168.9	171
PopA	118.3	118.3	164.6	185.8	?	?	169.7	189.6	150.7	150.7	146.5	146.5	172.7	185	?	?
PopA	106.1	122.1	?	?	183.5	199.9	162.1	162.1	151.8	158.4	144.3	144.3	166.5	184.9	?	?
PopA	?	?	185.7	193.2	183.9	200.4	?	?	147	153.9	?	?	184.9	189.1	?	?
PopA	108.8	127.7	197.1	199	196	202	161.6	193.5	153.9	158.3	?	?	166.3	184.8	170.9	170.9
PopA	102.9	106.5	?	?	183.5	197.8	?	?	?	?	?	?	166.6	189.2	?	?
PopA	118.2	125.9	180.1	202.7	183.8	196.1	179.6	198.8	151.8	151.8	144.2	144.2	184.7	184.7	166.7	171
PopA	118.1	129.6	?	?	162.8	198	155.8	163.9	151.6	151.6	131.9	144.2	166.3	184.7	166.4	166.4
PopA	118.2	125.8	?	?	183.5	197.8	?	?	147	151.7	144.4	146.7	?	?	167	167
PopA	116.1	125.7	187.5	189.5	196	198.1	186.9	186.9	153.8	153.8	144.2	144.2	166.2	184.6	?	?
PopA	?	?	?	?	?	?	191.8	191.8	?	?	131.9	144.4	?	?	166.9	168.9
PopA	?	?	?	?	?	?	157.6	157.6	150.7	150.7	144.3	144.3	?	?	169	171
PopA	?	?	?	?	183.9	196.3	167.7	189.8	147.3	151.9	144.3	144.3	?	?	168.8	171
PopA	118.4	126	182.3	195.7	183.8	194	177.2	184	?	?	144.2	146.4	?	?	166.8	173.1
PopA	?	?	?	?	198.4	198.4	189.6	194.3	147.2	147.2	146.4	146.4	166.5	189.1	166.6	170.9
PopA	126.2	128.1	187.9	201.1	?	?	186.1	189.9	?	?	144.2	146.5	187	191.2	?	?
PopA	118.1	127.6	180	193.3	198.3	204.4	?	?	144.7	151.6	144.2	146.4	?	?	169.2	169.2
PopA	106	125.7	174.3	176.3	183.7	198	?	?	?	?	144.2	144.2	166.5	189.1	170.8	170.8
PopA	125.9	125.9	?	?	183.5	197.9	159.7	163.6	147.1	151.7	144.5	144.5	?	?	166.8	166.8
PopA	118.1	125.8	180.1	193.3	183.8	183.8	162.1	171.6	137.5	147	135.8	144.2	?	?	166.6	168.8
PopA	?	?	?	?	200.2	200.2	?	?	?	?	144.2	144.2	184.8	188.9	?	?
PopA	102.5	128	166.8	166.8	?	?	?	?	?	?	132.4	146.8	?	?	171.3	173.3
PopA	116.3	129.8	178.1	178.1	?	?	164	169.5	147	151.7	144.2	144.2	166.3	184.7	168.8	170.8
PopA	106.2	127.8	174.4	174.4	183.7	200.1	164.2	184.2	151.8	151.8	?	?	166.5	189.2	169	171.2
PopA	?	?	?	?	?	?	193.1	193.1	?	?	131.8	131.8	172.5	172.5	169.3	169.3
PopA	125.9	127.8	?	?	183.5	197.8	160.2	160.2	150.8	153	144.4	144.4	185	189.1	166.8	169
PopA	?	?	?	?	?	?	160.2	186.3	?	?	131.9	144.2	166.3	184.7	162	168.3
PopA	103.3	116.3	?	?	183.7	193.9	177.6	190.1	147.1	154	131.9	144.2	166.3	166.3	166.6	170.9
PopA	?	?	?	?	183.7	197.9	161.8	189.8	150.9	155.4	?	?	166.4	184.9	?	?
PopA	116.3	127.6	?	?	183.8	183.8	?	?	147	151.7	144.2	144.2	166.4	166.4	168.8	170.8
PopA	106	125.7	?	?	183.7	195.9	169.8	189.6	137.4	151.6	146.4	146.4	166.3	172.4	?	?
PopA	?	?	208.3	210.2	198	200	?	?	144.5	146.9	144.2	144.2	?	?	169.3	171.3
PopA	?	?	?	?	183.7	196	184	189.2	151.6	151.7	144.2	144.2	188.8	188.8	167.1	169.3
PopA	?	?	?	?	?	?	?	?	?	?	144.3	146.4	166.2	184.7	168.9	171
PopA	106.2	126	197.1	200.9	183.7	197.9	183.3	186.9	150.9	159.6	?	?	166.6	166.6	?	?

PopA	121.8	127.7	164.6	191.5	183.7	195.9	166.8	177.7	144.6	151.7	144.3	154.9	172.6	185	?	?
PopA	123.9	125.8	187.8	210.2	183.8	196	189.8	191.6	?	?	132	132	185	189.1	166.7	170.9
PopA	?	?	183.8	191.4	198	200	164.4	194	147.1	151.7	137.8	144.2	166.5	185	167.2	169.3
PopA	116.2	125.7	181.8	187.6	183.7	200.1	?	?	147	147	144.2	148.5	166.5	185	?	?
PopA	126.1	126.1	189.7	210.6	?	?	193.7	193.7	?	?	?	?	?	?	166.7	166.7
PopA	116.4	127.8	?	?	183.5	197.8	163.9	189.6	145.9	145.9	144.3	144.3	166.5	185	166.8	171
PopA	103.3	127.8	180.2	187.8	?	?	169.4	191.5	143.5	143.5	144.3	146.5	?	?	168.9	168.9
PopA	103.4	106.2	?	?	183.7	200.1	186.3	186.3	144.7	151.7	?	?	166.5	181	171	171
PopA	?	?	?	?	?	?	169.7	189.4	?	?	131.9	141.1	180.6	184.8	171.5	171.5
PopA	116.5	126	185.9	193.5	184.1	198.4	162.2	166.2	146.2	150.9	?	?	?	?	169.1	169.1
PopA	125.9	127.7	?	?	183.6	197.8	?	?	146	153	132.1	146.4	186.1	186.1	171.4	171.4
PopA	103.3	103.3	176.3	178.2	179.6	198	164.2	167.9	?	?	?	?	?	?	168.8	168.8
PopA	103.7	116.6	?	?	183.6	197.9	164.3	178	152.3	152.3	?	?	166.6	166.6	168.9	168.9
PopA	106.1	118.2	?	?	196	204.1	170.3	178.2	138.8	151.7	131.9	144.2	166.5	185	167	167
PopA	112.4	128	184	204.7	183.9	198.1	164.2	195.5	150.8	150.8	144.3	144.3	?	?	166.8	171.1
PopA	?	?	?	?	?	?	164	164	?	?	146.5	146.5	166.5	172.8	?	?
PopA	106.1	125.8	187.5	195.2	196.5	198.4	167.5	175.3	139	151.7	146.4	146.4	166.4	185	166.6	168.8
PopA	103.4	127.8	?	?	183.6	197.9	?	?	151.7	151.7	144.4	144.4	?	?	166.8	168.9
PopA	106.3	118.4	187.8	189.8	198.1	198.1	157.8	157.8	147.3	152	144.1	146.4	166.3	166.3	171	171
PopA	122	127.9	?	?	?	?	159.7	185.6	151.7	151.7	144.3	146.5	166.5	181.7	168.9	170.9
PopA	103.3	125.8	182	199	183.7	200.1	162.2	164.1	139	162.5	137.8	146.4	?	?	158.4	158.4
PopA	106	125.7	193.3	199	195.9	197.9	177.6	189.6	151.6	162.4	144.2	144.2	185	189	171.9	174.7
PopA	125.9	127.9	180.3	182.2	183.9	198.3	160.4	164.3	136.7	151	146.3	146.3	166.2	166.2	166.6	166.6
PopA	109	127.9	?	?	184.1	204.4	186	196.4	147.3	151.9	137.8	144.4	167.6	186	168.9	171
PopA	?	?	164.7	189.6	183.5	197.8	163.9	163.9	146	150.8	144.4	146.5	176.4	176.4	166.8	169
PopA	118.2	125.8	187.6	191.5	195.9	195.9	164.1	186.9	139	154	144.2	146.3	174.4	174.4	170.9	172.9
PopA	129.7	129.7	?	?	?	?	183.2	186.9	147.2	151.8	144.2	144.2	184.7	184.7	168.8	168.8
PopA	?	?	170.5	187.6	198.2	198.2	153.3	179.6	129	156.1	144.2	146.4	174.8	189.1	168.8	171
PopA	125.8	127.8	180.1	180.1	?	?	180.1	183.9	150.5	150.5	144.4	146.6	?	?	166.8	166.8
PopA	106.1	129.7	164.7	189.6	198	204.1	157.8	177.2	147.2	147.2	137.7	146.4	166.2	176.5	168.8	168.8
PopA	103.4	127.8	?	?	183.6	202	163.6	189.7	145.9	150.7	144.3	146.5	166.5	185	?	?
PopA	106.1	122	166.6	187.7	196	198.1	167.5	181.1	147	151.6	144.2	144.2	166.4	184.9	166.6	170.9
PopA	102.6	127.8	?	?	?	?	?	?	144.8	151.8	144.3	144.3	166.3	188.9	?	?
PopA	118.4	126.1	174.3	191.5	183.9	198.3	160	177.8	?	?	132	144.3	188.8	188.8	168.9	168.9
PopA	?	?	?	?	183.8	198	164	187.4	147.2	151.8	131.9	144.1	185	185	169.3	169.3
PopA	118.2	127.8	180.1	199	192	204.1	187.5	196.8	147.2	151.8	144.2	146.3	?	?	166.5	166.5
PopA	?	?	?	?	?	?	164	164	150.7	150.7	144.4	144.4	167.5	177.1	166.9	171.2
PopA	102.5	125.9	187.6	187.6	?	?	169.8	189.7	147.2	151.8	132	146.5	185.1	187.1	?	?
PopA	106.3	128	164.6	191.5	183.7	197.9	162.1	187.2	150.8	150.8	144.3	144.3	?	?	166.9	169.1
PopA	106.1	125.9	170.4	191.4	183.7	204.1	162.1	177.5	147	153.9	144.2	146.4	166.2	174.5	166.6	172.9
PopA	?	?	?	?	200.1	204.1	177.9	186.3	137.4	146.9	?	?	166.5	180.9	168.7	170.7
PopA	?	?	?	?	?	?	163.8	185.6	?	?	?	?	166.5	166.5	166	168
PopA	103.4	116.4	191.5	202.8	183.8	183.8	157.8	179.2	147.1	154	?	?	166.3	184.8	166.6	168.7
PopA	102.9	102.9	193.8	212.5	192.6	204.8	?	?	?	?	?	?	166.3	186.8	?	?
PopA	127.8	127.8	180.3	201.1	198.1	198.1	190.2	190.2	146.1	146.1	144.2	146.3	184.9	186.9	171.2	171.2
PopA	106.4	128	180.2	195.4	198.5	198.5	162.3	169.8	?	?	131.9	144.2	176.5	188.8	166.5	166.5
PopA	118.4	126	?	?	198.5	198.5	?	?	?	?	?	?	180.7	188.9	167	169.2
PopA	118.5	126.1	188.1	190	183.8	183.8	164.3	167.8	?	?	144.2	146.5	186.5	188.6	?	?
PopA	102.7	118.3	170.6	182.2	196.2	200.3	159.9	167.7	146.1	146.1	141.1	144.2	166.3	190.9	166.8	166.8
PopA	126	126	?	?	183.6	183.6	163.9	169.7	150.8	150.8	144.4	144.4	166.4	185	169.1	169.1
PopA	115.6	127	?	?	183.7	198	164.2	164.2	?	?	131.9	144.3	177.5	186.4	167	171.3
PopA	116.6	122.2	176.6	212.6	180	198.5	186.3	196.6	?	?	137.8	144.2	184.8	184.8	166.5	171
PopA	116.5	129.8	210.4	212.4	?	?	?	?	136.8	146.4	144.1	144.1	172.6	184.8	166.7	169
PopA	116.5	129.9	189.7	191.5	196.2	200.2	171.9	187.3	146	150.8	137.8	144.2	166.5	184.9	169.1	169.1
PopA	106.2	125.9	?	?	183.8	198.1	164.4	164.4	?	?	?	?	166.4	172.6	167.1	169.2
PopA	116.6	118.4	?	?	198.4	200.5	164.2	164.2	?	?	144.2	144.2	180.7	186.8	171	171
PopA	118.4	126.1	180.2	187.8	184	204.6	?	?	?	?	137.9	144.2	166.3	166.3	170.9	170.9
PopA	125.8	127.7	174.2	197.1	197.9	197.9	183.9	185.9	?	?	131.9	141.1	185	189.1	170.8	170.8
PopA	111.1	116.6	165	178.5	198.5	200.6	160.1	190	?	?	131.9	137.8	166.3	174.5	166.4	166.4
PopA	106.5	118.5	180.5	182.3	184.1	196.6	179.5	198.5	?	?	144.2	146.4	184.8	184.8	166.6	168.9
PopA	106.2	127.7	170.4	172.3	198	198	177.9	190.1	?	?	146.4	146.4	186.8	186.8	166.6	166.6
PopA	115.6	125.2	185.8	191.5	183.7	204.1	162.2	189.9	?	?	?	?	166.4	166.4	?	?
PopA	125.9	127.8	?	?	183.8	198	164	183	151.8	151.8	144.6	144.6	166.4	187	166.9	166.9
PopA	105.3	116.4	170.5	189.6	183.8	198.1	164.1	189.7	?	?	144.2	144.2	166.4	186	?	?
PopA	103.5	118.3	?	?	183.8	198.2	?	?	?	?	137.8	144.2	166.3	166.3	166.8	171.3
PopA	?	?	?	?	183.6	197.9	162.1	193.5	?	?	144.4	144.4	174.9	190	?	?
PopA	147.2	147.2	?	?	183.6	199.9	164.1	189.8	125.9	125.9	?	?	166.4	185.8	166.8	169
PopA	122.2	126.2	?	?	183.7	198.1	?	?	?	?	131.9	144.2	166.6	188.4	?	?
PopA	?	?	?	?	184.4	202.9	185.6	196.5	?	?	?	?	166.3	174.6	?	?
PopA	?	?	189.6	193.4	196	200.1	183.4	193.5	139	151.7	135.8	146.4	166.5	189.1	169.3	169.3
PopA	103.6	127.9	199.1	201	184	198.3	162.4	164.3	?	?	144.2	144.2	184.7	184.7	171.3	171.3
PopA	125.9	129.7	?	?	198	200	?	?	143.6	146.1	132	144.3	186.9	188.9	166.6	171.1
PopA	116.1	125.7	?	?	183.9	196.1	161.9	189.1	151.7	151.7	137.9	144.2	?	?	166.6	170.8
PopA	106.4	122.2	182.4	193.8	183.8	183.8	164.3	193.7	?	?	137.8	144.2	166.6	176.7	158.6	169
PopA	103.5	125.9	182	183.9	196	196	?	?	?	?	144.2	144.2	172.5	184.8	169.1	169.1
PopA	122.2	126	187.8	193.5	183.8	200.3	?	?	?	?	131.8	144.2	186.7	188.7	166.8	169.1
PopA	?	?	185.7	187.5	183.7	198.1	157.5	161.5	142.2	160.3	146.4	146.4	166.5	189.2	?	?
PopA	125.9	129.7	?	?	183.7	200	160	173.7	?	?	144.2	146.2	166.3	188.9	169	169
PopA	?	?	180.2	187.7	?	?	160	170	?	?	137.8	144.3	166.5	185	166.8	173.1
PopA	106.3	127.8	187.7	189.6	198.2	200.2	?	?	136.7	150.8	144.2	144.2	176.7	184.9	166.9	169.1

