DMU

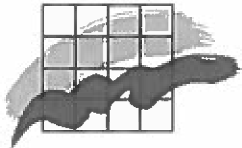
Ministry of Environment and Energy
National Environmental Research Institute

Population genetics: comparisons of different techniques in conservation projects

PhD thesis

Danmarks Miljøundersøgelser - BIBLIOTEKET
Grenåvej 12, Kalø, DK-8410 Rønde

3506906015



Ministry of Environment and Energy
National Environmental Research Institute

Population genetics: comparisons of different techniques in conservation projects

PhD Thesis
2001

BIBLIOTEKET
Danmarks Miljøundersøgelser
Kalø, Grenåvej 12, 8410 Rønde

Cino Pertoldi
National Environmental Research Institute
Department of Landscape Ecology

Aarhus University
Department of Ecology and Genetics

Data sheet

Title:	Population genetics: comparisons of different techniques in conservation projects
Subtitle:	PhD thesis
Author:	Cino Pertoldi
Department:	Department of Landscape Ecology
University:	Aarhus University Department of Ecology and Genetics
Publisher:	Ministry of Environment and Energy National Environmental Research Institute©
URL:	http://www.dmu.dk
Time of publication:	June 2001
Technical editor:	Kirsten Zaluski
Figures:	Henrik Rønnow, Bo Gaardmand
Please cite as:	Pertoldi, C. 2001: Population genetics: comparisons of different techniques in conservation projects. PhD thesis. National Environmental Research Institute. 144 pp. Reproduction is permitted, provided the source is explicitly acknowledged.
Keywords:	Genetics, population/quantitative, conservation, development, in-/stability, DNA, stress, mammals, insects
Editing completed:	22 June 2001
ISBN:	87-7772-621-9
Printed by:	Erik Vivelsted
Number of pages:	144
Circulation:	250
Price:	DKK 100,- (incl. 25% VAT, excl. freight)
Internet-version:	The report is also available as a PDF-file from NERI's homepage
For sale at:	National Environmental Research Institute Grenåvej 12-14, Kalø DK-8410 Rønde Tel.: +45 89 20 17 00 Fax: +45 89 20 15 15 e-mail: tpe@dmu.dk

Contents

Preface 5

Acknowledgements 5

Dansk resumé 6

Synopsis I

Population genetics: comparisons of different techniques in conservation projects 9

Conclusions 17

Recommendations for future directions 21

References 23

Published articles II

Extremely low mitochondrial DNA control-region sequence variation in the otter *Lutra lutra* population of Denmark. Hereditas 130:331-336.

Developmental stability in the Eurasian Otter (*Lutra lutra*) in Denmark. Ann. Zool. Fennici 34: 187-196.

Variation of skull morphometry of Eurasian otters (*Lutra lutra*) in Denmark and Germany. Ann. Zool. Fennici 35: 87-94.

Craniometrical variability and developmental stability. Two useful tools for assessing the population viability of Eurasian otter (*Lutra lutra*) populations in Europe. Biol. Jour. Linnean Society 70: 309-323.

Effect of the 1990 die-off in the northern Italian seas on the developmental stability of the stribed dolphin *Stenella coeruleoalba* (Meyen, 1833). Biol. Jour. Linnean Society 71: 61-70.

Allozyme variation in the Eurasian badger *Meles meles* in Denmark. J. Zool. 252:544-547.

Developmental instability in sexual reproducing and parthenogenetic populations of *Bacillus rossius rossius* and *Bacillus rossius redtenbacheri*. Evolutionary Ecology Research 3: 449-463.

Articles in press....................III

A New Method for Estimating Environmental Variability for Clonal Organisms, and the Use of Fluctuating Asymmetry as an Indicator of Developmental Instability. J. theor. Biol. 210: 000-000.

Effects of habitat fragmentation on the Eurasian badger (*Meles meles*) subpopulations in Denmark. (Italian Journal of Mammology) 105

Genetic consequenses of population decline in European otter (*Lutra lutra*): an assessment of microsatellite DNA variation in Danish otters from 1883 to 1993 112

Articles in prep....................IV

Analysis of the effects of different fertilisers on developmental stability in *Folsomia candida*....... 125

Developmental stability of wing size in *Drosophila melanogaster* under food stress 130

Preface

When I started my research project in 1997, I was pretty sure about which field my efforts should be directed towards: Conservation Biology, and of course, why not choose one of its most fascinating sub-disciplines: Population Genetics. Three years later, everything appears much more complicated than I would have expected. There are two principal reasons: firstly, that I have absorbed an extremely large bulk of information which has allowed me to become much more critical of myself and my enthusiasm when approaching a new research project. Therefore, also my approach methodologies have become much more prudent, understanding that sometimes a hasty investigation start may produce frustrating results. Secondly, that I have several times underestimated the enormous complexity of nature, and therefore the fascinating idea of trying to find some patterns in the indecipherable natural world began to become more and more difficult to realise. Hence, this PhD project is an attempt to develop and apply some methodologies together, in order to approach natures complexity by splitting up its components and their interaction. Below, I will present the work, which has been done during the PhD project period. When I present the works, I will always use the 1st plural person (we) as several persons, who are co-authors of the works, have collaborated. The high "biodiversity index" of the groups which have composed the list of the co-authors indicate how multidisciplinary the approaches are to the various problems which we have tried to solve. Therefore, I want to underline how important this coalition of several "brains" can be, and the importance of this kind of "aggregation" when approaching a conservation biology problem.

Acknowledgements

I would like to thank all the colleagues who have contributed to the various research projects and who have supported me in every sense during my PhD project, the list will be in random order as I have no ranking politics, but of course the women first: Kirsten Zaluski, Vibeke Simonsen, Jane Frydenberg, Karen-Lise Mensberg, Dorthe Meldrup, Sandra Cassotta, Laura Cassotta, Chiara Cassotta, Nadia Mucci, Paola Randi, Silvia Rapetti, Jane Uhd Jepsen, Camilla Håkansson, Doth Andersen, Pina Cassotta, Annie Sølling, Marianne Szygenda, Bodil Ehlers, Siri Østergaard, Benedicte Rønnow, Cecilia Mancusi, Kirsten Pedersen, Paola Nicolosi, Ditte Holm Andersen, Tina Fredsted, Signe Gammeltoft, Christina Weideick Kærsgaard, Letizia Marsili ...etc... Bo Gaardmand, Riccardo Navone, Luigi Cagnolaro, Freddy B. Christiansen, Lars Bach, Michael Møller Hansen, Ejner Eg Nielsen, Jørgen Bundgaard, Jens Mogens Olesen, Franco Pertoldi, Vittorio Lucchini, Valerio Scali, Aksel Bo Madsen, Bjarne Søgaard, Henrik Rønnow, Volker Loeschcke, Mogens Andersen, Erling Mørch, Bjørn Jacobsen, Louis Van De Zande, Ettore Randi, Mads Fjeldsø, Michele Cassotta, Paolo Guerra, Dave Parker, Emanuele Trezzi, Torsten Nygaard Kristensen, Thomas Bach Møller, Sandro Cavicchi, Anders Pape Møller, Dave Parker...etc...

Dansk resumé

Da jeg startede mit forskningsprojekt i 1997 var jeg rimeligt sikker på, hvad det skulle omhandle ... Conservation Biology, og selvfølgelig ... hvorfor ikke vælge en af dets mest fascinerende underdiscipliner: Populationsgenetik. Tre år senere ser alt langt mere kompliceret ud, end jeg kunne have forestillet mig.

Der er to hovedårsager: først at jeg har absorberet en temmelig stor bunke information som har udviklet min selvkritik, både personligt og vedr. min entusiasme, når jeg starter på et nyt forsøgsprojekt. Derfor er også mine opstartsmetodikker blevet mere rationelle under erkendelse af, at somme tider kan en forhastet start på undersøgelserne producere frustrerende resultater. For det andet, at jeg flere gange har underestimeret naturens enorme kompleksitet, og derfor blev den fascinerende ide om at finde bestemte mønstre i den udecifrerbare naturlige verden mere og mere besværlig at realisere. Dette PhD-projekt er derfor et forsøg på at udvikle og applikere nogle metodikker for at forsøge at forstå naturens kompleksitet ved at splitte den op i komponenter og deres interaktion. Nedenfor vil jeg præsentere det arbejde, som har været udført i forbindelse med PhD-projektperioderne. Jeg skriver i 1. person pluralis, da flere personer, som er medforfattere på arbejdet, har samarbejdet. Det høje biodiversitets-indeks i de grupper, som har komponeret listen af medforfattere, indikerer, hvor multidisciplinære de forskellige tiltag til problemområderne har været. Derfor vil jeg gerne understrege, hvor stort et udbytte man kan få af, at flere kapaciteter arbejder sammen, samt vigtigheden af en sådan konstellation, når man starter på et problemområde i Conservation Biology.