PopA	?	?	185.5	187.5	?	?	?	?	146.9	160.3	144.2	144.2	166.5	185	168.6	168.6
PopA	?	?	181.9	202.6	183.8	183.8	167.9	196.7	144.7	158.3	131.9	144.1	166.4	185	?	?
PopA	116.3	125.9	?	?	196	204	169.7	169.7	?	?	131.9	144.2	184.8	184.8	168.9	173.2
PopA	116.5	126.1	180.3	182.2	?	?	190	193.7	?	?	137.9	144.2	166.3	188.8	171	171
PopA	106.3	125.9	170.4	180.1	183.5	197.8	169.5	189.8	?	?	131.9	146.5	185	189.1	166.8	171
PopA	103.5	122	187.7	202.8	183.9	198.1	163.6	169.7	147.2	147.2	144.2	146.5	166.6	189.2	166.9	169
PopA	126.1	126.1	186.4	188.2	194.7	196.7	?	?	146	146	131.6	144.2	166.3	172.5	169	171.2
PopA	?	?	?	?	?	?	162.4	168.1	?	?	?	?	166.5	185.1	168.9	168.9
PopA	106.3	118.4	?	?	183.7	197.9	?	?	?	?	144.2	146.5	186	190.3	166.8	169
PopA	106.4	116.5	182.1	185.9	?	?	?	?	136.7	153.2	144.2	146.3	166.3	176.5	166.8	169.1
PopA	106.1	108.9	187.8	189.7	183.7	198	162.5	164.4	151.8	154	131.9	144.2	185	189.1	166.5	168.6
PopA	?	?	?	?	183.8	183.8	160	161.6	146.9	158.1	132	144.2	174.4	188.7	168.8	170.8
PopA	?	?	191.4	193.3	196	200	163.6	183.1	147	147	144.2	146.4	184.7	184.7	158.3	168.8
PopA	116.5	127.9	164.8	189.8	184	196.3	160.4	186.2	146.1	146.1	137.9	146.2	174.5	184.7	170.5	170.5
PopA	?	?	178.2	178.2	183.8	200.1	?	?	?	?	131.9	144.2	186	188.4	166.8	169
PopA	103.5	125.9	?	?	183.7	198	?	?	145	145	131.9	144.2	166.5	166.5	?	?
PopA	128	129.9	?	?	?	?	160.2	168.2	?	?	?	?	166.3	166.3	171.3	173.4
PopA	116.4	125.9	?	?	184.1	200.5	194.6	196.5	152	154.2	144.3	144.3	185	189.1	169.1	169.1
PopA	102.7	106.3	?	?	183.6	204	162.2	162.2	150.8	153	144.3	144.3	185	185	171	171
PopA	?	?	?	?	183.7	197.9	?	?	?	?	?	?	?	?	?	?
PopA	?	?	191.5	197.2	183.9	198.3	168.4	196.8	146.4	153.5	137.7	146.3	166.4	186.8	166.8	173.4
PopA	118.4	126	182	201	183.9	200.3	159.9	184.2	146	150.8	137.7	144.2	166.5	185	166.7	171.2
PopA	118.3	118.3	187.8	201	183.7	198.1	162.4	164.3	?	?	131.9	137.7	189.1	189.1	169.1	169.1
PopA	108.9	116.4	164.5	189.4	183.8	204.2	193.6	193.6	?	?	131.9	144.3	186.1	190.4	169	171.1
PopA	?	?	?	?	?	?	?	?	?	?	?	?	166.1	166.1	169.2	171.3
PopA	111.1	126.1	190.4	192.2	198.8	205	157.9	189.9	?	?	146.3	146.3	166.3	184.8	167.1	169.2
PopA	110.8	126	187.7	187.7	189.6	198.2	173.4	189.3	155.1	157.3	144.2	146.3	166.4	172.6	167	171.4
PopA	?	?	?	?	?	?	177.6	184.1	?	?	?	?	166.3	188.9	166.8	169.1
PopA	103.5	103.5	193.4	195.3	196.1	198.1	?	?	?	?	144.1	144.1	166.3	184.8	169.2	169.2
PopA	116.8	128.2	191.9	193.8	184.4	198.8	169.7	169.7	?	?	144.2	146.3	166.5	184.9	171.2	171.2
PopA	?	?	158.9	210.3	?	?	157.9	198.3	?	?	144.2	144.2	185.1	189.2	166.8	166.8
PopA	116.5	118.3	187.8	189.7	183.8	196.1	?	?	150.9	150.9	?	?	172.5	186.7	167.1	169.3
PopA	103.5	124	?	?	196.1	198.1	164.2	170.2	?	?	144.1	144.1	166.4	184.9	168.9	170
PopA	102.7	102.7	189.7	202.8	198.2	198.2	189.8	198.2	136.5	150.8	141.1	144.1	166.5	184.9	169.1	169.1
PopA	106.3	129.9	187.8	187.8	200.3	202.4	?	?	143.7	150.9	144.1	146.4	166.2	186.7	173.3	173.3
PopA	102.7	118.4	182.1	184	179.7	204.5	157.9	186.2	?	?	?	?	184.7	184.7	168.9	168.9
PopA	103.4	127.8	?	?	183.6	198	177.7	185.9	150.7	155.1	141.1	146.4	?	?	160	171
PopA	?	?	164.9	170.8	?	?	167.9	189.5	?	?	144.2	144.2	184.9	189.9	168.8	168.8
PopA	106.2	125.9	?	?	196.3	198.4	164.4	164.4	?	?	144.3	144.3	166.2	184.6	168.8	170.9
PopA	122.2	126.2	?	?	?	?	164.1	193.9	136.7	151.1	146.4	146.4	166.3	184.8	166.7	171.1
PopA	103.7	126.1	176.8	201.6	184.2	200.8	?	?	?	?	144.2	144.2	166.6	185	171.1	171.1
PopA	118.2	125.8	184.1	210.4	198.1	198.1	173.6	187.3	147.2	147.2	146.4	146.4	166.4	166.4	171	171
PopA	118.3	125.9	158.8	208.4	?	?	170	183.3	146	146	?	?	166.2	185.6	166.9	169.1
PopA	121.6	125.7	?	?	183.8	183.8	194.8	196.7	147.1	151.7	137.8	144.2	166.5	166.5	169.5	169.5
PopA	?	?	182.1	202.9	?	?	160.4	187.3	146.1	146.1	146.4	146.4	184.7	188.7	168.9	171
PopA	106.1	118.2	174.2	185.7	183.7	198	162.1	181.7	147	147	137.8	146.3	166.6	185	166.5	170.8
PopA	125.9	127.9	?	?	183.6	197.9	166.3	193.9	146.2	150.9	137.8	144.3	?	?	169	171.1
PopA	?	?	?	?	183.4	199.7	164	189.5	147.1	158.5	144.4	144.4	166.5	166.5	?	?
PopA	106.4	126	193.6	197.4	184	198.5	164.3	196.5	?	?	144.2	146.4	184.8	184.8	?	?
PopA	118.3	127.9	?	?	198.3	198.3	164.3	196.8	136.9	136.9	144.2	144.2	184.8	186.9	169.1	169.1
PopA	109.1	127.9	183.9	191.6	198.4	200.5	?	?	?	?	132	137.9	166.3	184.7	171	173.2
PopA	?	?	?	?	193.9	202	177.9	190.1	151	151	144.2	146.4	172.4	188.8	168.9	168.9
PopA	118.3	125.9	187.9	193.7	183.8	198.2	161.9	184.2	150.9	155.4	144.2	146.4	166.3	172.5	166.9	169.1
PopA	106.6	128.1	182.6	184.5	198.9	198.9	164.2	186.2	136.7	153.1	?	?	166.4	184.8	?	?
PopA	?	?	?	?	183.6	197.8	171.8	198.1	150.8	155.2	146.5	146.5	166.5	166.5	169.1	171
PopA	109.2	116.5	?	?	198.4	200.5	160.3	164.3	143.8	146.3	132	137.9	184.8	186.8	169.1	171.3
PopA	126.1	128	?	?	183.9	196.3	170	170	146.3	146.3	144.1	144.1	166.5	185	168	170.4
PopA	106.5	122.3	?	?	183.8	198.1	160	201.9	?	?	?	?	166.5	186	?	?
PopA	102.6	118.2	182	182	183.6	197.8	177.7	189.8	146.2	155.3	131.8	144.4	166.6	166.6	166.8	168.9
PopA	122.1	125.9	195.3	197.2	200.1	200.1	?	?	?	?	144.2	144.2	166.3	188.9	166.8	166.8
PopA	106.3	106.3	?	?	183.8	198.3	164.2	170	?	?	144.2	146.3	166.2	184.7	173.1	173.1
PopA	105.9	125.7	189.4	193.2	183.8	200	168.2	183.5	147.1	151.6	141.1	144.3	176.6	189.1	169.4	171.4
PopA	118.4	118.4	189.8	191.7	184.1	184.1	?	?	146.3	151	144.2	144.2	176.5	184.8	166.7	169
PopA	125.8	127.7	185.8	195.2	183.8	196	169.9	196.4	146	153	?	?	166.4	172.9	?	?
PopA	106.2	118.3	185.9	189.7	196	198.1	160	160	151.7	151.7	144.2	146.4	166.2	166.2	168.7	168.7
PopA	127.9	127.9	?	?	?	?	164.2	164.2	?	?	137.7	144.2	184.6	186.7	166.6	168.9
PopA	?	?	?	?	?	?	?	?	?	?	131.8	146.2	166.3	166.3	?	?
PopA	118.1	118.1	?	?	?	?	157.8	183.3	137.6	151.8	144.3	144.3	166.3	188.8	169.3	169.3
PopA	103.4	108.8	?	?	183.7	196	164.1	164.1	151.8	151.8	131.9	144.2	166.3	172.5	167	169.3
PopA	?	?	180.2	191.6	?	?	170.2	170.2	?	?	144.2	146.4	166.3	184.9	166.7	168.9
PopA	118.4	126	185.9	187.8	198.2	204.3	?	?	?	?	132	144.3	166.2	166.2	166.5	168.8
PopA	106.1	106.1	164.6	176.2	198.1	200.1	164.4	187.4	138.8	153.9	131.9	144.1	166.3	188.7	166.6	168.6
PopA	?	?	?	?	?	?	196.5	196.5	146.1	146.1	144.2	146.4	166.3	166.3	166.5	166.5
PopA	118.4	122.1	166.8	186.1	184	184	164.3	186.2	136.7	151	131.9	137.9	166.3	188.8	162.5	162.5

PopB	?	?	?	?	?	?	164	167.5	146.8	151.5	144.2	146.4	184.7	186.8	?	?
PopB	125.8	127.6	186	187.9	198.4	200.4	160.1	193.6	137.5	146.9	137.8	137.8	184.8	184.8	172	172
PopB	116.3	118.1	?	?	?	?	?	?	137.6	153.9	?	?	?	?	171.8	171.8
PopB	122.1	127.9	?	?	183.3	195.5	?	?	137.4	144.4	144.1	154.8	166.5	185	157.3	171.7
PopB	106	118	170.2	171.7	?	?	?	?	144.5	146.8	144.2	144.2	?	?	167.8	172.2
PopB	122.1	126	176.5	193.7	183.7	183.7	?	?	151.6	153.8	144.2	144.2	172.6	185	169.9	169.9
PopB	116.2	125.8	?	?	183.3	183.3	190.1	190.1	146.9	151.5	131.8	131.8	172.6	188.8	171.7	171.7
PopB	102.2	106	140.9	179.9	195.6	195.6	161.9	177.4	137.4	151.6	131.8	144.1	166.2	184.7	167.3	167.3
PopB	106.1	116.3	174.4	200.8	?	?	157.8	164	146.9	146.9	138.9	144.8	?	?	166.5	168.7
PopB	112.3	129.5	174.3	176.2	?	?	164.3	186.2	151.5	151.5	144.4	144.4	166.5	174.8	169.6	171.6
PopB	105.8	118	187.4	189.3	183.3	197.6	183.7	185.6	151.5	151.5	144.2	146.4	166.5	185	169.1	171.1
PopB	125.9	125.9	?	?	183.4	195.6	167.9	167.9	151.5	151.5	131.8	144.3	166.3	188.9	166.8	166.8
PopB	125.7	127.6	181.8	181.8	193.5	195.5	161.9	167.5	156	156	137.7	144.3	166.3	188.8	167.3	173.7
PopB	?	?	?	?	?	?	?	?	?	?	144.3	144.3	184.7	188.8	168.9	171
PopB	?	?	?	?	?	?	?	?	151.6	151.6	144.3	144.3	166.3	184.8	167.7	172
PopB	125.7	125.7	?	?	184	204.5	157.8	160.1	146.8	153.8	144.2	146.4	166.2	184.8	167.7	167.7
PopB	125.6	125.6	186	187.8	?	?	185.9	185.9	146.8	146.8	?	?	166.3	172.5	?	?
PopB	125.6	125.6	?	?	191.8	197.9	183.3	185.3	151.5	151.5	?	?	184.9	186.8	?	?
PopB	116.1	129.4	158.7	196.9	189.4	197.5	?	?	137.3	151.5	131.7	148.7	166.3	184.7	167.2	169.5
PopB	118	125.7	?	?	183.3	197.7	162.4	189.6	137.4	146.9	144.3	146.5	?	?	167.4	167.4
PopB	116.2	125.6	174.7	186.3	198.7	198.7	166.3	187.3	128.9	151.6	144.3	144.3	184.7	184.7	169.9	171.9
PopB	106	125.7	199.6	199.6	?	?	164.3	179.4	137.5	151.4	144.3	146.5	?	?	167.2	167.2
PopB	103.3	125.7	?	?	197.7	197.7	160	163.9	137.4	151.5	131.8	144.2	180.8	184.8	167	169.1
PopB	105.9	108.7	164.4	187.5	183.2	183.2	?	?	?	?	131.8	144.2	?	?	167.4	169.5
PopB	106.1	125.8	199.1	210.3	183.4	195.6	?	?	137.5	156.1	144.1	144.1	?	?	166.6	168.8
PopB	127.9	127.9	208.2	210.1	?	?	159.9	159.9	146.8	153.7	146.4	146.4	166.6	185.1	169.4	169.4
PopB	117.9	129.4	?	?	?	?	163.9	190	146.8	151.4	137.9	144.3	184.6	188.7	171.8	171.8
PopB	125.7	127.6	205.4	205.4	197.7	199.7	?	?	151.7	158.3	144.2	144.2	172.5	184.8	169.8	174
PopB	102.3	117.9	187.7	189.5	?	?	189.5	198	151.4	153.7	144.2	144.2	166.2	188.8	?	?
PopB	116.4	127.8	180.4	188.1	183.4	203.8	164.1	189.9	147.1	151.7	144.2	144.2	176.7	185	?	?
PopB	?	?	182	195.4	183.9	183.9	160	160	?	?	144.3	144.3	?	?	168.9	171
PopB	103.4	125.8	188.1	212.9	?	?	167.6	191.5	137.4	158.2	131.8	144.4	166.6	189.1	167.4	167.4
PopB	106.2	127.8	178.5	197.7	?	?	?	?	?	?	144.3	144.3	166.3	184.7	170.1	172.2
PopB	125.5	127.4	193.7	205.1	?	?	157.6	185.8	146.8	146.8	144.3	144.3	184.6	188.7	167.5	167.5
PopB	103.3	106	?	?	?	?	161.9	166.2	147.2	151.9	131.8	131.8	174.6	184.8	166.5	166.5
PopB	?	?	?	?	?	?	?	?	137.4	151.4	144.2	144.2	184.8	184.8	167.5	167.5
PopB	106	127.6	?	?	?	?	?	?	151.4	153.7	132	144.2	166.2	188.7	167.6	171.9
PopB	106	127.7	?	?	183.8	196.1	?	?	?	?	131.9	141.2	166.2	172.4	?	?
PopB	106.3	120.1	187.7	195.3	?	?	157.5	189.6	151.5	155.9	146.5	146.5	184.6	186.6	167.5	171.7
PopB	103.3	103.3	?	?	183.3	203.7	189.7	193.4	?	?	131.8	137.6	?	?	167.5	171.7
PopB	103.3	125.8	193.4	193.4	?	?	162.1	162.1	146.9	151.6	135.7	144.3	166.4	189	168.8	168.8
PopB	118.3	127.8	176.6	212.8	197.8	203.9	177.7	196.3	152	152	146.4	146.4	166.3	184.8	171.2	171.2
PopB	105.8	127.5	170.3	191.2	197.5	197.5	163.8	163.8	153.7	155.9	144.3	144.3	166.5	172.7	167.3	169.5
PopB	125.7	127.6	?	?	?	?	?	?	151.5	160.2	144.3	146.4	?	?	171.8	173.9
PopB	103.2	112.4	187.5	193.1	183.1	199.5	181.6	185.7	151.5	151.5	144.2	144.2	?	?	169.3	169.3
PopB	106.2	125.9	?	?	?	?	?	?	151.7	151.7	131.9	144.2	166.5	166.5	169.7	169.7
PopB	121.9	129.5	?	?	183.4	187.4	162.2	164	152	152	144.2	144.2	166.5	185	168.6	170.7
PopB	122.1	129.9	170.6	172.5	198	204.1	161.9	185.8	155.9	162.3	146.4	146.4	166.1	184.6	166.4	168.5
PopB	125.9	129.7	187.6	189.6	?	?	168	177.7	144.7	158.3	139	144.8	172.7	185	169.8	171.9
PopB	106	125.8	178.1	210.2	183.4	203.7	164	167.8	151.6	151.6	144.2	144.2	176.7	189.1	171.7	171.7
PopB	116.1	125.6	179.9	185.7	?	?	161.5	169.6	128.8	137.3	131.8	144.3	166.2	166.2	169.5	169.5
PopB	125.7	125.7	164.6	180.1	?	?	164	175.7	156	156	144.2	146.4	174.7	184.9	?	?
PopB	118.2	125.8	188.2	203.5	?	?	185.9	189.7	137.6	156.2	144.2	144.2	166.6	185	173.9	175.7
PopB	125.8	127.7	?	?	183.4	199.7	163.9	183.9	137.4	151.6	131.8	146.5	184.9	184.9	169.6	171.6
PopB	118	118	164.7	178.2	?	?	177.4	189.5	137.3	151.4	144.3	144.3	166.1	184.7	167.4	171.8
PopB	125.8	125.8	210.7	210.7	195.7	195.7	?	?	147	151.7	131.8	144.4	166.4	189	169.7	171.8
PopB	?	?	183.8	187.7	?	?	160.1	164	146.9	151.3	144.4	146.6	?	?	169.7	171.8
PopB	105.9	127.6	?	?	196.6	198.6	177.5	196.1	?	?	132.1	144.3	?	?	171.9	174
PopB	?	?	174.2	180	?	?	164.3	164.3	146.8	146.8	144.2	144.2	166.2	188.8	167.3	171.5
PopB	106.3	126	198.9	210.2	183.7	198.2	?	?	146.7	151.4	137.9	144.3	186.4	186.4	?	?
PopB	103.3	120	178.2	197.1	183.4	197.6	171.5	171.5	144.8	151.9	144.3	144.3	166.3	184.8	168.7	170.8
PopB	102.4	127.7	180.1	189.5	195.6	203.7	161.1	193.4	144.5	147	139.7	144.3	185	189.1	167.5	171.7
PopB	106.2	116.4	188.1	190	185.1	198.5	167.2	171.3	137.6	151.9	146.2	146.2	184.9	184.9	170.3	174.7
PopB	118.3	122.1	164.7	178.3	?	?	177.7	183.3	147.1	156.2	131.8	144.3	166.4	166.4	169.8	169.8
PopB	106.1	118.2	164.6	183.8	197.7	197.7	162	167.8	146.9	146.9	144.2	146.4	166.4	166.4	169.6	171.7
PopB	116.1	118	211.8	213.8	183.2	195.5	163.8	189.5	137.6	151.9	144.2	144.2	176.4	184.6	166.3	170.5
PopB	106.2	125.9	?	?	183.5	195.6	170.1	177.7	151.7	151.7	131.9	144.2	166.5	166.5	167	169.1
PopB	106.1	127.7	178.2	178.2	?	?	175.7	183.2	153.8	158.1	144.2	144.2	180.7	184.9	169.5	169.5
PopB	?	?	178.2	178.2	183.4	197.8	?	?	151.7	153.9	144.2	146.5	166.4	174.6	169.7	171.8
PopB	121.4	125.2	182.1	185.9	?	?	162.2	189.8	151.8	158.4	144.3	144.3	166.5	185	167.5	167.5
PopB	118.2	121.9	187.8	210.3	195.8	197.8	170	186	137.6	154.1	?	?	166.4	166.4	170	170
PopB	118	121.7	195.7	195.7	183.2	183.2	?	?	153.7	153.7	144.3	146.5	184.9	189	167.2	171.4
PopB	103.1	105.9	?	?	?	?	161.9	161.9	146.9	151.6	?	?	166.4	188.9	167.8	170
PopB	102.4	118.2	164.6	187.6	?	?	?	?	151.9	154.1	131.7	148.7	185	189.1	169.7	169.7