Emnerne, der er dækket i denne PhD-afhandling, strækker sig over en omfattende reeksamination af interaktionen mellem genetik, demografi og forskellige typer af stokasticitet. Afhandlingen illustrerer, at

forsigtighedsprincippet er essentielt for forståelsen af data baseret på forskellige typer af genetiske markører, da sådanne markører kan blive associeret med genotyper, som er fuldstændig forskellige fra dem, der er involveret i fremtidig respons på selektion, og samtidig understrege vigtigheden af kvantitativ genetik (QG) i undersøgelserne som et uundværligt støtteredskab i Conservation Biology projekter.

Flere faktorer gør sig gældende ved fald i populationerne, men de principielle faktorer, der diskuteres i denne afhandling er de antropogene, såsom landskabets udvikling og forurening: Disse faktorer er hovedårsag til trussel for populationerne og evt. udryddelse. Disse primære antropogene faktorer har hårdtslående økologisk og genetisk effekt, som bidrager til risikoen for udryddelse. I afhandlingen bringes en diskussion af rollen af økologiske faktorer, som der tillige har været for metapopulationsdynamik, men ihukommende at landskabets udvikling forårsager habitatfragmentering og isolation af små populationer samt intensificering af metapopulationsdynamik. Ødelæggelse af habitater er den største trussel, da det bidrager til risikoen for udryddelse af halvdelen af de truede fugle i verden og tre fjerdedele af de truede pattedyr i australasien og amerika (Groombridge 1992).

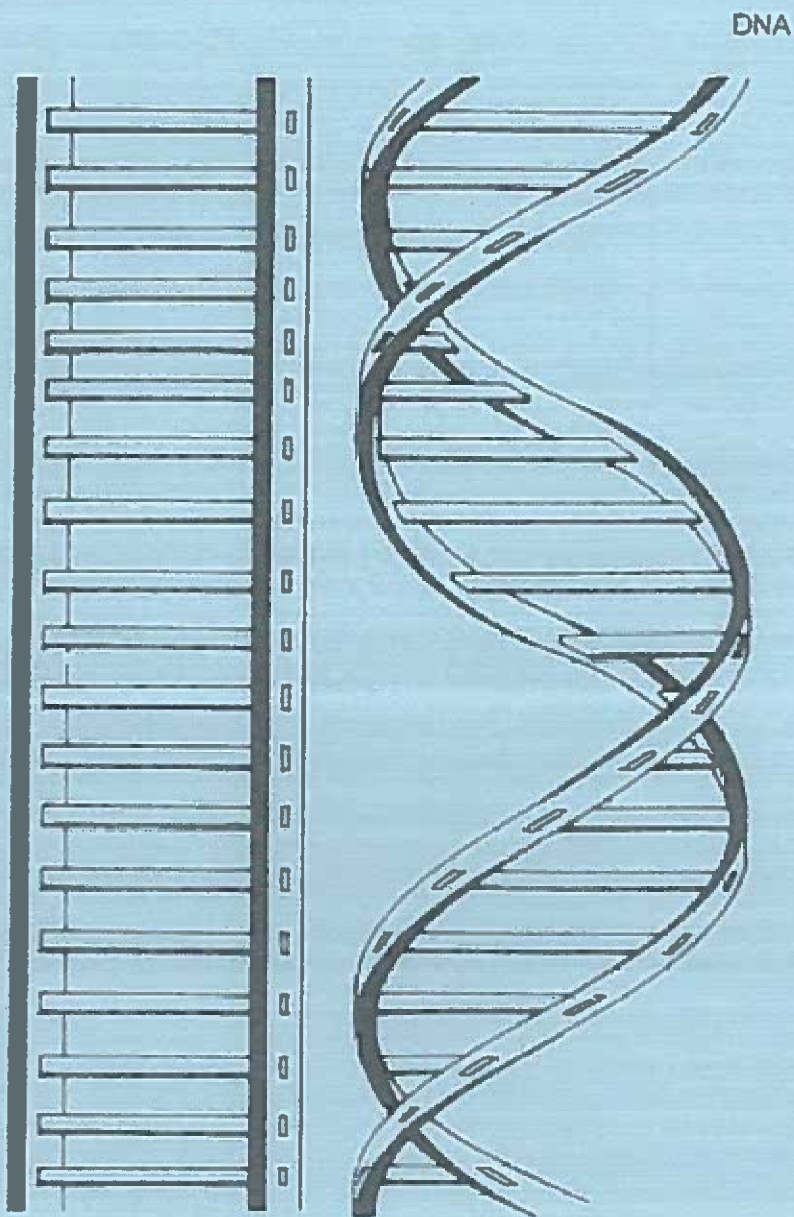
Vi forsøger også at diskutere vigtigheden af indavlens negative konsekvenser i naturen, og hvordan disse kan registreres, ved studier af naturligt forekommende populationer, ved hjælp af traditionelle molekylære markører og ved QG undersøgelser. Omend vi stadig accepterer potentialet af indavl i populationer i fangenskab, må vi i stigende grad erkende, at når degenerering er blevet signifikant i den vilde natur, så er det sandsynligt, at populationen vil falde tilbage til næsten udryddelse af enkle demografiske årsager. Tabet af genetisk diversitet kan ofte betragtes som et symbol snarere end en trussel, og ydermere må vi huske på, at arter og populationer inden for arter kan variere substantielt i graden af degenerering (Soulé 1980). Erfaringsmæssigt bevis på destruktivt degenererende effekt er imidlertid blevet leveret fra laboratorieeksperimenter, og ydermere er der leveret bevis på det faktum, at degenerering kan være langt strengere i naturlige miljøer end laboratoriepopulationer (Jiménez *et al.* 1994). Vi vil gerne minde om, at omend det ikke diskuteres i denne afhandling, så bliver også planterne berørt af habitatfragmenteringen; af de ca. 250.000 plantearter som vi ved er uddøde i historisk tid, er næsten 1.000 faktisk uddøde i det sidste århundrede, og mere end 60 gange det antal uddør måske i løbet af de næste 50 år (Raven 1987). På populationsplan, som er vores hovedfokus, ser situationen endda værre ud. Faktisk har Hughes, Daily & Ehrlich (1997) for nylig forsøgt at estimere antallet af populationer, der pt. bebor jordkloden. De beregnede, at hvis hver art blev delt op i ca. 220 populationer, er jorden beboet af mellem 1,1 og 6,6 milliarder populationer. Desværre, hvis vi antager at populationsudryddelse er en enkel lineær funktion af habitattab (direkte konsekvens), så taber vi 1.800 populationer pr. time (1,6 millioner om året). Disse skræmmende data fremmer forståelsen for, hvor vigtigt og hvor bydende nødvendigt det er at behandle problematikken omkring populationstab.

De forskellige forsøg er grundigt beskrevet i vedlagte artikler, publicerede såvel som in press og submittede.