PopB	103.2	125.6	159	201.2	?	?	163.9	183.3	147	151.6	137.8	144.2	184.8	188.9	169.9	169.9
PopB	?	?	?	?	?	?	?	?	?	?	?	?	174.5	188.8	?	?
PopB	125.6	125.6	189.5	197.1	?	?	167.3	189	?	?	?	?	?	?	167.4	169.6
PopB	106	121.8	164.4	164.4	183.3	183.3	164.2	183.6	151.5	155.9	144.3	146.5	166.4	189	166.4	168.5
PopB	126	126	164.5	185.8	183.4	197.6	162.4	189.6	146.9	151.6	144.3	144.3	166.4	184.8	169.1	169.1
PopB	164.8	189.9	?	?	?	?	?	?	137.5	151.5	144.3	144.3	184.8	188.9	166.7	166.7
PopB	125.6	127.5	210.6	212.5	179.6	183.7	169.8	185.1	137.4	151.6	137.7	144.3	166.4	184.9	167.7	167.7
PopB	105.9	125.6	205.1	205.1	?	?	170	193.7	146.8	151.5	139.8	144.2	166.5	185	166.5	168.6
PopB	?	?	?	?	?	?	?	?	137.5	146.8	144.3	144.3	184.8	188.9	?	?
PopB	106	106	164.7	189.5	?	?	164	183.9	144.5	153.8	131.7	146.3	185	187.1	167.5	169.6
PopB	125.7	127.6	182	189.7	183.8	198.1	167.7	183.1	147.3	151.9	144.2	144.2	166.2	184.8	167.6	169.7
PopB	125.7	127.6	181.8	187.6	?	?	163.9	183.2	146.8	146.8	146.5	146.5	?	?	171.7	171.7
PopB	?	?	?	?	?	?	159.9	188.5	128.9	146.8	?	?	166.2	184.7	169.7	169.7
PopB	125.8	127.8	185.6	198.9	?	?	162	163.9	144.4	155.9	135.9	144.3	184.7	186.7	167.7	169.8
PopB	?	?	?	?	?	?	?	?	151.5	151.5	?	?	?	?	167.6	167.6
PopB	116.2	125.6	?	?	195.8	202	165.9	169.8	?	?	144.3	148.7	?	?	?	?
PopB	108.9	125.7	182	189.5	187.5	199.7	164	181	129	137.6	144.2	146.5	166.3	184.8	166.5	170.8
PopB	116.2	121.9	185.8	187.6	195.7	197.8	164	177.1	137.5	160.4	144.3	146.5	166.5	187	171.7	171.7
PopB	106.3	127.8	174.5	176.3	184	184	160.1	164.2	137.4	151.5	131.8	131.8	?	?	?	?
PopB	?	?	?	?	195.7	197.7	?	?	137.6	151.8	144.3	144.3	166.5	185.1	169.8	171.9
PopB	?	?	187.9	195.4	183.5	197.7	177.5	189.5	137.3	151.5	132	154.9	176.6	184.8	171.9	171.9
PopB	106.3	127.9	176.4	191.6	183.5	195.8	181.5	194	151.8	151.8	131.8	144.3	166.6	185.1	169.9	169.9
PopB	102.6	118.4	182.3	189.9	?	?	189	189	152	152	144.1	146.3	172.6	188.9	167.9	170.1
PopB	103.4	118.2	174.3	200.8	183.4	183.4	157.7	163.7	137.5	151.6	?	?	166.6	189.1	166.5	170.8
PopB	125.6	125.6	?	?	?	?	165.9	185.7	137.3	151.4	?	?	?	?	169.6	171.8
PopB	106	125.7	181.8	185.8	195.5	203.7	?	?	151.5	156	144.3	144.3	?	?	171.7	171.7
PopB	106	127.6	164.5	198.8	?	?	?	?	151.5	153.7	144.3	146.5	166.6	166.6	168.5	168.5
PopB	?	?	?	?	?	?	?	?	?	?	?	?	166.2	184.7	169.8	169.8
PopB	103.6	126	?	?	196.1	196.1	152.4	169.9	151.4	155.8	144.3	144.3	172.4	184.8	?	?
PopB	106.1	125.7	?	?	198	200	?	?	151.4	151.4	137.9	146.5	184.6	188.7	167.5	167.5
PopB	?	?	?	?	?	?	?	?	147	153.8	139.8	144.4	166.5	166.5	167.5	169.7
PopB	118	125.6	170.7	182.3	?	?	163.8	169.9	144.5	151.5	144.3	144.3	166.5	189	?	?
PopB	121.7	127.6	170.4	185.8	195.7	203.7	162	170	152	154.2	137.8	144.2	166.4	166.4	166.6	168.8
PopB	106.2	125.8	182	187.7	195.8	203.8	164.1	167.6	151.7	160.5	141.1	144.2	172.5	184.8	171.9	171.9
PopB	?	?	?	?	?	?	163.6	169.1	147.3	147.3	131.8	144.2	166.3	184.8	?	?
PopB	103.1	125.7	188.2	211	196	198.1	163.8	169.9	147	151.5	144.3	144.3	166.4	172.5	167.6	173.9
PopB	102.7	125.1	180.2	189.7	?	?	159.8	169.7	151.6	153.8	144.2	146.5	184.8	186.8	?	?
PopB	?	?	?	?	?	?	160.6	184.5	151.6	151.6	144.1	146.4	166.5	166.5	?	?
PopB	116.5	126	164.9	182.3	197.8	199.9	159.8	179.1	146.9	151.6	131.9	137.8	174.5	184.8	166.6	170.9
PopB	?	?	?	?	183.7	183.7	162.1	185.8	146.8	158.1	135.8	144.3	166.2	188.7	167.6	171.9
PopB	126	126	182.4	212.9	?	?	?	?	146.9	146.9	144.4	144.4	184.8	188.9	168.7	170.8
PopB	108.8	118.2	187.8	210.3	?	?	183.3	191.7	137.6	147.3	144.3	146.5	166.4	186.9	169.8	169.8
PopB	106.2	123.9	?	?	?	?	159.6	183.7	?	?	146.3	146.3	184.8	184.8	171	171
PopB	118.3	127.8	187.7	210.3	183.5	195.7	201.9	201.9	138.9	147.6	144.3	146.7	166.4	172.6	169.8	171.9
PopB	118.4	126	165	186.2	?	?	?	?	151.9	156.4	137.8	144.2	166.5	166.5	167.8	172
PopB	106	106	190	212.8	?	?	?	?	137.4	151.4	131.8	144.3	?	?	171.6	171.6
PopB	?	?	170.8	212.8	?	?	?	?	137.3	137.3	131.9	144.3	189.1	189.1	168.6	168.6
PopB	102.4	127.7	208.4	210.2	?	?	?	?	137.4	158.3	131.8	146.5	166.5	185	171.8	171.8
PopB	?	?	164.6	189.7	183.8	204.4	?	?	146.9	151.5	137.8	144.3	174.5	188.9	167.7	172
PopB	103.5	125.9	?	?	?	?	?	?	137.3	151.4	144.3	146.5	?	?	169.7	171.7
PopB	106.3	118.3	190	190	?	?	?	?	151.5	153.7	131.9	144.2	166.6	189.1	169.9	171.9
PopB	118.4	127.9	164.4	164.4	?	?	?	?	146.8	151.5	131.8	144.3	184.9	188.8	167.7	172
PopB	116.3	125.9	178.3	178.3	?	?	?	?	137.3	151.5	132	144.3	166.3	190.8	167.6	171.9
PopB	102.3	102.3	190	203.4	183.2	197.6	?	?	137.4	151.5	131.9	141.2	?	?	169.5	169.5
PopB	118.1	125.7	181.8	185.7	183.3	183.3	159.9	163.6	146.7	146.7	137.7	144.3	184.8	186.7	171.6	173.7
PopB	?	?	191.5	193.4	198.3	200.3	?	?	?	?	144.4	144.4	166.2	184.7	171.9	173.9
PopB	126	126	184.2	190.1	?	?	?	?	151.6	156	131.8	144.2	166.3	184.8	166.4	166.4
PopB	108.6	127.6	?	?	?	?	160	189.6	147	153.8	144.3	144.3	184.9	184.9	?	?
PopB	118.3	127.9	180.3	193.5	198	204	171.9	198.4	144.9	151.9	144.2	146.4	166.3	188.9	169	169
PopB	106	106	?	?	?	?	167.6	169.7	146.8	158	144.2	144.2	166.3	166.3	167.4	171.6
PopB	118.2	121.8	164.5	164.5	197.6	197.6	?	?	151.7	156.1	144.2	146.3	166.4	166.4	169.5	171.7
PopB	121.8	125.7	?	?	?	?	177.4	189.6	?	?	144.3	146.4	?	?	?	?
PopB	106	127.6	180.2	182.1	183.8	183.8	185.8	185.8	128.9	153.7	132	144.3	184.8	184.8	171.8	173.9
PopB	125.6	125.6	184	201	183.7	195.9	161.5	176.9	146.9	146.9	144.3	146.5	166.3	190.8	166	169.1
PopB	105.9	129.5	186.1	191.8	?	?	?	?	137.3	137.3	?	?	166.3	186.6	?	?
PopB	116.1	120	164.9	186.2	?	?	189.6	195.2	128.9	158.2	144.2	144.2	166.4	184.8	?	?
PopB	103.6	116.5	?	?	193.6	195.7	184	184	153.9	153.9	131.9	144.2	166.4	189	166.6	168.8
PopB	?	?	?	?	?	?	?	?	137.3	151.5	131.9	144.2	184.8	188.8	?	?
PopB	105.9	121.9	164.5	189.5	?	?	163.9	175.6	137.4	151.5	144.3	146.5	189	189	168.6	168.6
PopB	106	112.4	182.2	197.6	?	?	181	189.5	137.4	151.6	144.2	146.5	166.5	184.9	170	172.1
PopB	105.9	127.6	185.7	187.5	?	?	?	?	137.4	146.9	144.2	144.2	189	189	168.7	168.7
PopB	103.1	118	184.1	195.5	?	?	163.8	167.4	137.4	146.9	144.4	144.4	?	?	170.1	170.1
PopB	118.2	127.8	193.8	203.3	200.1	200.1	167.3	180.7	152	156.3	?	?	166.2	184.7	169.1	169.1
PopB	118.4	125.9	174.5	174.5	?	?	?	?	146.9	153.7	132	144.4	166.3	184.7	169.9	169.9

PopC	?	?	?	?	?	?	185.8	185.8	?	?	?	?	?	?	?	?
PopC	116.2	123.8	178.2	187.6	200.2	204.2	183.3	189.6	151.3	151.3	131.8	146.4	166.6	187.1	?	?
PopC	112.3	118.2	159.2	178.6	183.7	200.1	159.8	163.9	146.6	151.3	131.8	144.1	166.3	184.8	169.8	171.8
PopC	?	?	184.1	193.6	?	?	163.8	175.5	147.2	147.2	144.2	146.3	166.4	188.9	167.8	172.1
PopC	?	?	?	?	184.1	192.3	183.3	193.4	146.7	146.7	144.3	146.4	166.5	185.1	169.7	171.8
PopC	122.2	128	164.9	180.5	183.7	198	?	?	151.9	156.2	144.3	144.3	166.3	166.3	171.9	171.9
PopC	118	125.7	170.7	191.9	?	?	?	?	144.3	146.7	144.1	148.5	166.2	166.2	169.7	169.7
PopC	?	?	170.7	187.9	?	?	?	?	156.3	156.3	144.2	146.4	166.4	186.9	?	?
PopC	102.5	118.2	174.7	180.5	196.5	198.5	173.9	198.6	151.5	153.7	131.8	144	166.5	186.9	167.8	167.8
PopC	?	?	174.4	189.7	198.5	198.5	164.3	190.1	146.8	151.5	144	144	166.6	181	172	172
PopC	?	?	189.9	197.4	190.4	199.7	188.2	198.3	137.6	147.2	131.9	152.8	166.2	166.2	170.6	172.8
PopC	?	?	172.7	176.6	?	?	?	?	147.3	152	144.2	144.2	184.8	184.8	167.9	170
PopC	124.1	126	187.6	208.2	183.9	196.2	189.7	191.5	137.1	137.1	131.8	131.8	184.8	188.9	167.5	171.8
PopC	103.5	125.9	?	?	?	?	158.1	190	151.3	151.3	146.5	146.5	166.3	172.5	167.5	169.7
PopC	?	?	180.4	189.9	183.8	198.1	177.4	185.6	153.6	153.6	144.2	144.2	172.4	188.7	167.3	171.6
PopC	106	116.3	187.9	189.8	184.2	198.6	169.9	177.7	146.6	155.8	131.7	144.1	184.7	184.7	169.8	171.9
PopC	118	118	185.9	201	183.7	183.7	158	183.4	137.4	151.4	144.2	144.2	166.2	188.7	169.7	171.8
PopC	118	121.8	?	?	?	?	?	?	146.7	151.3	144.1	144.1	?	?	?	?
PopC	106.4	126	186	195.6	196.2	202.4	189.8	189.8	137.6	147.3	131.8	144.2	166.2	184.6	171.8	171.8
PopC	121.5	125.9	170.4	202.7	183.8	198.2	170	177.1	146.7	151.3	131.8	146.3	166.6	189.2	167.6	169.7
PopC	?	?	170.8	170.8	198	198	164	189.7	146.6	155.7	144.3	146.4	185	189.1	169.9	171.9
PopC	?	?	176.6	191.8	188.5	202.2	184.1	193.6	151.9	151.9	131.9	144.2	166.3	184.8	170.1	170.1
PopC	?	?	187.9	202.9	198.2	198.2	177.7	177.7	146.8	151.4	131.9	131.9	187	187	?	?
PopC	106.2	106.2	166.7	178.4	?	?	194.4	196.2	151.3	153.5	144.3	146.5	166.3	184.8	?	?
PopC	125.8	125.8	182.2	187.9	183.9	183.9	159.8	170	146.8	151.4	131.8	137.7	185	185	172	172
PopC	118.4	122.1	186.1	201.2	?	?	181.5	184.2	151.4	151.4	144.3	144.3	166.2	184.7	166.6	170.9
PopC	?	?	?	?	?	?	?	?	146.7	146.7	131.8	144.2	184.8	188.7	167.5	171.8
PopC	?	?	187.7	187.7	196.3	198.4	?	?	137.3	151.3	144.2	144.2	166.3	172.4	169.8	171.8
PopC	109.1	125.9	121.9	127.9	?	?	162.4	177.4	142.6	147.4	144.1	144.1	166.6	174.9	167.8	167.8
PopC	?	?	184.2	193.5	183.9	198.1	169.8	184	144.3	151.3	131.7	137.6	184.7	184.7	171.8	173.9
PopC	106.3	126	178.5	210.6	184.7	206.9	163.7	167.5	152.1	152.1	144.2	144.2	176.4	188.8	171.2	171.2
PopC	118.3	125.1	176.5	201	188	188	160.1	164.4	146.8	160.2	141.1	144.3	184.7	184.7	167.5	171.8
PopC	102.6	118.3	199.3	201.3	?	?	?	?	147.3	152	144.2	144.2	184.8	188.8	170	172.1
PopC	?	?	?	?	?	?	?	?	?	?	144.2	146.3	184.7	184.7	?	?
PopC	?	?	187.8	193.6	184	198.4	?	?	151.9	158.5	146.5	146.5	185.1	189.1	167.6	171.9
PopC	109	127.9	159.1	180.4	?	?	164.2	187.3	151.3	151.3	144.2	144.2	172.5	188.8	166.7	166.7
PopC	?	?	?	?	?	?	?	?	?	?	?	?	184.8	186.8	172.2	172.2
PopC	110.5	117.9	164.5	164.5	?	?	?	?	137.3	144.3	144.1	154.7	189.2	191.2	167.4	169.6
PopC	103.6	125.9	180.3	201.2	?	?	164.3	183.6	144.7	147.1	131.9	144.2	166.4	188.9	172.2	172.2
PopC	118.3	129.8	174.7	191.9	183.8	204.3	160.1	160.1	151.9	151.9	131.7	144.1	172.4	188.8	167.9	170.2
PopC	103.3	103.3	193.5	193.5	?	?	?	?	146.7	157.9	144.2	144.2	166.4	188.8	169.9	169.9
PopC	?	?	190	203	183.7	202	?	?	137.7	151.9	137.7	137.7	185	189	170.1	172.1
PopC	?	?	159.3	180.6	?	?	163.5	163.5	151.8	158.4	144.3	146.4	166.4	166.4	?	?
PopC	116.3	121.9	?	?	198	200	163.6	171.5	146.7	151.3	137.7	146.3	166.3	184.8	170	170
PopC	?	?	?	?	?	?	?	?	147.1	151.8	?	?	?	?	?	?
PopC	116.3	125.7	189.8	210.7	?	?	172.1	190	137.3	151.3	131.8	146.4	166.3	184.8	170	172.1
PopC	103.3	106	?	?	183.8	200.2	157.8	181.8	146.7	151.3	?	?	166.3	166.3	?	?
PopC	?	?	189.8	193.6	184.1	198.3	169.9	186	151.5	151.5	137.7	144.1	166.3	188.9	?	?
PopC	?	?	176.1	197	183.6	183.6	?	?	146.6	151.3	?	?	166.2	184.7	167.6	169.7
PopC	106.1	122	?	?	?	?	158.2	162	146.6	160	135.7	144.1	184.9	188.7	167.6	171.9
PopC	126	127.8	164.7	193.5	184.1	204.6	185.9	189.6	146.6	151.2	144.3	146.5	186.8	190.9	171.9	171.9
PopC	106.4	125.2	165	191.8	196.1	198.1	?	?	146.8	151.4	144.3	144.3	188.8	188.8	159.7	167.8
PopC	103.2	105.1	164.7	187.7	196.2	198.2	157.8	164	151.3	151.3	144.2	146.3	166.2	188.8	167.6	169.7
PopC	102.6	116.4	164.8	166.7	?	?	167.6	186.6	137.7	147.3	144.2	144.2	184.8	184.8	167.8	167.8
PopC	118.3	122.1	178.5	188	?	?	161.8	163.9	147	147	144.2	144.2	189.1	189.1	172.3	172.3
PopC	103.6	125.3	180.2	204.8	196	198	157.8	162.2	144.3	151.4	135.7	144.1	184.7	188.8	166.7	171
PopC	?	?	191.8	199.4	183.8	198.1	162.2	162.2	144.4	151.4	144.1	146.3	166.3	188.9	169.9	169.9
PopC	103.6	126	184.2	203.2	?	?	189.3	193.6	144.8	152	144.1	146.4	166.3	188.8	167.9	170
PopC	?	?	?	?	?	?	?	?	?	?	137.8	144.2	176.6	184.9	?	?
PopC	106	116.2	185.8	212.2	183.8	196.1	183.3	189.7	146.8	146.8	135.7	144.3	?	?	166.5	170.9
PopC	118	121.8	174.3	174.3	183.8	198.2	181.1	183.1	137.3	146.8	144.1	144.1	184.7	188.8	?	?
PopC	?	?	166.9	166.9	?	?	163.9	177.7	151.3	151.3	131.8	144.1	166.3	184.8	169.9	171.8
PopC	103.2	125.6	?	?	?	?	189.7	193.4	151.3	151.3	144.1	146.2	172.4	188.6	167.5	169.7
PopC	?	?	186	188	?	?	?	?	147.1	147.1	131.6	144.2	166.5	172.7	167.8	172.1
PopC	?	?	191.7	193.7	?	?	?	?	137.6	156.2	144.1	144.1	166.4	166.4	167.9	170
PopC	106.3	125.9	?	?	?	?	158.1	164.3	151.5	151.5	144.1	146.2	176.5	176.5	166.7	173
PopC	125.6	125.6	164.9	164.9	?	?	181.1	185.9	151.8	151.8	144.3	144.2	180.6	184.6	167.6	169.7
PopC	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
PopC	108.8	112.4	188.1	188.1	?	?	190.1	193.8	142.4	147.4	131.8	144	184.8	190.9	171.9	171.9
PopC	106.2	106.2	180.2	197.3	198.3	198.3	159.9	159.9	144.5	151.5	144.3	144.3	184.7	188.8	167.7	167.7
PopC	?	?	?	?	?	?	?	?	?	?	131.8	144.2	166.3	184.8	?	?
PopC	?	?	174.4	189.6	198.5	198.5	?	?	137.3	153.5	131.9	137.8	166.4	166.4	?	?
PopC	118.3	126	182	189.7	183.8	198.1	?	?	137.6	152	144.3	144.3	166.2	166.2	167.5	173.9
PopC	?	?	185.8	185.8	?	?	?	?	146.7	151.3	144.3	144.3	166.4	176.6	169.9	172

PopD	116.3	125.8	210.3	212.2	193.9	196	?	?	137.4	137.4	?	?	184.6	190.7	166.8	171
PopD	106	118.1	178.3	195.3	195.9	197.9	?	?	144.4	151.5	?	?	166	176.3	171	171
PopD	118.1	127.6	185.7	189.5	197.8	199.8	?	?	137.3	137.3	146.4	146.4	166.1	184.5	166.6	170.9
PopD	125.7	129.5	191.4	193.3	199.9	203.9	?	?	137.2	146.7	?	?	166	166	170.1	172/27
PopD	106.1	125.8	166.5	176.2	197.9	201.9	?	?	153.7	155.9	?	?	184.6	188.6	166.6	170.9
PopD	?	?	185.8	191.5	197.8	199.9	?	?	137.4	153.8	137.7	144.2	166.2	184.9	166.7	173.1
PopD	102.3	121.9	187.6	191.4	197.8	203.8	?	?	153.6	153.6	137.7	146.4	166.2	166.2	168.8	171
PopD	?	?	201.3	203.1	183.5	183.5	163.8	185.7	147.4	152.1	131.8	144.2	166.4	166.4	166.5	170.8
PopD	106	118.2	180.1	187.6	183.5	195.8	?	?	151.5	155.9	137.8	144.3	184.7	188.8	166.6	170.6
PopD	?	?	?	?	184.8	184.8	?	?	151.9	158.4	?	?	166.3	166.3	166.9	166.9
PopD	105.9	118	164.4	164.4	183.4	203.8	179.6	189.9	151.9	158.4	144.2	144.2	166	184.5	166.5	170.8
PopD	?	?	165.1	186.3	?	?	163.8	189.4	147.3	152	144.2	146.4	166.4	176.7	171.2	171.2
PopD	?	?	187.8	189.7	197.9	202	?	?	137.6	152	?	?	184.9	189	171	173.1
PopD	106	108.8	164.5	164.5	183.5	183.5	175.5	197.4	137.2	154	131.9	146.4	188.6	188.6	168.8	170.8
PopD	?	?	191.7	193.6	183.8	198.2	165.8	185.7	152	152	144.1	146.3	166.3	188.8	?	?
PopD	106.1	118.3	191.9	201.3	?	?	?	?	151.6	153.8	?	?	172.7	172.7	?	?
PopD	?	?	185.9	187.7	183.6	195.9	?	?	137.4	147	?	?	166.2	188.9	166.8	171
PopD	106.1	106.1	185.7	187.6	183.5	183.5	?	?	151.4	151.4	144.2	146.4	166.1	166.1	166.7	168.8
PopD	116.3	118.1	170.4	174.2	183.6	197.8	?	?	146.8	146.8	144.2	144.2	166	184.4	167.9	172.1
PopD	?	?	164.8	174.6	183.5	197.9	?	?	144.8	147.2	?	?	166.3	188.8	166.5	168.8
PopD	122	127.7	164.6	200.8	?	?	?	?	?	?	?	?	184.4	188.5	170.9	170.9
PopD	103.2	116.2	199	210.2	183.5	199.8	?	?	151.8	151.8	146.4	146.4	166.2	185.2	?	?
PopD	125.8	129.7	178.2	178.2	195.9	199.9	?	?	137.2	137.2	?	?	166	184.5	169.8	169.8
PopD	?	?	166.9	186.1	?	?	?	?	151.8	154.1	?	?	166.4	166.4	167.9	172.2
PopD	116.4	125.9	164.7	189.7	183.7	183.7	?	?	151.5	153.7	?	?	166.2	166.2	167.7	167.7
PopD	106.1	125.7	166.5	180	183.6	197.8	?	?	137.6	147.3	?	?	176.2	184.5	167.6	169.8
PopD	?	?	164.6	193.3	183.5	183.5	?	?	147.3	151.9	?	?	176.2	184.5	169.9	171.9
PopD	?	?	174.3	193.4	197.9	197.9	?	?	147.1	147.1	?	?	166.1	184.8	166.6	166.6
PopD	103.3	127.8	195.2	202.7	183.6	183.6	?	?	146.7	151.4	144.2	144.2	166.5	166.5	166.7	171
PopD	116.2	125.8	189.6	199	197.8	199.8	?	?	147.3	147.3	146.4	146.4	166.1	184.6	166.7	168.8
PopD	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
PopD	?	?	?	?	195.8	195.8	?	?	151.4	155.8	?	?	166.2	188.6	166.6	168.9
PopD	?	?	?	?	186	202.4	?	?	146.7	151.4	?	?	166.5	189.1	167.8	170
PopD	116.2	121.8	178.1	187.6	199.7	203.8	?	?	151.4	151.4	146.4	146.4	166	186.5	170.9	170.9
PopD	?	?	187.7	199.1	183.6	183.6	?	?	151.5	153.8	144.2	144.2	166.4	184.9	171	171
PopD	106.1	125.7	182	185.9	183.5	197.8	?	?	137.7	147.4	?	?	186.6	186.6	169.9	172
PopD	?	?	193.9	201.4	?	?	?	?	152.1	152.1	131.9	137.8	166.3	184.9	?	?
PopD	118.1	127.6	162.6	185.8	183.5	183.5	?	?	151.4	153.6	144.2	144.2	166	184.5	166.6	171
PopD	?	?	180	202.7	197.8	201.8	?	?	144.4	146.7	144.3	144.3	184.5	184.5	166.5	166.5
PopD	?	?	164.6	191.6	202.4	204.4	189.9	193.8	146.8	146.8	144.2	146.4	166.1	172.2	168.9	170.9
PopD	?	?	184	185.8	183.5	183.5	?	?	144.8	151.8	144.2	144.2	165.9	184.4	170.1	172.2
PopD	103.3	118.1	199	204.7	183.6	197.9	?	?	128.8	156	?	?	166.1	184.5	167.8	169.9
PopD	122	127.7	191.4	193.3	195.8	197.7	?	?	144.2	146.6	144.2	146.4	166	188.5	166.7	168.8
PopD	?	?	?	?	?	?	159.6	169.7	?	?	131.8	144.1	166.3	166.3	?	?
PopD	?	?	180.3	180.3	183.6	203.9	?	?	147.3	152.1	144.2	144.2	184.8	184.8	?	?
PopD	?	?	191.5	193.4	183.5	203.9	?	?	137.4	151.5	?	?	184.5	188.6	166.6	166.6
PopD	?	?	170.7	193.7	200.5	202.7	159.6	169.4	147.3	156.4	131.9	144.2	166.2	166.2	167.9	170
PopD	103.4	118.2	186.3	186.3	183.6	183.6	?	?	146.8	153.7	?	?	166.2	188.6	172	172
PopD	?	?	?	?	184.7	200.2	?	?	?	?	131.8	144.1	166.4	166.4	171.3	171.3
PopD	118.2	123.9	164.7	191.6	202	204.1	189.9	193.7	146.9	146.9	?	?	166.3	172.5	166.7	168.9
PopD	118.1	125.7	174.3	193.3	?	?	?	?	146.7	151.3	144.2	146.4	166.1	186.5	172	172
PopD	?	?	?	?	?	?	?	?	144.3	151.4	?	?	166.2	188.8	?	?
PopD	106.1	122	185.8	187.7	195.8	195.8	?	?	151.4	151.4	146.4	146.4	166.1	186.6	158.4	170.9
PopD	?	?	?	?	198	200	169.8	193.3	137.6	147.2	144.2	144.2	185	185	169.8	169.8
PopD	?	?	170.9	170.9	197.8	203.9	175.5	197.6	146.7	153.6	144.2	144.2	166	166	174	174
PopD	120.1	125.8	174.2	191.4	183.8	183.8	?	?	152	156.4	?	?	166.1	166.1	169.8	171.8
PopD	103.3	121.9	164.7	187.6	183.5	199.8	?	?	128.8	146.7	?	?	166	166	166.8	168.8
PopD	118.2	127.7	187.9	191.7	183.5	199.8	?	?	144.3	151.3	137.8	141.1	184.5	184.5	166.7	168.8
PopD	?	?	?	?	183.9	183.9	?	?	147.3	147.3	?	?	?	?	?	?
PopD	125.8	127.8	174.3	176.2	183.6	197.9	?	?	151.4	151.4	144.2	146.4	166.1	166.1	?	?
PopD	?	?	201.4	203.2	?	?	?	?	147.3	154.2	144.1	144.1	172.6	188.9	169.2	171.3
PopD	118.2	127.7	189.7	197.1	183.6	183.6	162.2	189.9	146.8	153.8	131.9	144.3	180.5	188.5	166.6	171
PopD	116.2	129.6	170.5	178.3	183.7	197.9	?	?	146.8	151.4	144.3	148.6	166.1	176.3	158.5	171.1
PopD	125.8	127.7	180	202.6	196.3	198.3	?	?	?	?	?	?	166.1	172.4	?	?
PopD	106.1	125.8	178.2	191.5	204	204	?	?	147.4	152.1	?	?	184.6	184.6	170	172.2
PopD	103.4	122	?	?	195.9	200	?	?	137.4	146.8	?	?	185.1	187.1	169.1	169.1
PopD	?	?	166.4	166.4	197.8	197.8	?	?	152	154.1	?	?	166.1	184.5	167.8	167.8
PopD	106	118.1	?	?	197.8	197.8	?	?	146.7	153.6	144.3	146.4	180.4	184.4	167.5	171.8
PopD	106	118.1	164.5	191.4	192.1	198.3	?	?	137.4	151.5	?	?	166.2	166.2	167.6	169.8
PopD	?	?	164.9	203.2	184.8	184.8	?	?	138.9	145.4	144.1	144.1	172.6	174.6	?	?
PopD	118.1	125.7	164.6	164.6	183.5	183.5	?	?	151.9	156.4	146.4	146.4	?	?	?	?
PopD	?	?	208.7	210.6	?	?	?	?	139	139	?	?	166.5	185.1	167.8	174.2
PopD	116.3	125.8	178.3	212.1	183.6	183.6	?	?	144.8	151.9	?	?	166.2	184.6	167.8	167.8
PopD	?	?	185.9	187.8	200	200	162.2	186.2	146.9	146.9	?	?	184.7	188.9	?	?