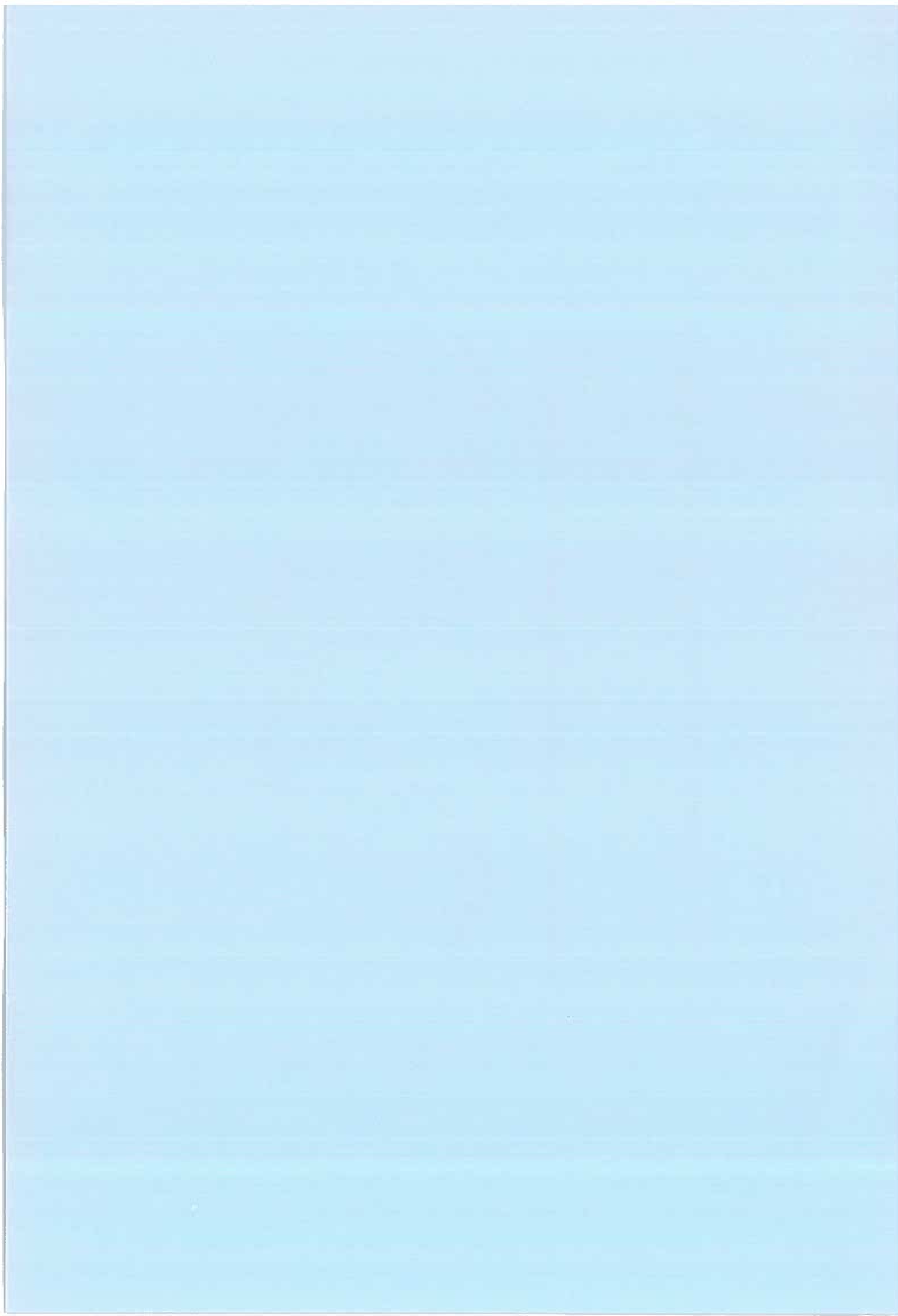
Vores generelle anbefaling baseret på alle de undersøgelser, der er præsenteret i denne afhandling er, at flere teknikker (forskellige molekylære teknikker, forskellige QG analyser) bør implementeres ved start på et projekt i Conservation Biology. Populationsgenetik forbliver en hjørnesten i Conservation Biology, men imidlertid bør flere hypoteser testes i et forsøg på at undgå på forhånd givne formodninger og for at bevare et rumligt tidsmæssigt syn på problematikken omkring Conservation Biology.

Konkluderende må vi understrege, at argumenterne diskuteret i denne afhandling og de præsenterede resultater kun er toppen af et isbjerg, hvis bund repræsenterer et større antal akkumulerede nulresultater, som skal publiceres efterfølgende. Ydermere må vi også understrege, at flere projekter er igang i øjeblikket, men de er ikke diskuteret i denne afhandling grundet pladsmangel. Det som delfiner og bananfluer har til fælles har åbnet vores øjne for den verden af muligheder, der er til rådighed, når modelleringsprincipperne anvendes på den nuværende viden om DNA og populationsgenetik.

Synopsis



In fact, the two DNA strands are twisted around each other to make a double helix.



Population genetics: comparisons of different techniques in conservation projects

The topics covered in this PhD dissertation comprise a comprehensive re-examination of the interaction between genetics, demography and different types of stochasticity. This PhD illustrates that caution is essential in interpreting data based on different kinds of genetic markers, as such markers may be associated with genotypes that are completely different from those involved in some future responses to selection, and at the same time underline the importance of quantitative genetics (QG) investigations as an indispensable complementary tool in conservation biology projects.

Several factors are responsible for population decline, however the principal factors discussed in this PhD are the anthropogenic ones, such as land development and pollution that are the primary causes of extinction. These primary anthropogenic factors have ramifying ecological and genetic effects, that contribute to extinction risk. The role of ecological factors, such as metapopulation dynamics, has been discussed, bearing in mind that land development causes habitat fragmentation and isolation of small populations, and intensification of metapopulation dynamics. Habitat destruction is the biggest threat as it contributes to extinction risk of half of the endangered birds of the world and three-quarters of the threatened mammals of Australasia and the Americas (Groombridge 1992).

We also try to discuss the importance of inbreeding depression in nature and how it can be detected when studying natural populations, with the help of traditional molecular markers and with QG investigations, however, although we still accept the potential of inbreeding in captive populations, we increasingly recognise, that by the time inbreeding depression is important in the wild, it is likely that populations will decline to extinction for simple demographic reasons. Loss of variability can indeed several times be considered a symptom rather than a cause of danger, and furthermore, we must bear in mind, that species and populations within species differ substantially in magnitude of inbreeding depression (Soulé 1980). Empirical evidence of inbreeding deleterious effects have, however, been provided from laboratory experiments, and furthermore, evidence of the fact that inbreeding depression may be more severe in natural environments than laboratory populations has been proved (Jiménez *et al.* 1994). Although not discussed in this PhD dissertation, also plants are affected by habitat destruction effects. In fact, of the roughly 250.000 plant species known to have gone extinct in historical times, almost 1.000 have become extinct in the last century, and more than sixty times that amount may become extinct in the next fifty years (Raven 1987). At the population level, which is our main focus, the situation appears even worse. Hughes, Daily & Ehrlich (1997) recently attempted to estimate the number of populations currently inhabiting earth. They estimated that if each species is divided into approximately 220 populations, then earth is occupied by between 1.1 and 6.6 billion populations. Unfortunately, if we assume that population extinction is a simple linear function of habitat loss, then we are losing 1.800 populations per hour (1.6 million annually). These terrifying data help us to understand how important and urgent the problematics of population loss is.

The apparently easiest way for having a good start of a conservation focused PhD project was to analyse the genetic variability of the D-loop control region of the mitochondrial DNA (mtDNA) of 30 otters from the endangered Danish population: The first DNA-based genetic marker system that could be routinely applied to surveys of genetic variation in natural populations was animal mitochondrial DNA (mtDNA). This molecule was amenable to analysis by methods that were widely available in the early 1980s for manipulating plasmid DNA. mtDNA sequence differences could be detected as restriction fragment length polymorphisms (RFLPs) (Lansman *et al.* 1981). More recently, the introduction of the polymerase chain reaction (PCR) has nearly eliminated the need to directly purify specific DNA sequences. Improvements in DNA sequencing methods have made it feasible to determine the exact nucleotide sequence of amplified regions of mtDNA for large numbers of individuals (Murray 1989).

In many important aspects, mtDNA polymorphisms are quite different from allozyme polymorphism. Animal mtDNA is generally inherited maternally as a single linkage unit of about 15 kb (Lansman *et al.* 1981). Mutation rates are often higher for animal mtDNA than for nuclear genes (Vawter & Brown 1986), and there are generally many more detectable polymorphisms than there are for single allozyme loci (Avise & Lansman 1983). Perhaps most significantly, detailed characterization of sequence differences can be used to infer genealogical relationships and estimate divergence time (reviewed in Avise *et al.* 1987). The high mutation rate of the D-loop region was one of the principal reasons why we chose this technique, as we have *a priori* suspected that the genetic variability could be

low in the Danish population because of its small population size.

Mucci, N., **Pertoldi, C.,** Madsen, A. B., Loeschcke V. and Randi, E. 1999. Extremely low mitochondrial DNA control-region sequence variation in the otter *Lutra lutra* population of Denmark. Hereditas 130: 331-336.