PopD	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
PopD	106	116.2	199.5	199.5	?	?	?	?	146.8	151.5	144.2	144.2	180.5	184.6	167.6	167.6
PopD	?	?	?	?	198.2	198.2	?	?	151.9	154.1	?	?	?	?	167.5	167.5
PopD	118.1	125.8	?	?	183.9	196.4	?	?	151.4	151.4	?	?	174.3	184.6	167.8	172
PopD	106	121.9	164.5	187.7	183.4	199.7	?	?	152	152	?	?	165.9	188.5	172.1	172.1
PopD	?	?	174.2	197.1	?	?	?	?	137.7	147.3	?	?	?	?	?	?
PopD	?	?	?	?	?	?	?	?	138.9	147.6	?	?	166.3	184.8	172.2	172.2
PopD	103.4	106.1	183.9	200.9	195.9	204	?	?	147.3	152	?	?	166.2	188.7	173.9	173.9
PopD	?	?	167	191.9	?	?	?	?	147.3	152	144.2	144.2	188.8	188.8	?	?
PopD	?	?	187.7	200.9	183.6	195.9	?	?	151.3	157.9	?	?	166.2	184.6	167.8	172
PopD	?	?	167	197.5	183.8	198.2	?	?	147	151.6	?	?	?	?	?	?
PopD	?	?	164.9	190	182.7	197.1	167.7	169.2	151.8	151.8	?	?	166.5	166.5	166.8	166.8
PopD	?	?	172.4	193.4	183.6	195.8	?	?	142.4	152	?	?	?	?	167.8	172
PopD	108.9	127.8	164.7	199.1	197.8	197.8	?	?	151.5	156	?	?	166.5	185	166.7	166.7
PopD	?	?	164.8	200.9	184	184	?	?	149.7	152	?	?	188.6	188.6	167.8	167.8
PopD	?	?	?	?	183.9	183.9	?	?	147.2	151.8	?	?	?	?	169.9	171.9
PopD	?	?	164.6	185.8	183.9	183.9	175.6	197.5	151.5	151.5	?	?	184.9	184.9	168.8	168.8
pop = E																
PopE	?	?	?	?	?	?	?	?	146.9	151.6	144.2	146.5	?	?	?	?
PopE	?	?	159.3	165	?	?	?	?	?	?	?	?	?	?	167.4	171.6
PopE	?	?	172.1	174.1	?	?	?	?	146.7	151.4	?	?	?	?	?	?
PopE	?	?	?	?	?	?	?	?	?	?	144.2	144.2	166.4	180.6	169.8	171.8
PopE	118.2	125.8	?	?	?	?	?	?	137.3	151.3	?	?	?	?	167.3	171.6
PopE	?	?	165.1	203.2	197.9	197.9	178.1	190	?	?	?	?	?	?	167.6	171.9
PopE	?	?	?	?	?	?	?	?	146.7	146.7	?	?	?	?	?	?
PopE	?	?	193.2	200.7	183.4	183.4	?	?	128.8	128.8	?	?	?	?	167.4	169.6
PopE	103.3	118.2	199.5	205.2	184	198.2	?	?	?	?	144.3	144.3	?	?	167.7	169.8
PopE	?	?	190.2	190.2	198.3	200.3	?	?	?	?	?	?	?	?	167.5	171.8
PopE	?	?	?	?	197.6	203.7	?	?	128.7	144.3	?	?	166.3	188.8	173.7	173.7
PopE	?	?	?	?	196.3	196.3	?	?	?	?	?	?	?	?	?	?
PopE	103.3	118.2	?	?	197.6	197.6	?	?	151.4	151.4	?	?	166.3	188.9	167.3	167.3
PopE	?	?	?	?	?	?	?	?	137.3	146.8	?	?	?	?	171.7	171.7
PopE	?	?	?	?	?	?	?	?	?	?	144.2	144.2	?	?	?	?
PopE	?	?	?	?	?	?	?	?	?	?	?	?	?	?	169.8	169.8
PopE	?	?	185.7	200.8	183.5	197.8	?	?	151.5	151.5	?	?	?	?	167.5	167.5
PopE	?	?	?	?	?	?	?	?	?	?	144.2	144.2	?	?	?	?
PopE	?	?	187.7	189.6	195.6	197.7	?	?	137.4	151.5	?	?	?	?	167.4	167.4
PopE	?	?	?	?	183.4	183.4	?	?	?	?	?	?	?	?	?	?
PopE	?	?	?	?	183.4	183.4	?	?	?	?	?	?	?	?	?	?
PopE	?	?	?	?	?	?	?	?	?	?	142.1	144.2	?	?	?	?
PopE	?	?	178.7	184.5	184	198.3	186.6	190.3	?	?	?	?	?	?	?	?
PopE	?	?	?	?	183.4	183.4	?	?	137.3	155.8	?	?	?	?	?	?
PopE	106	118.1	189.6	210.2	?	?	?	?	137.5	137.5	144.2	146.4	?	?	?	?
PopE	?	?	?	?	?	?	?	?	137.4	147	?	?	?	?	171.8	173.9
PopE	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
PopE	103.5	122	?	?	197.8	199.8	?	?	?	?	?	?	?	?	169.7	171.8
PopE	112.7	125.9	?	?	183.4	183.4	162	189.7	?	?	132	144.4	?	?	169.7	171.8
PopE	?	?	?	?	?	?	?	?	?	?	131.9	131.9	166.3	184.8	173.9	173.9
PopE	116.3	125.8	174.2	180	?	?	?	?	146.9	151.6	137.9	144.2	166.2	186.7	?	?
PopE	122	126	?	?	183.5	183.5	?	?	151.5	151.5	?	?	?	?	171.8	171.8
PopE	?	?	174.7	193.7	204.1	204.1	164.4	190.2	?	?	?	?	?	?	169.8	171.9
PopE	106	125.7	?	?	?	?	?	?	?	?	131.9	144.2	184.7	184.7	167.5	169.8
PopE	?	?	?	?	?	?	?	?	?	?	?	?	166.3	188.7	?	?
PopE	116	125.5	?	?	?	?	?	?	137.3	137.3	131.9	146.6	?	?	?	?
PopE	?	?	180	193.3	194.1	204.2	?	?	?	?	?	?	?	?	169.8	171.9
PopE	?	?	?	?	198.4	200.7	?	?	146.8	151.5	146.4	146.4	166.4	172.5	169.7	169.7
PopE	?	?	165.1	170.9	184	198.2	164.6	186.5	?	?	144.2	144.2	184.6	186.7	167.8	170
PopE	?	?	165	178.6	197.9	197.9	?	?	?	?	?	?	?	?	167.4	171.8
PopE	?	?	?	?	?	?	?	?	?	?	144.3	146.5	?	?	?	?
PopE	103.2	116.3	158.7	187.6	?	?	?	?	151.6	151.6	144.3	146.4	184.7	184.7	169.7	171.7
PopE	?	?	199	210.3	?	?	?	?	147.1	151.7	144.3	146.4	166.3	184.7	?	?
PopE	?	?	?	?	?	?	?	?	?	?	142.1	144.3	?	?	?	?
PopE	?	?	186.2	190.2	193.7	197.7	161.7	176.2	146.8	151.4	?	?	?	?	169.6	171.6
PopE	106.3	126	181.9	197	197.7	197.7	167.9	183.9	151.5	155.8	144.3	144.3	?	?	167.5	169.7
PopE	?	?	?	?	?	?	?	?	146.9	149.2	?	?	?	?	?	?
PopE	102.6	125.9	?	?	183.5	203.8	160.1	160.1	137.4	151.5	?	?	166.5	188.3	169.7	171.8
PopE	125.8	125.8	181.8	189.6	183.4	183.4	?	?	151.5	155.9	135.9	146.5	166.4	190.4	167.4	171.7
PopE	?	?	165	165	183.8	198.1	162.5	164.5	?	?	?	?	?	?	169.9	169.9
PopE	116.3	118.2	?	?	183.4	197.7	?	?	142.1	144.4	?	?	184.7	186.7	168.6	168.6
PopE	?	?	183.8	210	198.2	204.3	158.4	170.1	?	?	132	144.3	184.7	184.7	167.9	170.1
PopE	?	?	184.3	197.5	196	198	?	?	?	?	?	?	?	?	167.5	167.5
PopE	?	?	178.8	203.5	184	198.3	?	?	149.3	151.6	?	?	?	?	167.6	173.9
PopE	?	?	?	?	197.7	197.7	189.7	189.7	144.5	146.8	?	?	?	?	167.5	169.7
PopE	118.4	126	170.5	170.5	183.5	197.8	?	?	?	?	?	?	?	?	167.9	170.1

PopE	106.2	125.9	174.3	178.2	197.7	197.7	157.7	169.6	151.5	158.1	?	?	166.6	186.1	166.6	168.8
PopE	118.1	120	?	?	?	?	?	?	147	147	132	146.4	166.2	188.8	171.8	171.8
PopE	121.9	125.8	178.2	189.6	?	?	?	?	147	151.7	144.3	144.3	184.8	184.8	167.7	171.9
PopE	102.5	125.1	177.9	198.8	183.3	199.6	163.8	187.6	146.7	151.4	137.8	144.2	?	?	171.5	173.6
PopE	106.1	127.7	180	185.7	183.4	197.6	159.6	167.4	137.4	155.7	143.9	146	?	?	171.6	173.8
PopE	?	?	?	?	?	?	?	?	?	?	?	?	?	?	169.7	169.7
PopE	118.2	125.8	185.8	187.6	183.3	183.3	157.6	162	146.8	146.8	?	?	166.3	184.9	170.7	170.7
PopE	?	?	183.8	185.7	183.3	197.7	?	?	137.4	146.7	?	?	174.6	186.8	169.1	171.2
PopE	103.2	118	185.6	195.1	?	?	?	?	151.5	155.9	144.2	144.2	172.3	174.4	169.7	171.7
PopE	106.2	125.8	?	?	183.4	183.4	?	?	137.4	151.5	144.3	144.3	?	?	167.5	171.7
PopE	118.3	127.8	?	?	183.4	197.7	?	?	146.8	146.8	144.3	146.5	166.3	188.9	166.4	170.7
PopE	?	?	186.4	186.4	198.3	200.3	190.3	190.3	144.5	151.5	144.3	146.4	166.1	188.7	?	?
PopE	116.3	127.8	?	?	?	?	?	?	144.6	153.9	144.2	146.5	172.4	184.7	?	?
PopE	?	?	172.1	211.9	?	?	?	?	137.3	137.3	146.3	146.3	166	184.6	169.6	169.6
PopE	103.2	125.7	187.6	198.9	?	?	?	?	128.8	144.5	131.8	146.5	166.3	186.7	169.7	169.7
PopE	105.9	125.7	?	?	?	?	?	?	137.3	146.9	144.3	144.3	166.1	166.1	169.7	173.8
PopE	?	?	193.3	212.1	183.5	183.5	162	185.3	?	?	135.8	144.3	166.6	186.4	167.5	171.7
PopE	116.2	125.8	164.6	193.3	?	?	?	?	147	151.6	137.8	144.3	166.3	184.8	167.6	169.8
PopE	?	?	188	188	183.8	198.1	?	?	?	?	132	132	?	?	169.5	169.5
PopE	?	?	?	?	?	?	?	?	147	151.6	144.2	144.2	166.4	184.8	169.8	171.9
PopE	125.9	125.9	187.5	193.2	183.4	183.4	163.9	163.9	146.8	151.4	?	?	184.7	186.8	?	?
PopE	106.2	125.8	191.4	198.9	197.7	199.7	169.8	185.3	146.8	155.9	144.3	144.3	166.6	166.6	171.7	171.7
PopE	105.9	121.8	?	?	?	?	?	?	137.4	146.8	144.3	146.5	?	?	169.8	171.9
PopE	?	?	?	?	183.7	200	?	?	?	?	144.2	144.2	167.5	184.9	169.9	171.9
PopE	122	129.8	164.6	185.8	?	?	163.9	189.6	146.8	151.5	144.3	146.5	166.3	176.6	?	?
PopE	116.1	125.7	186.2	190	196	200.1	164.5	164.5	?	?	144.3	144.3	?	?	167.4	169.5
PopE	105.9	125.6	181.7	210.1	?	?	?	?	137.4	146.8	144.3	146.4	166	184.6	169.6	173.8
PopE	106.2	116.4	?	?	?	?	?	?	151.7	153.9	144.2	144.2	184.8	184.8	?	?
PopE	?	?	?	?	?	?	?	?	137.4	151.4	144.3	146.4	?	?	167.6	169.7
PopE	117.9	125.6	?	?	?	?	?	?	146.7	155.8	144.2	144.2	166.2	184.6	171.8	171.8
PopE	106.3	116.5	182	197.1	183.6	197.8	164.1	175.8	146.8	153.7	?	?	?	?	168.8	168.8
PopE	?	?	?	?	?	?	?	?	128.9	146.8	132	144.3	?	?	?	?
PopE	125.7	129.4	?	?	?	?	?	?	137.3	137.3	144.3	144.3	166.1	184.6	?	?
PopE	103.3	125.8	?	?	?	?	?	?	152.5	152.5	132	137.9	172.4	184.7	167.6	169.7
PopE	?	?	?	?	183.3	203.7	?	?	?	?	144.2	144.2	166.4	166.4	167.4	169.5
PopE	102.2	125.7	172.3	181.8	?	?	?	?	144.5	153.7	137.9	144.2	166.2	166.2	166.5	166.5
PopE	?	?	176.1	200.8	195.7	195.7	163.8	185.8	146.8	151.4	137.8	144.3	166.4	186.1	167.3	167.3
PopE	103.5	127.7	187.7	210.2	195.6	195.6	177.7	190.6	144.4	151.5	137.8	144.2	?	?	169.8	171.8
PopE	109	118.3	164.6	174.3	183.4	199.7	185.8	185.8	146.8	158.1	?	?	166.6	166.6	171.7	173.9
PopE	?	?	176.2	187.8	?	?	?	?	151.6	153.8	131.9	144.2	166.2	184.7	171.9	174
PopE	125.8	125.8	164.6	189.5	183.4	199.7	?	?	128.8	151.4	141.1	144.2	166.7	186.3	169.7	169.7
PopE	103.5	118.2	?	?	183.5	195.8	164	187	151.5	151.5	?	?	?	?	166.5	168.8
PopE	122	125.7	164.6	164.6	?	?	?	?	147	151.6	144.3	144.3	166.2	180.7	166.6	172.9
PopE	103.5	127.9	180.1	180.1	195.8	201.8	162	185.8	144.4	146.9	143.5	143.5	?	?	167.4	167.4
PopE	118.2	127.7	?	?	?	?	?	?	137.6	151.7	131.8	146.3	166.3	184.8	167.6	171.8
PopE	?	?	?	?	?	?	?	?	?	?	?	?	166.3	166.3	?	?
PopE	103.3	118.2	?	?	?	?	?	?	147	147	144.2	144.2	172.3	172.3	?	?
PopE	125.8	125.8	181.9	187.7	183.4	183.4	163.9	185.9	151.5	151.5	145.5	145.5	169.6	169.6	?	?
PopE	?	?	?	?	183.6	183.6	?	?	?	?	?	?	166.3	188.9	166.9	169.1
PopE	122	129.6	?	?	?	?	?	?	158.1	162.3	132.1	146.5	166.2	174.5	167.6	171.8
PopE	?	?	?	?	197.7	197.7	?	?	146.8	155.9	135.8	144.2	166.3	176.6	166.4	170.8
PopE	125.9	125.9	?	?	?	?	?	?	128.8	151.5	?	?	166.7	202.8	167.5	171.8
PopE	?	?	187.6	189.5	197.7	197.7	?	?	137.4	137.4	?	?	166.6	186.3	167.5	167.5
PopE	102.2	106	185.6	193.1	?	?	?	?	151.4	151.4	144.3	144.3	166.2	166.2	169.7	171.8
PopE	102.6	118.3	?	?	197.8	197.8	?	?	146.8	151.5	?	?	?	?	168.7	168.7
PopE	?	?	?	?	?	?	?	?	?	?	?	?	?	?	167.5	169.6
PopE	106	125.8	?	?	?	?	?	?	?	?	146.4	146.4	166.2	184.7	?	?
PopE	125.7	129.5	?	?	?	?	?	?	144.5	146.8	137.8	146.4	166.2	166.2	167.6	171.9
PopE	102.3	125.7	174.1	176	?	?	?	?	128.8	146.8	144.2	144.2	166.2	188.7	171.8	171.8
PopE	106	127.6	?	?	?	?	?	?	146.8	158	144.3	146.4	166.2	188.7	169.8	169.8
PopE	118.2	125.8	170.6	189.6	?	?	?	?	147	147	144.2	144.2	166.3	184.8	169.8	171.9
PopE	102.5	122	187.5	202.6	?	?	167.8	183.2	137.3	146.8	?	?	166.4	184.9	168.6	168.6
PopE	125.8	125.8	?	?	199.7	203.8	185.8	185.8	128.7	151.3	?	?	?	?	166.3	170.6
PopE	?	?	?	?	?	?	?	?	146.7	151.4	144.2	144.2	?	?	167.5	171.9
PopE	125.9	129.9	185.7	200.8	183.5	197.7	198.1	199.9	151.5	155.8	137.9	146.6	?	?	171.8	171.8
PopE	?	?	192	195.7	184.1	198.2	?	?	153.7	160.2	132	144.3	184.6	188.7	167.6	169.7
PopE	?	?	178.5	178.5	198.1	198.1	184.4	190.2	?	?	?	?	?	?	?	?
PopE	?	?	191.4	199	179.4	199.7	?	?	137.4	151.5	?	?	166.6	166.6	?	?
PopE	103.1	125.5	185.6	200.7	?	?	?	?	151.5	155.9	144.3	146.4	184.7	188.6	169.6	171.7
PopE	109.1	125.7	178.2	189.6	?	?	?	?	146.9	153.9	144.3	146.5	172.4	184.7	167.5	171.7
PopE	?	?	?	?	?	?	?	?	?	?	137.9	144.2	184.8	184.8	167.6	169.7
PopE	?	?	212.1	212.1	198.2	198.2	183.8	190.2	151.7	153.9	131.9	144.2	184.7	188.8	167.6	167.6
PopE	?	?	?	?	183.5	201.8	?	?	146.8	146.8	?	?	?	?	167.5	169.7
PopE	?	?	158.8	174.3	183.5	183.5	161.6	193.4	144.3	151.5	144.3	146.4	?	?	167.5	171.8

8.4. Genepop results: HWE probability test

Results from GENEPOP

Tue Jan 22 08:38:53 WST 2013

Genepop version 4.2: **Hardy-Weinberg test**

File: 083853 (Bufobufo)

Number of populations detected: 5
Number of loci detected: 8

Estimation of exact P-Values by the Markov chain method.

Markov chain parameters for all tests:
Dememorization: 100
Batches: 1000
Iterations per batch: 1000
Hardy Weinberg: Probability test
****

==
Results by locus
==

Locus "Bbufu11"

POP	P-val	S.E.	W&C (Fis estimates)	R&H (Fis estimates)	Steps
A571m	0.0206	0.0024	-0.0406	0.0073	147808 switches
B778	0.6470	0.0076	0.0121	-0.0137	161388 switches
C513m	0.3234	0.0092	-0.0047	-0.0003	54273 switches
D460m	0.4816	0.0079	-0.1110	-0.0670	87238 switches
E503	0.4964	0.0074	-0.1021	-0.0677	153988 switches

All (Fisher's method):
Chi2: 13.7589
Df : 10.0000
Prob : 0.1843

Locus "Bbufu49"

POP	P-val	S.E.	W&C (Fis estimates)	R&H (Fis estimates)	Steps
A571m	0.0181	0.0026	0.0108	0.0142	78795 switches
B778	0.0000	0.0000	0.1473	0.1399	60211 switches
C513m	0.0246	0.0036	0.0742	0.0674	31323 switches
D460m	0.0059	0.0017	0.0642	0.0586	37507 switches

```
E503        0.1057  0.0072   0.1014  0.0856  40866 switches

All (Fisher's method):
 Chi2:    Infinity
 Df   :    10.0000
 Prob :    High. sign.

Locus "Bbufu62"
------------------------------------------
                          Fis estimates
                         ---------------
POP         P-val   S.E.    W&C     R&H     Steps
----------- ------- ------- ------- ------- ------
A571m       0.0089  0.0016  -0.1035 -0.0269  59480 switches
B778        0.5826  0.0099   0.1035  0.0238  46404 switches
C513m       0.0140  0.0018   0.0177  0.1211  59381 switches
D460m       0.0070  0.0010   0.1902  0.1206  99170 switches
E503        0.0054  0.0011   0.1333  0.0544  66528 switches

All (Fisher's method):
 Chi2:    39.4193
 Df   :    10.0000
 Prob :    0.0000

Locus "Bbufu65"
------------------------------------------
                          Fis estimates
                         ---------------
POP         P-val   S.E.    W&C     R&H     Steps
----------- ------- ------- ------- ------- ------
A571m       0.0006  0.0002   0.1308  0.1069  99155 switches
B778        0.0398  0.0044   0.0745  0.0984  59875 switches
C513m       0.0912  0.0064   0.0630  0.0339  37889 switches
D460m       0.0069  0.0015  -0.0093 -0.0008  34272 switches
E503        0.0001  0.0001   0.1716  0.1505  35732 switches

All (Fisher's method):
 Chi2:    54.8446
 Df   :    10.0000
 Prob :    0.0000

Locus "Bbufu24"
------------------------------------------
                          Fis estimates
                         ---------------
POP         P-val   S.E.    W&C     R&H     Steps
----------- ------- ------- ------- ------- ------
A571m       0.0700  0.0047   0.0964  0.0507  93121 switches
B778        0.1097  0.0055   0.0046  0.0010 113603 switches
C513m       0.8859  0.0038   0.0616  0.0556 138571 switches
D460m       0.4843  0.0104   0.0694  0.0516  54426 switches
E503        0.3784  0.0093   0.0245  0.0246  79160 switches

All (Fisher's method):
 Chi2:    13.3736
 Df   :    10.0000
 Prob :    0.2035

Locus "Bbufu46"
------------------------------------------
```

```
                    Fis estimates
                   ---------------
POP        P-val   S.E.    W&C     R&H     Steps
----------- ------- ------- ------- ------- ------
A571m       0.3031  0.0075   0.0174  0.0039 103184 switches
B778        0.1009  0.0045   0.0024  0.0729 101042 switches
C513m       0.2672  0.0077   0.0597  0.0362  46951 switches
D460m       0.1889  0.0049   0.1304  0.0198  67303 switches
E503        0.1143  0.0053   0.1078  0.1569  59353 switches

All (Fisher's method):
 Chi2:    17.2853
 Df   :    10.0000
 Prob :    0.0683

Locus "Bbufu54"
-----------------------------------------
                    Fis estimates
                   ---------------
POP        P-val   S.E.    W&C     R&H     Steps
----------- ------- ------- ------- ------- ------
A571m       0.0638  0.0033   0.0401  0.0452 192958 switches
B778        0.9249  0.0029  -0.0794 -0.0267 163216 switches
C513m       0.2197  0.0060   0.0478  0.0684 109653 switches
D460m       0.0672  0.0033   0.0780  0.0624 113523 switches
E503        0.3786  0.0081  -0.0652 -0.0184  95433 switches

All (Fisher's method):
 Chi2:    16.0333
 Df   :    10.0000
 Prob :    0.0987

Locus "Bbufu15"
-----------------------------------------
                    Fis estimates
                   ---------------
POP        P-val   S.E.    W&C     R&H     Steps
----------- ------- ------- ------- ------- ------
A571m       0.0000  0.0000   0.1778  0.1476 148608 switches
B778        0.0084  0.0010   0.1625  0.0633 156794 switches
C513m       0.3466  0.0037   0.1543  0.0751 269986 switches
D460m       0.0489  0.0023   0.1104  0.1410 151964 switches
E503        0.0174  0.0007   0.1094  0.1470 601742 switches

All (Fisher's method):
 Chi2:    46.2858
 Df   :    10.0000
 Prob :    0.0000

==========================================
    Results by population
==========================================


 Pop : A571m
-----------------------------------------
                    Fis estimates
                   ---------------
locus      P-val   S.E.    W&C     R&H     Steps
----------- ------- ------- ------- ------- ------
```

Bbufu11	0.0206	0.0024	-0.0406	0.0073	147808 switches
Bbufu49	0.0181	0.0026	0.0108	0.0142	78795 switches
Bbufu62	0.0089	0.0016	-0.1035	-0.0269	59480 switches
Bbufu65	0.0006	0.0002	0.1308	0.1069	99155 switches
Bbufu24	0.0700	0.0047	0.0964	0.0507	93121 switches
Bbufu46	0.3031	0.0075	0.0174	0.0039	103184 switches
Bbufu54	0.0638	0.0033	0.0401	0.0452	192958 switches
Bbufu15	0.0000	0.0000	0.1778	0.1476	148608 switches

All (Fisher's method):
Chi2 : 73.8355
Df : 16.0000
Prob : 0.0000

Pop : B778

Fis estimates
----

locus	P-val	S.E.	W&C	R&H	Steps
Bbufu11	0.6470	0.0076	0.0121	-0.0137	161388 switches
Bbufu49	0.0000	0.0000	0.1473	0.1399	60211 switches
Bbufu62	0.5826	0.0099	0.1035	0.0238	46404 switches
Bbufu65	0.0398	0.0044	0.0745	0.0984	59875 switches
Bbufu24	0.1097	0.0055	0.0046	0.0010	113603 switches
Bbufu46	0.1009	0.0045	0.0024	0.0729	101042 switches
Bbufu54	0.9249	0.0029	-0.0794	-0.0267	163216 switches
Bbufu15	0.0084	0.0010	0.1625	0.0633	156794 switches

All (Fisher's method):
Chi2 : Infinity
Df : 16.0000
Prob : High. sign.

Pop : C513m

Fis estimates
----

locus	P-val	S.E.	W&C	R&H	Steps
Bbufu11	0.3234	0.0092	-0.0047	-0.0003	54273 switches
Bbufu49	0.0246	0.0036	0.0742	0.0674	31323 switches
Bbufu62	0.0140	0.0018	0.0177	0.1211	59381 switches
Bbufu65	0.0912	0.0064	0.0630	0.0339	37889 switches
Bbufu24	0.8859	0.0038	0.0616	0.0556	138571 switches
Bbufu46	0.2672	0.0077	0.0597	0.0362	46951 switches
Bbufu54	0.2197	0.0060	0.0478	0.0684	109653 switches
Bbufu15	0.3466	0.0037	0.1543	0.0751	269986 switches

All (Fisher's method):
Chi2 : 31.0253
Df : 16.0000
Prob : 0.0134

Pop : D460m

Fis estimates
----

locus	P-val	S.E.	W&C	R&H	Steps

```
Bbufu11     0.4816  0.0079  -0.1110 -0.0670  87238 switches
Bbufu49     0.0059  0.0017   0.0642  0.0586  37507 switches
Bbufu62     0.0070  0.0010   0.1902  0.1206  99170 switches
Bbufu65     0.0069  0.0015  -0.0093 -0.0008  34272 switches
Bbufu24     0.4843  0.0104   0.0694  0.0516  54426 switches
Bbufu46     0.1889  0.0049   0.1304  0.0198  67303 switches
Bbufu54     0.0672  0.0033   0.0780  0.0624 113523 switches
Bbufu15     0.0489  0.0023   0.1104  0.1410 151964 switches

All (Fisher's method):
 Chi2 :    47.8134
 Df   :    16.0000
 Prob :    0.0001

 Pop : E503
-----------------------------------------
                       Fis estimates
                      ---------------
locus       P-val   S.E.    W&C     R&H     Steps
----------- ------- ------- ------- ------- ------
Bbufu11     0.4964  0.0074  -0.1021 -0.0677 153988 switches
Bbufu49     0.1057  0.0072   0.1014  0.0856  40866 switches
Bbufu62     0.0054  0.0011   0.1333  0.0544  66528 switches
Bbufu65     0.0001  0.0001   0.1716  0.1505  35732 switches
Bbufu24     0.3784  0.0093   0.0245  0.0246  79160 switches
Bbufu46     0.1143  0.0053   0.1078  0.1569  59353 switches
Bbufu54     0.3786  0.0081  -0.0652 -0.0184  95433 switches
Bbufu15     0.0174  0.0007   0.1094  0.1470 601742 switches

All (Fisher's method):
 Chi2 :    51.3871
 Df   :    16.0000
 Prob :    0.0000
============================================
 All locus, all populations
============================================
All (Fisher's method) :
 Chi2 :    Infinity
 Df   :    78.0000
 Prob :    High. sign.

Normal ending
```