Our first research approach started immediately with a frustrating result, in fact the fashionable idea of screening the mtDNA of one of the most endangered carnivores in Europe, the Eurasian otter, immediately gave a strange result: genetic variability nearly equal to zero in the Danish otter population. Therefore, given the high variability of the D-loop region found in other mammal populations (Vawter & Brown 1986), we could only hypothesize that an extreme recent population bottleneck had happened in the Danish otter population. This population bottleneck should have eroded the Danish otter genetic variability. As a logical consequence mtDNA variability might have been reduced to an even lower level than nuclear DNA variability, due to the smaller effective population size (N_e) of the mtDNA genome as compared to nuclear DNA (Avise & Lansman 1983). But that was not the case (see below). Furthermore, it has been suggested that the low variability which was found, could be due to the presumably low N_e/N ratio of the otters (Frankham 1995) and/or to the possibility that the Danish otter population structure resembles a metapopulation. Hedrick (1996) showed that certain forms of metapopulation structure can result in N_e that are only a small fraction of the census size. Direct evidence of selective differences among mtDNA haplotypes has however been found in *Drosophila* (Hutter & Rand 1995), hence we cannot also exclude the possibility that one or several selective events could have reduced the mtDNA diversity.

Frustrated by this result we attempted another approach and began to follow another fashionable stream: a QG approach applied to a craniometric investigation of the Danish otter skulls: Craniometric analyses have been used for some time to provide information on relationships between populations (e.g. Manning 1971, Huson & Page 1980). A debate is still going on as to the relative importance of genetic and environmental influences on patterns of quantitative variation (Atchley *et al.* 1981). However, morphological similarity cannot be taken to indicate genomic similarity. Our investigation was further expanded by using another technique that consists in measuring an estimator of developmental stability (DS) at the individual or population level: the fluctuating asymmetry (FA) in bilateral traits. FA reflects small, random deviations from symmetry in otherwise bilaterally symmetrical characters (Ludwig, 1932; Van Valen, 1962, Leary & Allendorf 1989; Parsons 1990; Møller & Swaddle 1997). Minor deviations from perfect symmetry are thought to represent a direct measure of the ability of individuals to control development (Zakharov 1992, Møller & Swaddle 1997). Both environmental and genetic stresses are known to affect FA:

FA tends to become elevated under stress (Leary & Allendorf 1989). Stress factors known to raise FA include various chemicals including polluted habitats (Weiner & Rago 1987), pesticides (Valentine & Soulé 1973), extreme temperatures (Siegel & Doyle 1975, Sciulli *et al.* 1979) and food deficiency either in terms of quality or quantity (Parsons 1990). Also severe restriction of nutrients to mothers during pregnancy causes asymmetry in skeletal traits of offspring (Sciulli *et al.* 1979).

The genetic factors that can affect FA can be summarised into at least five categories: hybridisation that disrupts co-adapted gene complexes, loss of genetic variation, degree of protein heterozygosity, episodes of directional selection, and genes mutation (see Møller and Swaddle 1997 for a review). In summary, FA is apparently the easiest way to determine the degree of stress that a population is experiencing or has experienced (Møller and Swaddle 1997 and references therein).

We started our investigation with measuring several traits on otter skulls from the Natural History Museum of Aarhus and the Zoological Museum of Copenhagen.

Pertoldi, C., Loeschcke, V., Madsen, A. B. and Randi E. 1997. Developmental stability in the Eurasian Otter *Lutra lutra* in Denmark. Ann. Zool. Fennici 34: 187-196.

We found that the skull of the otters became more skewed and smaller with time. For females this tendency was not so evident as for males, suggesting a higher susceptibility of the males to stress. An attempt to correlate the contaminant level which was measured in the otter tissues with their skulls' FA gave no results, indicating that many other factors could have contributed to hiding this relationship, or just simply that contaminants do not increase FA in otters, even though they reduce the individual fitness, in the extreme case leading to sterility (Jensen *et al.* 1977). However, the lack of correlation between FA and contaminants concentration was an unfortunate result, as the urgent need for an economic technique that can quantify the effect of con-

taminants in nature is required. Agricultural and industrial pollution have had both localised and global effects. Long-lasting pesticides, such as DDT, become concentrated in terrestrial and aquatic food chains, and have endangered several birds of prey. About 2.5% of mammals of Australasia and the Americas and 4% of the endangered birds of the world are at risk from pollution (Groombridge 1992). These numbers underestimate the extent of morbidity mortality, and fertility impairment caused by pesticides in many non-endangered species. However, the previous investigation results are very interesting because they show that something had happened in the Danish declining otter population. Unfortunately, however, we could not say if FA had increased because of an erosion of genetic diversity and/or because of the deterioration of the habitat conditions. A continuation of the previous investigation was undertaken when we obtained the possibility of measuring skulls from a presumed healthy otter population, from an east German region (Lusatia).

Pertoldi, C., Madsen, A. B., Randi, E., Braun, A. and Loeschcke, V. 1998. Variation of skull morphometry of Eurasian otters *Lutra lutra* in Denmark and Germany. Ann. Zool. Fennici 35: 87-94.

In this investigation, the most important results were the findings that in both the Danish and Lusatian population, FA was negatively correlated with the male skull size (suggesting that the male skulls are under directional selection). This could be interpreted in the way that big body size (which is strongly correlated with skull size) in a male otter is a good indicator of its general condition and its fitness (see reviews in Møller & Pomiankowski 1993a, 1993b, Møller & Swaddle 1997). The positive correlation between body size and FA found in females, could then indicate the non-adaptive function of a big body size in females and the increased energetic cost associated with a bigger body size. The increased variance with time of Danish female skulls has been explained by a recent population bottleneck and/or increased environmental stress. In fact, a bottleneck can reduce variation in quantitative characters as a consequence of a reduction in additive genetic variance (V_a) (Carson & Templeton, 1984). From classical QG theory we know that if the assumption of the simple models hold, loss in heterozygosity should be mirrored by a proportional loss in V_a at the expected rate of 1/2Ne per generation. Hence, we should expect a reduction of the phenotypic variance (V_p), which in a constant environment can be considered a reliable estimator of V_a. However, if the variance expressed in a given character is due to non-additive interactions, such as epistasis or dominance, a bottleneck can increase the variance of QG characters (Bryant *et al.* 1986, Goodnight 1987). Bilateral symmetry can also be disrupted by founder events and inbreeding, leading to an increase in FA (Waddington 1957, Leamy 1984). There is also empirical evidence for increased V_p in presence of stressing factors (Imasheva *et al.* 1997). Therefore, we could not easily determine the causes of the increased V_p. Some considerations were also done about the discovery of a strong reduction with time of the degree of sexual dimorphism of the skull length between the Danish males and females (no significant reduction of the sexual size dimorphism was detected for the Lausitzian population). The sexual size dimorphism has been suggested to have some consequences on otter diet, with an increase in dietary overlap between sexes (intersexual competition) because of the reduction in male size that have reduced the male hunting capacity to bigger prey (Lynch & O' Sullivan 1993). At the same time the possibility of an increase in interspecific dietary competition with smaller sympatric Mustelid species like the American Mink *Mustela vison* was also suggested.

After these results we put all our efforts into the FA studies. We obtained the data of all the available skulls of otter, which have been collected in Europe during the last two centuries. We found the same patterns as were found for the Danish and the Lusatian otter populations: increased FA and skull reduction with time (especially in male skulls) detected in the presumed endangered populations and non significant changes in the presumably healthy population:

Pertoldi, C., Loeschcke, V., Braun, A., Madsen, A. B. and Randi, E. 2000. Craniometrical variability and developmental stability. Two usefull tools for assessing the population viability of Eurasian otter *Lutra lutra* populations in Europe. Biol. J. Linn. Soc. 70: 309-323.

It was speculated about the possibility that a reduced sexual dimorphism was produced by a relaxation of the selective pressure, because of a reduced density of individuals. However, some other possibilities have been discussed: for example Frankel & Soulé (1981) suggested that a 10% increase in the inbreeding coefficient may result in a 10% reduction in traits closely associated with fitness such as lon-

gevity, fecundity and body size. Furthermore, the possibility of genotype-environment interactions was also discussed. Turelli (1988) has argued that the dynamics of quantitative genetic parameters cannot be predicted only from models of maintenance of heritable variation, because genetic variances, covariances and correlations, for example, can change with environmental conditions if there are genotype-environment interactions.