8.5. Tables of allelic frequencies for each locus:

```
 Locus:
Bbufu11
------------------
   Pop     Alleles
Genes
           -------------------------------------------------------------------------------------- -----
           103   105   107   109   111   113   117   119   121   123   125   127   129   131
A571m      0.108 0.004 0.119 0.029 0.013 0.004 0.087 0.146 0.002 0.070 0.016 0.238 0.132 0.031 446
B778       0.112 0.000 0.161 0.015 0.000 0.012 0.058 0.112 0.009 0.064 0.003 0.291 0.133 0.030 330
C513m      0.133 0.008 0.142 0.033 0.017 0.017 0.075 0.150 0.017 0.092 0.017 0.258 0.033 0.008 120
D460m      0.121 0.000 0.198 0.017 0.000 0.009 0.095 0.164 0.009 0.069 0.017 0.181 0.095 0.026 116
E503       0.136 0.000 0.121 0.024 0.000 0.015 0.073 0.107 0.005 0.063 0.000 0.350 0.083 0.024 206

 Locus:
Bbufu49
------------------
   Pop     Alleles
Genes
           ------------------------------------------------------------------------------------------------------------------------------------------------------------------
           160   166   168   172   174   176   178   180   182   184   186   188   190   192   194   196   198   200   202   204   206   208
216
A571m      0.015 0.053 0.018 0.024 0.006 0.036 0.027 0.053 0.068 0.062 0.024 0.059 0.154 0.074 0.059 0.065 0.030 0.024 0.02
B778       0.007 0.113 0.007 0.033 0.011 0.036 0.022 0.047 0.062 0.069 0.029 0.077 0.106 0.091 0.022 0.040 0.022 0.029 0.026
C513m      0.019 0.074 0.031 0.056 0.006 0.049 0.031 0.031 0.062 0.031 0.025 0.056 0.117 0.086 0.062 0.080 0.006 0.031 0.03
D460m      0.005 0.130 0.035 0.045 0.005 0.050 0.015 0.050 0.055 0.020 0.010 0.085 0.090 0.065 0.080 0.060 0.010 0.015 0.04
E503       0.017 0.098 0.006 0.040 0.017 0.046 0.029 0.052 0.040 0.063 0.040 0.115 0.080 0.069 0.017 0.057 0.017 0.017 0.040

 Locus:
Bbufu62
------------------
   Pop     Alleles
Genes
           ---------------------------------------------------------------------------------- -----
           163   179   183   185   187   189   191   193   195   197   199   201
203
A571m      0.004 0.009 0.350 0.002 0.004 0.002 0.011 0.015 0.104 0.322 0.113 0.013 0.050 460
B778       0.000 0.010 0.347 0.005 0.010 0.005 0.005 0.020 0.153 0.296 0.066 0.010 0.071 196
C513m      0.000 0.000 0.368 0.028 0.028 0.009 0.009 0.000 0.104 0.321 0.075 0.028 0.028 106
D460m      0.000 0.000 0.362 0.037 0.005 0.000 0.005 0.005 0.112 0.261 0.101 0.037 0.074 188
E503       0.005 0.005 0.396 0.000 0.000 0.000 0.010 0.021 0.099 0.328 0.078 0.010 0.047 192

 Locus:
Bbufu65
------------------
   Pop     Alleles
Genes
           ---------------------------------------------------------------------------------------------------------------------------------------------- -----
           158   160   162   164   166   168   170   172   174   176   178   180   182   184   186   188   190   192   194   196   198   200
202
A571m      0.035 0.068 0.068 0.172 0.013 0.039 0.072 0.017 0.007 0.007 0.066 0.017 0.011 0.066 0.074 0.044 0.098 0.009 0.04
B778       0.033 0.074 0.096 0.163 0.019 0.067 0.067 0.019 0.000 0.011 0.056 0.011 0.019 0.081 0.089 0.007 0.107 0.011 0.026
C513m      0.067 0.090 0.060 0.157 0.015 0.015 0.045 0.022 0.007 0.007 0.067 0.000 0.045 0.075 0.067 0.045 0.134 0.007 0.05
D460m      0.000 0.132 0.053 0.053 0.053 0.079 0.079 0.000 0.000 0.079 0.053 0.053 0.000 0.000 0.079 0.000 0.132 0.000 0.07
E503       0.042 0.052 0.094 0.146 0.021 0.052 0.031 0.000 0.000 0.031 0.042 0.000 0.010 0.052 0.167 0.042 0.177 0.000 0.021
```

Locus: Bbufu24

Pop	128	136	138	140	142	144	146	148	150	152	154	156	158	Genes
A571m	0.003	0.052	0.038	0.003	0.049	0.276	0.000	0.404	0.084	0.029	0.041	0.015	0.006	344
B778	0.020	0.151	0.006	0.008	0.039	0.204	0.000	0.360	0.089	0.073	0.036	0.011	0.003	358
C513m	0.000	0.096	0.000	0.015	0.071	0.293	0.000	0.394	0.061	0.040	0.020	0.010	0.000	198
D460m	0.009	0.118	0.018	0.009	0.059	0.259	0.009	0.355	0.095	0.045	0.014	0.005	0.005	220
E503	0.037	0.132	0.000	0.004	0.059	0.290	0.007	0.338	0.040	0.051	0.033	0.004	0.004	272

Locus: Bbufu46

Pop	132	136	138	140	142	144	146	148	152	154	Genes
A571m	0.144	0.015	0.090	0.015	0.006	0.521	0.202	0.004	0.000	0.002	466
B778	0.144	0.011	0.052	0.014	0.009	0.583	0.164	0.011	0.000	0.011	348
C513m	0.161	0.016	0.068	0.005	0.000	0.583	0.151	0.005	0.005	0.005	192
D460m	0.093	0.009	0.065	0.009	0.000	0.556	0.259	0.009	0.000	0.000	108
E503	0.089	0.018	0.067	0.004	0.013	0.576	0.219	0.000	0.009	0.004	224

Locus: Bbufu54

Pop	166	168	172	174	176	180	184	186	188	190	Genes
A571m	0.392	0.004	0.048	0.020	0.028	0.016	0.273	0.072	0.129	0.018	502
B778	0.345	0.000	0.054	0.021	0.024	0.006	0.321	0.048	0.170	0.012	336
C513m	0.356	0.000	0.041	0.005	0.031	0.015	0.289	0.036	0.206	0.021	194
D460m	0.453	0.000	0.042	0.014	0.028	0.014	0.252	0.047	0.140	0.009	214
E503	0.426	0.005	0.053	0.021	0.016	0.011	0.237	0.095	0.105	0.032	190

Locus: Bbufu15

Pop	158	160	166	168	170	172	174	Genes
A571m	0.015	0.004	0.287	0.362	0.287	0.043	0.002	470
B778	0.003	0.000	0.295	0.351	0.307	0.042	0.003	336
C513m	0.000	0.012	0.294	0.265	0.388	0.041	0.000	170
D460m	0.011	0.005	0.371	0.215	0.360	0.038	0.000	186
E503	0.000	0.000	0.294	0.338	0.304	0.064	0.000	296

8.6. Allele frequency/null alleles. CERVUS

Allele	freq		5,	2013,	at	0.46111	pm					
****			****									
Locus	k N		HObs	HExp	PIC	NE-1P	NE-2P	NE-PP	NE-I	NE-SI	HW	F(Null)
Bbufu11	14	609	0.885	0.854	0.838	0.452	0.289	0.121	0.037	0.333	NS	-0.0191
Bbufu49	25	574	0.871	0.943	0.939	0.209	0.117	0.024	0.006	0.281	NS	0.0389
Bbufu62	13	571	0.727	0.75	0.713	0.641	0.463	0.272	0.099	0.4	NS	0.0147
Bbufu65	23	498	0.825	0.923	0.917	0.271	0.157	0.04	0.011	0.292	NS	0.0553
Bbufu24	13	696	0.731	0.771	0.741	0.601	0.423	0.229	0.082	0.385	NS	0.0257
Bbufu46	10	669	0.601	0.629	0.589	0.772	0.597	0.405	0.178	0.48	NS	0.0186
Bbufu54	10	718	0.737	0.742	0.704	0.652	0.475	0.283	0.104	0.406	NS	0.001
Bbufu15	7	729	0.595	0.702	0.642	0.73	0.567	0.401	0.149	0.436	***	0.0817

8.7 Kinship Matrix

	A003m	A007m	A009m	A010f	A011m	A012m	A015f	A016m	A017f	A018m	A020f	A021m	A022f
A003m	r												
A007m	-0.0623												
A009m	-0.0816	0.144											
A010f	-0.0305	0.0014	-0.1152										
A011m	0.104	-0.0192	0.1102	-0.0763									
A012m	-0.1015	0.451	0.2539	0.3446	-0.2161								
A015f	-0.1356	-0.0976	-0.2267	-0.0815	-0.1878	-0.0183							
A016m	0.0137	0.3615	-0.2934	0.0611	-0.4493	1	-0.5836						
A017f	0.0908	-0.4438	-0.1078	-0.2369	0.0379	-0.3299	-0.2508	0.217					
A018m	0.0251	-0.1434	0.0246	-0.0914	-0.1108	-0.314	0.0909	-0.4017	0.3442				
A020f	-0.0796	0.1712	-0.0053	-0.1155	-0.047	0.1669	-0.1225	0.2571	0.2909	-0.0599			
A021m	0.0336	-0.2027	-0.1147	0.089	0.1526	-0.3481	-0.3195	-0.2266	0.3497	0.0754	-0.0354		
A022f	0.1057	0.1021	-0.0649	0.0897	-0.1118	0.1466	-0.3096	0.4188	-0.0957	-0.0299	-0.1573	0.0747	
A023m	-0.1595	-0.0809	-0.1748	0.119	-0.1389	-0.0592	0.2177	-0.2069	0.1982	0.0187	0.1574	0.0289	-0.0715
A028f	0.226	-0.0049	0.1437	-0.2772	0.0905	-0.16	-0.0815	0.0821	0.3282	-0.0986	0.1689	0.1128	-0.0197
A029m	0.1638	0.129	0.2069	-0.3265	0.0534	-0.1397	-0.0593	-0.338	-0.059	-0.0429	-0.0718	-0.0761	-0.1075
A034f	-0.0444	0.0224	-0.0247	0.12	-0.1083	0.5132	-0.1722	0.117	-0.3543	-0.0932	-0.3257	-0.1479	0.1245
A035m	0.0834	0.1575	0.2826	-0.1295	-0.1441	0.2084	0.0072	0.0883	-0.1291	0.0563	0.03	-0.0656	-0.0897
A038m	-0.0098	0.0596	0.2902	-0.0408	0.2315	0.1028	-0.1441	0.5389	0.3631	0.2487	0.0008	0.1965	0.1118
A039m	0.1715	0.0851	-0.1433	-0.0031	0.1504	0.1556	0.1474	-0.2721	-0.1305	-0.1229	0.0305	0.0672	-0.0302
A040m	-0.0603	0.18	-0.1379	-0.0295	-0.0264	0.0765	-0.1005	-0.1562	-0.0905	-0.0838	0.1059	0.238	-0.0472
A053m	-0.3885	-0.4908	0.2672	0.1458	0.0622	-0.3958	0.2902	-0.6724	x	-0.2623	-0.5002	0.3087	-0.4908
A056m	0.0963	-0.0897	-0.2448	0.0125	-0.0725	-0.0088	-0.167	0.6268	0.3026	0.0699	-0.0324	0.2042	0.1468
A057m	0.2247	0.1202	0.1386	-0.107	0.1866	0.152	-0.205	0.2028	-0.1032	-0.1438	0.2885	-0.0352	0.2885
A058m	-0.0584	-0.0878	-0.3039	-0.0488	-0.2294	0.0541	-0.0787	0.4434	0.4391	-0.1659	0.0949	0.0825	-0.0022
A061m	-0.0667	-0.2642	-0.1512	-0.0198	0.2832	-0.406	-0.3056	-0.0549	0.4642	-0.0461	0.1756	0.3406	-0.047
A062m	-0.0237	-0.263	-0.2937	0.0172	0.0255	-0.3763	0.0466	-0.2542	0.2351	-0.0383	0.0725	0.2255	-0.1083
A063m	0.1275	-0.2549	-0.0095	0.1167	0.1787	-0.325	-0.2688	-0.0002	0.4909	0.0251	0.1517	0.3787	0.0243
A067f	-0.1286	0.4864	0.0286	-0.1119	-0.182	0.0823	-0.1688	0.5832	-0.3572	0.0987	-0.176	-0.1936	0.2

A023m	A028f	A029m	A034f	A035m	A038m	A039m	A040m	A053m	A056m	A057m	A058m	A061m	A062m	A063m
-0.0414														
0.2083	0.2806													
-0.0341	-0.0697	0.1595												
-0.1137	0.1677	0.0336	-0.0608											
-0.2218	0.1774	-0.0682	-0.0291	0.1908										
-0.1497	0.0822	0.0323	-0.0135	-0.2209	-0.0687									
0.0553	-0.1042	-0.0395	-0.0814	-0.0064	-0.0208	0.0409								
-0.2646	-0.1224	-0.4113	-0.1046	-0.1056	-0.1224	0.1197	-0.1409							
0.0967	-0.004	-0.0038	-0.168	-0.0025	0.2184	-0.1536	0.0796	-0.6591						
0.1349	-0.0057	-0.0319	-0.2238	-0.0183	0.0116	-0.1318	0.1276	-0.5002	0.0859					
-0.2432	-0.2281	-0.411	-0.4438	-0.2696	-0.0596	-0.0894	0.1265	0.0349	0.4209	0.0277				
-0.003	-0.0331	-0.3321	-0.2525	-0.0949	0.1886	-0.0451	0.0959	0.2399	0.2493	0.0492	0.2503			
0.2128	-0.0682	-0.0044	-0.3118	-0.2872	-0.0507	-0.2014	-0.0045	-0.6919	0.1347	-0.0291	0.2332	0.0642		
-0.087	0.2428	-0.2543	-0.1282	-0.161	0.2456	0.0208	-0.0636	0.6782	0.0317	0.0313	-0.0737	0.2888	-0.1172	
-0.0731	-0.0837	-0.0243	-0.1312	-0.0041	-0.0613	-0.1166	0.015	0.1019	0.0355	0.0959	-0.0001	-0.106	-0.1491	-0.0201