Habitat destruction and fragmentation restrict dispersal, and eliminate for many species the most important mechanism for population persistence in response to long-term climate alterations: change of geographic distribution. We already know that in response to previous periods of global warming and cooling associated with glacial cycles, species often changed their geographic range while maintaining essentially the same phenotype except perhaps, for changes in body size (Coope 1979). The fragmentation phenomenon could apparently not look so dangerous for the otters: as they have shown in the previous investigation to have preserved a high phenotypic plasticity, despite their low genetic variability this evolutionary strategy should allow this Mustelid to survive also in suboptimal habitats and to quickly adapt to environmental change. Changes in the creode (initially under the influence of changes in the conditions of development and then at the expense of rearrangement of the genotype) could be considered to be a possible pathway of evolutionary transformation known as the "Baldwin effect" (Shiskin 1992). There is however, a maximum rate of directional and random environmental change that a population can tolerate by adaptive evolution without becoming extinct, depending on the amount of genetic variability it can maintain. Rapid extreme environmental changes, such as anthropogenic global warming, will place a premium on genetic variability and adaptability of many populations in fragmented environments during the coming centuries. Therefore, we cannot ignore the fact that current rates of environmental change are causing extinction several orders of magnitude faster than any mass extinction in the past (Lynch 1996).

The general thought that FA could be used to discover some of nature's hidden patterns became more and more exiting, and suddenly another opportunity appeared; a big sample size of skull of the striped dolphin (*Stenella coeruleoalba*) which have been collected in the Mediterranean basin became available for measuring:

Pertoldi, C., Podestá, M., Loeschcke, V., Schandorff, S., Mancusi, C., Nicolosi, P., Marsili, L. and Randi, E.. 2000. Effect of the 1990 die-off in the Northern Italian seas on the developmental stability of the striped dolphin *Stenella coeruleoalba* (Meyen, 1833). Biol. J. Linn. Soc. 71: 61-70.

A big bulk of the striped dolphin skulls stem from a period in which a big epizootic has collapsed its Mediterranean population, therefore we were presented with a unique opportunity. To see if the individuals found dead during the epizootic have a higher FA as compared to the individuals which have been collected before and after the epizootic. In fact, if we could find higher FA in the epizootic period skulls, we could conclude that FA is a really good indicator of the overall individual's condition and disease susceptibility, as we expected that during an epizootic, the individuals which will die should be those in worse health conditions. However, when looking with more attention to the data, we discovered that the mean FA of the post epizootic period was lower than the mean FA of the epizootic period, but was also significantly lower than the mean FA of the pre-epizootic period. Epizootic could therefore have had a selective purging effect on the dolphin population, which could mean that FA could have an hereditary component. If part of FA is hereditable it will of course be dangerous to try to correlate FA only with the environmental factors. Low level of heritability also helps to confirm the assumption that FA can be used as an indicator of environmental conditions. If FA is hereditable, it would not provide a reliable bioassay. For FA to have evolutionary significance it must be related to fitness and have a heritable basis. The heritability of FA is currently the subject of some debate (for conflicting views see Leamy 1997a, Leamy 1997b). However, most researches agree that FA often has a heritable component, but that this is typically small (Fowler & Whitlock 1999). The dolphin investigation was also interesting as it contrasted the idea of Pape Møller that also the trait with a directional asymmetry (DA) can be used for monitoring the environmental stress, as the striped dolphin skull traits which shows that DA did not follow the patterns showed by the traits with true FA. Also in this investigation we attempted to correlate the contaminant level which was measured in the dead dolphins, with their skull FA, but despite the extremely large number of toxicant isomers which was used in our correlation tests no relationships were found.

Then we got into another interesting FA debate: how much can the genome variability play a role in determining the DS of an individual? The fitness

advantage of heterozygosity has been proposed to be the primary device that maintains genetic diversity in natural populations (Lewontin 1974), yet the mechanism behind this advantage is still poorly understood and is a topic of much debate (e.g. Houle 1989, 1994). From an empirical point of view, many researchers have suggested a positive relationship between protein heterozygosity and suggested fitness-indicators, such as growth rate (Zouros *et al.* 1980). Heterozygosity has also often been associated with high feeding rates (Garton 1984, Garton *et al.* 1984) and increased viability (Farris & Mitton 1984). Therefore, there are some data that support the idea that heterozygotes have a general fitness advantage over homozygotes, and also that the link between heterozygosity could be causal as enzyme heterozygosity increases metabolic efficiency (Garton 1984, Garton *et al.* 1984). Therefore, if all individuals have the same energetic input, highly heterozygous individual lower routine metabolic costs should leave more energy to invest in growth, reproduction, or any other activity (Mitton & Grant 1984).

It is not clear how heterozygotes apparently gain this fitness advantage over more homozygous individuals. It may be related to differences in metabolic and enzymatic efficiency. Mitton (1995) has suggested that it may be related to differential rates of protein turnover between homozygous and heterozygous individuals. There have been numerous findings to indicate that individuals that are more heterozygous at enzyme loci show less deviation from the phenotypic mean (Livshits & Kobyliansky 1984) and are usually less asymmetric than more homozygous individuals from the same randomly mating population (Leary *et al.* 1983, Zouros & Foltz 1987). It was Lerner's (1954) initial model that first predicted an inverse relationship between heterozygosity and V_P. He surmised that heterozygotes can produce a greater variety of biochemical products and hence can canalise their development better under a wide range of environmental conditions. As FA represents a measure of developmental homeostasis, and heterozygotes have a canalisation advantage, we may expect to find a negative relationship between heterozygosity and FA.

In order to test some of the aforementioned cited hypotheses we chose for our investigation a peculiar insect, the stick insect (*Bacillus rossius*):

Pertoldi, C., Loeschcke, V. and Scali, V. (2001): Developmental stability in sexually reproducing and parthenogenetic populations of *Bacillus rossius rossius* and *Bacillus rossius redtenbacheri*. Evolutionary Ecology Research, 3:1-15.

Measuring FA in two sub-species of parthenogenetically and totally homozygotic stick insects compared to the sexual populations of the two sub-species, has shown to be a significantly higher FA in the totally homozygotic populations indicating that the homozygotic populations are much more unstable as compared to the sexual equivalent. Unfortunately, the sexual populations do not live in sympatry, but the parthenogenetic populations of both sub-species live in a more degraded environment as compared to the sexual ones. Therefore, we could not estimate the relative contribution of the genetic and environmental factors, to DI. Furthermore, our results did not support Lerner's conjecture. The reasons why we did not find Lerner's expected pattern, could be that body size in the two sub-species *rossius* and *redtenbacheri* is a sexually selected trait, and therefore is under directional selective forces that could have confounded the expected pattern, or more simply the relationship between FA and heterozygosity is too weak. Furthermore, we should expect that the more asymmetric parthenogenetic females of the *redtenbacheri* sub-species should also show a higher V_P, but that was not the case. These findings support therefore Waddington's hypothesis (Waddington 1957), who suggested that separate mechanisms are responsible for the effect of stress on V_P and on developmental instability (DI). The results obtained in this investigation allow us to reflect about the costs associated with a reduced heterozygosity which can typically happen in small populations in drift-mutation balance. In a crowding experiment with laboratory *Drosophila* the abiotic stressing factor was the reduced food amount. This laboratory experiment could give us some indications about the possibility of detection in natural periods of food shortage or overcrowding:

Bundgaard J., **Pertoldi C.** and Lefranc A. Larval crowding and developmental stability of wing size in *Drosophila melanogaster* Meigen (in prep.).

Some clear patterns were found: individuals which were grown under more crowded conditions showed higher FA as compared to those which were grown under more optimal conditions. Furthermore, at both

crowding levels, a significant reduction in wing size and an increase in FA and V_p were found for flies emerging during the late eclosion stage as compared to those which eclosed during the two earlier stages. The increased V_p also opened another interesting debate: In a sexually reproducing population ($V_g > 0$), V_p is given by: $V_p = V_g + V_{env} + (GxE) + cov(GE) + DI$, where (GxE) is the genotype environment interaction and cov(GE) is the covariance between genotypic and environmental sources of variance. The interaction term expresses the extent to which genotypic variants differ in their sensitivity to environmental effects. The cov(GE) has long been recognised as a confounding source of experimental error (for instance, when the fastest-growing animals are given the best diet). This term (cov(GE)), probably contributed to the increased V_p together with the increased DI. Also in this investigation we run into a big problem: the dependence of FA on the size of the trait. The trait measured (wing length) showed an extremely high phenotypic plasticity in these experiments, creating several problems with FA indexes and phenotypic variability indexes.

Incapable of separating the two components (genetic and environmental) which contemporarily affect FA and V_p and aware of the confounding effect that V_g and its interactions (GxE and cov(GE)) can have in QG investigations, we tried to find laboratory populations in which all organisms were genetically identical, in order to measure only the environmental components effect:

Pertoldi, C., Kristensen, T. N. and Loeschcke, V. A New Method for Estimating Environmental Variability for Clonal Organisms, and the Use of Fluctuating Asymmetry as an Indicator of Developmental Instability. Journal Theor. Biol. (in press), and an unpublished report using as a model organism the clonal collembola *Folsomia candida* is enclosed in this PhD dissertation.

This investigation, is probably one of the most important results obtained in this PhD project as we discovered a new method to quantify and partition out the environmental components and calculate the effect that V_{env} has on FA and V_p,. Furthermore, it emerged that despite the attempt to minimise the environmental variability we detected in 1/3 of the samples in this experiment an uncontrolled environmental component, which should alert us about the general thought that laboratory experiments can be conducted under controlled conditions. In this investigation, a discussion of the two principal methods used to estimate DS has been undertaken. Waddington (1957) proposed that two processes ensure phenotypic constancy: canalisation and DS. The former is thought to ensure phenotypic constancy in populations in spite of genetic and environmental variation, whereas the latter is believed to operate in given genetic and environmental conditions. In populations, the easiest way to appraise canalisation may be by estimating inter-individual variance and DS by intra-individual variance, which is often estimated by the level of FA in bilaterally symmetrical organisms. The genetic bases of DS and canalisation are for the most part unknown even though an important number of studies have focused on this subject in the last few decades (see Wagner *et al.* 1997, for canalisation and Clarke 1993, for DS). No convincingly genetic mechanism for DS has been proposed until now. In contrast different hypotheses involving genetic and molecular mechanisms of canalisation have already been suggested. Thoday (1958) proposed that modifier genes could be responsible for the maintenance of the canalisation of a trait, and recent findings of heat-shock protein HSP-90 mutants in *Drosophila* have provided the first molecular evidence for such a mechanism (Rutherford & Lindquist 1998). Two principal methods are commonly employed for the estimation of DS or DI. Some studies use V_p (Zouros *et al.* 1980, Livshits & Kobliansky 1984), even if the estimate can be blurred by V_g and V_{env}, while other studies use FA (Møller & Swaddle 1997 and references therein). When utilising methods that are based on V_p as an estimator of DS, then situations that satisfy the criteria for detection of DS are incredibly rare. Populations have to be genetically homogeneous ($V_g = 0$) and there can be no variation in environmental conditions across the habitat range of species ($V_{env} = 0$). Furthermore, if a trait is highly canalised and the peak of the size-fitness function is very narrow, there will be a little range in trait size variance between populations, and differences will be difficult to detect. Given the fact that full life table experiments for all the species will be extremely time and money consuming and given the urgent need of obtaining as much information as possible about the stress responses of the largest number of species available, we have to consider alternative methods. One of these methods is to measure developmental stability (DS) of the species in presence of stressors. Unfortunately (for our purpose), natural populations generally comprise large numbers of genetically diverse individuals inhabiting environments, which are both spatially and temporally heterogeneous (Stearns 1992). Thus in order to credibly extrapolate from individuals to field populations, it is necessary to understand how genetic and environmental factors in the field po-

tentially modify responses measured in the laboratory. This means that the genetic basis for stress tolerance can only be properly assessed by separating and quantifying the effect of both genes and environment and, most importantly, their interaction on the expression of tolerance. However, when studying stress tolerance, individuals, even from the same population, may have different phenotypic responses. Therefore, new investigations should be conducted employing clonal organisms. What however emerged from our study is that even when eliminating the genetic variability we still have the problem of the within population V_{env}, which can alter the estimates of the population DS. We can therefore easily conclude that use of clonal organisms to test toxicity of contaminants is the easiest way to do it, and the possibility of partitioning out V_{env} with the methods we discovered could also increase the replicability of these tests.

We chose two molecular markers to investigate the genetic structure of the Danish badger population: We know that finest resolution is achieved at the level of the family by techniques such as DNA fingerprinting (Jeffreys *et al.* 1988), but the badger has proven to be a frustrating stumbling block for several groups attempting to use this technique, However, the badgers are a good example of a species in which population size is large, but genetic variability is inexplicably low also at the minisatellites level. The two principal reasons of the low genetic variability are probably those previously cited: a metapopulation dynamics and/or a low N_e/N. Unaware of the results obtained by the other groups, we decided to try the minisatellites which are markers with close to 100% heterozygosity. Heterozygosity could therefore not be used as an index of genetic variation, however, heterozygosity is not the only way to measure genetic variability, and other measures such as the total number of alleles can be more sensitive to the effects of population decline (Leberg 1992). The process of allele loss is seen most dramatically in the complex, bar-code like banding patterns of DNA fingerprints.

Pertoldi C., Loeschcke V., Madsen A. B., Randi E. and Mucci N. Effects of habitat fragmentation on the Eurasian badger (*Meles meles*) subpopulations in Denmark. Italian Journal of Mammology (in press).

The minisatellites investigation show some interesting patterns but unfortunately, the extremely high freshness of the tissues which was required for making the fingerprints have limited the sample size available for the investigation. We therefore decided also to perform an allozyme investigation, as some genetic variability using electrophoresis has been reported for the British badgers (Evans *et al.* 1989).

Pertoldi, C., Loeschcke, V., Madsen, A. B., and Randi, E. 2000. Allozyme variation in the Eurasian badger *Meles meles* in Denmark. Journal of Zoology, London. 252:14-17.

The conclusions of the two previous papers regarding the genetic variability of the Danish Eurasian badgers is that the Danish badger populations are highly fragmented at the regional level: The reasons can be several, however, we can suspect that roads and intensive agricultural practice are the primary consequence of the territory fragmentation. Population subdivision substantially reduces the correlation in environmental stochasticity among localities. For example, when considering one large continuous reserve *versus* several small distant reserves of the same total area, subdivision can increase the mean time of extinction (T). Thus in a case when single populations are subject to major catastrophes, occurring randomly among populations, then population subdivision can clearly be advantageous for persistence. Subdivision can also increase persistence in the presence of catastrophic epidemics, not only by reducing the transmission of epidemics among localities, but in some cases by reducing their frequency because many epidemics require a threshold density. In the long term, however, at least for the small and isolated population in a mutation-drift balance, the accumulation of deleterious mutations will be dangerous. What is probably a major threat for the Danish badger population is the harvesting effect of traffic accidents which is estimated to eliminate around 10% of the Danish badger population every year (Åris 1995). Unexploited vertebrate populations fluctuate already through time with coefficients of variation in the range 20% to 80% or more (Pimm 1991). Exploited populations (such as the Danish badger populations) are also highly variable (Myers, Bridson & Barrowmann 1995) due not only to environmental stochasticity, but also because of the constant-rate harvesting due to road accidents which tend to reduce population stability (May *et al.* 1978). The traffic harvesting effect, together with the already present environmental stochasticity effects, could be the primary reasons for the low genetic variability found in the Danish badger population as compared to the genetic variability found in populations in other countries where the road fatalities harvesting effect is not so strong. Furthermore, demographic stochasticity can create a kind of Allee effect, which is a phenomenon particularly common

and relevant for populations with a highly complex social structure as the badger structure is. In populations below certain low treshold, most population trajectories tend to decrease, resulting in a high probability of extinction (Lande 1998). Individuals may suffer reduced fitness from insufficient cooperative interactions with conspecifics. Positive assortative matings, should however not constitute a problem for the badgers, as inbreeding is usually deleterious in species that normally outbreed, whereas, when inbreeding is part of the natural social system of a species, the genetic load is usually low and inbreeding depression is far less severe. The Eurasian badger has a restricted male juvenile dispersal (Cheeseman *et al.* 1987) and forms highly stable social groups (Kruuk 1978). Therefore, one may expect that the demographical consequences of fragmentation outweigh the genetical consequences of finite population size supporting the contention (Gilpin 1987) that ecological effects may be more significant than genetic effects, at least in the short term, whereas in the long term the genetical consequences will be increasingly important.

The biggest problem of the previous allozyme investigation is that only one locus was variable and the confidence of Wright's statistic which could be attached was therefore also low (however, see below a discussion of the interpretation of F_{ST}). We are, however, a little bit concerned about the fact that what we have detected at the molecular level in our badger investigation, is probably a more optimistic picture than it really is. In fact, we would underline the possibility that the genetic data that we can obtain at the present time could be further confounded by the refugee effect: When habitat is lost, individuals of many species are able to flee and restablish themselves in other sites. Such cases have been studied with refugee models of genetic population structure. The general effects are a reduction of the genetic differentiation among demes (F_{ST}), and an increase of the additive genetic variance (V_a) within every deme. These effects accumulate for as many generations as habitat continued to be lost (Porter 1999). Furthermore, we cannot exclude further subdivision of the subpopulations we have defined in our investigations. A low dispersal rate, on the order of few individuals exchanged among populations per generation, is sufficient to prevent much genetic differentiation at quasi neutral loci, such as most molecular genetic polymorphism (Crow & Kimura 1970). In contrast, adaptive differences among populations can be maintained by natural selection even under a high level of dispersal and gene flow (Endler 1977). Lack of differentiation or low differentiation among populations at molecular genetic loci does not imply lack of adaptive differences. It could therefore be a serious mistake to manage populations in different environments as a single unit, simply because low or no molecular differentiation among them has been detected, especially if morphological, behavioural and physiological characteristics in which the populations might be adaptively differentiated have not been investigated.

The molecular investigations helped us in understanding how the Danish badger population structure is organised at the macrogeographical level, but, unfortunately no clear information was obtainable at the microgeographical level due to the low genetic variability which was found, which for example does not allow us to build a pedigree of a clan. At present days a work using microsatellite primers specifically built for badgers, is in progress but also here the variability looks too low for microgeographical investigation. Any conclusion about the size that a patch should have in order to sustain a stable population could therefore not be drawn from our molecular investigation. However, we know that the rate of dispersal into unsuitable regions determines the minimum size of a geographically isolated patch of suitable habitat, known as the critical patch size. With random dispersal, lethal surroundings, and a low intrinsic rate of increase (which is low for badgers), the critical patch size is much larger than the average individual dispersal distance (Kierstead & Slobodkin 1953). The genetic investigations indicated a low genetic variability, which supports the fact that there is isolation among patches, due to increasing fragmentation of the landscape. However, also the need of knowing the past history of a population could help us to plan future conservation efforts. Until a few years ago, the only available information of the past history of a population could be obtained from the hunters, but most of the time we were dealing with anecdotal knowledge, which does not go back more than 50-70 years, whereas the new PCR techniques allow us to obtain data which would have been impossible to obtain in other ways. In the next paper, we describe how modern genetic techniques may be applied to extract and examine the DNA of individuals that have been dead for many years. Theoretical studies suggest that DNA is unlikely to survive intact more than about 100,000 years. However, even over this time period, the evolutionary questions that can be addressed are far reaching and include systematics, paleoecology, the origin of diseases, and evolutionary processes at the population level. The use of genetic data has greatly expanded evolutionary studies of extant organisms, with one major limitation: History must be reconstructed by extrapolation from current genetic patterns rather than directly observed in the fossil record. Population genetics cannot deal well with the confounding effects of natural selection, genetic drift, gene flow, and population history without resorting to simplified

models and assumptions (Templeton *et al.* 1995). Systematists utilize character state data and phylogenetic methods to reconstruct evolutionary history, but this approach is fraught with problems caused by character reversals and parallelisms (Swofford *et al.* 1996). The questions addressed by ancient DNA research concern any issue that benefits from a direct historical perspective. These areas include (*a*) systematics (Höss *et al.* 1996); (*b*) changes in genetic diversity as a function of time and environmental change (Hardy *et al.* 1995, Thomas *et al.* 1990); (*c*) migration and admixture (Roy *et al.* 1996) and (*e*) ecology and paleoecology (Kohn & Wayne 1997). Reconstructing the past population relationships of endangered species is also important for reintroduction programmes and therefore Museum collections have become a source of DNA from populations that are difficult to sample or are extinct.

Pertoldi, C., Hansen, M. M., Loeschcke, V., Madsen, A. B., Jacobsen, L. and Baagoe, H. Genetic consequences of population decline in European otter *Lutra lutra*: An assessment of microsatellite DNA variation in Danish otters from 1883 to 1993. Proc. Royal Soc. B. (in press).

Despite the fact that microsatellites evolve in an unexpected fashion that is likely to confound their use in measuring genetic distance (see below), the results obtained in the previous investigation seem to be extremely interesting. Pairwise F_{ST} values and assignment tests showed small but significant genetic differences between the present population and extinct otters from other regions in Denmark (but see below the discussion about the reliability of F_{ST}). Another interesting observation is that the Danish otter population did not seem to have undergone a population bottleneck as has been previously suggested, also if some rare alleles have been lost with time (but see below the discussion about the importance of preservation of rare alleles for conservation purposes). The method applied for detecting a bottleneck is based on the fact that following a bottleneck (lasting less than 2Nb generations, where Nb is the effective population size at the time of the bottleneck) a population will show a higher level of apparent heterozygosity than expected, based on a population in mutation-drift equilibrium (Cornuet & Luikart 1996). This is due to the sudden loss of rare alleles at the time of the bottleneck, and the resulting deficit of alleles relative to the number of heterozygotes. However, the detection of a bottleneck with molecular markers, has a limitation, as it requires the screening of several loci and several individuals. Furthermore, to loose a large fraction of its genetic variance measured by heterozygosity in molecular genetic polymorphism or heritable variance in quantitative characters, a population reduced to a small effective size must remain small for at least $2N_e$ generations. This fact further reduced the possibility of detecting a strong population reduction especially if a rapid population recovery has followed the bottleneck. It is however, still unclear whether biologically plausible population bottlenecks will have been sufficiently severe to be detectable using current techniques. It is therefore possible that measurements of FA and V_p can be considered to be more sensitive in order to detect population reductions, also if they are both contemporarily affected by environmental and genetic components.

Conclusions

Our general conclusion based on all the investigations which have been presented in this PhD dissertation is that when approaching a conservation project, several techniques (different molecular techniques, different QG analyses) should be employed. Population genetics remains a cornerstone of conservation biology, however, several hypotheses have to be tested, trying to avoid *a priori* assumptions and having a spatio temporal vision of the conservation problematiques which are going to be approached. We tried to find some answers to our questions from the results obtained in our investigations and from the progress made by other groups of researchers during the last years:

How important is inbreeding depression for the endangered populations?

It is often assumed that by measuring current levels of heterozygosity, the genetic "health" of a population can be measured. But we have to distinguish two different kinds of threats: first, there is a threat posed by loss of genetic variability, a danger that is thought to be manifested in terms of lowering a population's ability to adapt to novel threats, such as pathogens, parasites and competitors (O'Brien 1994). Quite separate are the detrimental effects of inbreeding depression. Here, population size reduction creates a founder effect in which rare deleterious recessive traits have the opportunity to change in frequency. While many of these recessives are lost, some may drift to high frequency and become expressed in the homozygous state, causing a reduction in individual fitness (Saccheri *et al.* 1996). There are two important problems, which complicate any attempts to infer risk of inbreeding depression from measurements of genetic variability. First

loss of variability and inbreeding depression affects populations at different times and exert their influences over very different time scales. While loss of variability through neutral genetic drift takes place over many generations, the effects of inbreeding depression tend to be felt at, or soon after, the population declines (Keller *et al.* 1994). Consequently, immediately after a population crash there may be severe inbreeding depression even though little variability has been lost. This increase in homozygosity, coupled with an increased expression of deleterious recessive alleles, often leads to fitness and viability reductions. What could be more determinant in small populations, is the incapacity of a response to the selective forces as they are mainly governed by a drift-mutation balance, and when the chance of accumulation of deleterious alleles is higher.

How important is it to plan long term conservation strategies?

Some serious doubts are beginning to arise about the long term conservation strategies. In fact, even the population sizes necessary to allow for an adaptive response to future environmental changes are likely to be far smaller than those necessary to protect populations against ecologically driven extinction in the face of typical levels of environmental variability. Also the question about how large populations must be to retain evolutionary potential is still under debate, with N_e fluctuating from 500 to 5,000 (see Franklin & Frankham 1998 for discussions), however also an N_e of 500 is often an unlikely number to maintain for endangered populations. In addition, there are several reasons to doubt that loss of genetic diversity will cause populations to become endangered, although lack of genetic diversity may increase the threat to populations that are already endangered (Holsinger & Witt 1997).

How important is preservation of rare alleles?

We have seen in the last investigation that the Danish otter population has lost some rare alleles with time, but rare alleles contribute very little to variation in fitness among individuals and have little to do with any immediate response to natural selection. Fisher's fundamental theorem of natural selection shows that the response of any population to natural selection is directly proportional to the V_a in fitness. Alleles occurring in moderate frequencies contribute most to determining the amount of V_a present, because genotypic differences among individuals within a population are accounted for primarily by differences in those alleles.

Which are the limits and advantages of molecular marker investigations as compared to the QG ones, and which are their applications?

The level of genetic diversity can be assessed investigating non-metric and metric morphological traits, random amplified polymorphic DNA (RAPD), microsatellites, allozymes, minisatellites and sequencing of mitochondrial and nuclear DNA (Avise 1994). All these techniques serve as suitable surrogate for estimating adaptive genetic diversity and population genetic structure, but sometimes molecular surveys must be misleading, as there are several good theoretical reasons to doubt that a strong connection will normally be found between levels of molecular and adaptive genetic diversity within populations.

Population genetic processes governed by the mating system, genetic drift and gene flow are expected to affect the major part of markers in the same way leading to similar genetic structure, however the impact of selection may be different. The questions which the different molecular techniques can answer are however different: Can the genetic variability at the molecular markers really be considered as a good estimator of the N_e? As several factors contribute to the relationship between genetic variation and population size in wildlife (Frankham 1996), no real confident conclusions can be drawn about the size of a population which has been investigated for genetical diversity. However, new methodologies have been developed (Schwartz *et al.* 1999). The relationship between genetic variation and N_e should be strongest for neutral genetic markers and poorest for the most strongly selected markers; non-coding nuclear DNA should show the best relationship, followed by allozymes, QG genetic variation for peripheral characters and mtDNA, with QG variation for reproductive fitness characters showing the weakest relationship. Hence, we can easily see why microsatellites loci are increasingly used in population genetics studies as most are neutral and highly polymorphic which make them suitable for the N_e estimates (Brudford & Wayne 1993). Microsatellites are tandemly-repeated simple sequences, usually less than 100 base pairs altogether and with di-tri or tetranucleotide repeat units. Polymorphism is manifested as different numbers of repeats, probably generated by a mixture of stepwise mutations and occasional deletions, limited by an upper reflecting boundary. Although some microsatellite loci occur in expressed regions of the genome and are thus susceptible to selection pressures, the overwhelming majority are located outside these areas. Another logical question is if genetic variation at the molecular markers reflect the QG variability. But, the evolutionary dynamics of molecular markers and adaptative traits are likely to be quite different simply because the rate at which new variants are introduced is also quite different (Lynch 1996). Nucleotide se-

quence and restriction-site variants are introduced at a rate of 10^{-8} to 10^{-5} per generation. New polygenic variants, on the other hand, arise at a rate of 10^{-4} to 10^{-3} per generation. Hence V_a in polygenic traits will rebound much more rapidly following a population bottleneck than will diversity of molecular markers. The fact that molecular marker loci will provide little insight into conditions at loci underlying adaptive variation unless a large fraction of the former are tightly linked to the relevant quantitative-trait loci, make the lack of molecular divergence among populations uninformative, as it cannot exclude local adaptations. Large quantitative trait variation has been found even when levels of gene flow, estimated with allozymes is high (Loughee & Handford 1992). This fact has important implications, in fact, conservation biologists assign population distinctiveness by classifying populations as Evolutionary Significant Units (ESU is a population that merits separate management and has a high priority for conservation). Historically, this classification has included both ecological and genetic data, but because of an increasing interest for the molecular technique the new criteria for the determination of an ESU are based exclusively on molecular data. This is a clearly wrong approach which has to be changed with the reintroduction of the classical morphological investigations. Hence, together with the classical Wright's F-statistics, other QG indexes should be employed. Differences in population means of the traits can be tested with an ANOVA for single traits, or MANOVA for multivariate analysis. Alternatively a Wilk's lambda, which is sensitive to differences in mean and in the ratio of the characters can provide a more clear separation among populations. Furthermore, other two indexes, the average taxonomic distance and Mahalonobis distance (D^2) (Manly 1986) can also be employed. However, we must bear in mind that also natural selection on a polygenic trait can be weak, in fact selection on QG variation is weak for characters peripheral to reproductive fitness itself (Falconer & Mackay 1996). For an N_e smaller than few hundred individuals, the expected amount of variation for a quantitative character is nearly independent of the strength of selection and is largely a result of mutation-drift balance. In fact, in polygenic characters the forces of selection are distributed over a large number of loci, rendering the selective forces on specific loci small enough to be overwhelmed by random genetic drift. However, if N_e increases, the equilibrium level of genetic variance will gradually switch towards a selection-mutation balance.

QG investigations, can also help us to understand which selective pressures are acting or have acted on the populations we are investigating. Some general patterns of how different characters behave in presence of different selective pressures have been found (Møller & Swaddle 1997). For example, secondary sexual characters have been hypothesised to demonstrate higher V_p between and within individuals as compared to ordinary morphological traits. Phenotypic variation is generally reduced in the sex with more intense sexual selection (males) (Pomiankowski & Møller 1995). Long-term directional selection would produce an increase in both the number of genes and the average effect of each locus on the trait. Traits subject to long-term stabilising selection would demonstrate the opposite effect, restricting the effect of environmental and genetic factors on the expression of the genotype (Pomiankowski & Møller 1995).

The phenotypic coefficient of variation is strongly positively correlated with the additive genetic and the residual coefficient of variation for secondary sexual characters (Pomiankowski & Møller 1995). Hence, traits with a high degree of V_p also have a high degree of genetic variation. Empirical studies of phenotypic variation in secondary sexual characters and ordinary morphological traits, elucidating the relative strength of sexual and natural selection, could thus contribute considerably to an understanding of the factors involved.

We have to introduce another important index for conservation biologists: the traits heritability (h^2). The h^2 of a trait is the fraction of the V_p that has an additive genetic basis. h^2 has a zero-to-one range and it can be viewed as the efficiency with which a population responds to natural selection.

It is clear from the review made by Fowler & Whitlock (1999) that the large scale declines in h^2 predicted in classic theory (due to loss of V_a) are not incompatible with small scale increases in V_P as seen in studies of FA. One of the simplest short-term conservation strategies is to try to maintain a certain level of h^2 (as adaptive responses to future environmental conditions will often have a polygenic basis). Two main factors govern h^2, V_a and V_{env}, which are not unlinked, as an increased homozygosity leads to increased susceptibility to V_{env}, which in turn then will also contribute to a loss of h^2. Hence, there are two main possibilities in order to increase h^2: increase V_a or decrease V_{env}. We know how we can increase V_a, but we do unfortunately not really know how we can reduce V_{env} in nature. But some interesting laboratory experiments could be done, for example by measuring

how V_{env} behaves in presence of different biotic and abiotic components. Unfortunately, both the quantitative parameter and molecular variation estimates are subject to a big statistical sampling error. But despite that, more importance should be given to the QG investigations, also if the QG investigations are limited by the fact that when phenotypes are a function of multiple genetic and environmental effects, it is essentially impossible to ascertain an individual genotype from its outward appearance. However, QG studies provide a basis for identifying sources of variation contributing to individual differences.

A separate discussion apart should be devoted to the allozyme techniques: The traditional allozyme investigation has the advantage or disadvantage that a great deal of protein polymorphism is presumably adaptive (Nevo 1983) and therefore, differences in genetic diversity may also be due to a variety of ecological factors. Hence, the possibility of the use of non neutral markers to detect local adaptation could be utilized, however several problems are afflicting the allozyme techniques: The molecular nature of allozyme alleles is critical to understanding selection acting upon them. If alleles are mixtures of many segregating and recombining amino acid polymorphisms that only share isoelectric points or net charge, then their study in the absence of sequence input will be problematic. One would like to treat individual electromorphs as mutationally unique, but this may not be realistic, and the charge-state model predicts that loci with many electromorphs will possess intraclass mutational heterogeneity. Furthermore, only a small proportion (30% or less) of the variation present at a locus is generally detectable by allozyme analysis, primarily because only differences generated by charged aminoacid residues are resolved by electrophoresis.

An additional problem which can be raised when using allozyme technique is the interpretation of the heterozygosity value (H). In fact, the H value can be determined at a few highly polymorphic loci on many individuals of a population which are heterozygous at largely the same loci. Or, alternatively, if H is based on many moderately polymorphic loci, the individuals of a population are largely heterozygous at different loci and recombination can create a wide array of phenotypes (Hartl *et al.* 1994). However, it is important to note that other factors may be more important if alternative definitions of the evolutionary potential are defined instead, as the maximum deviation possible from the current mean. Then allelic diversity may be a much stronger predictor of such potential than H (James 1971). Allozymes are however still considered an excellent technique when you want to assess the gene-flow among populations. Wright's island model of population structure was used to relate the distribution of allozyme alleles in populations to N_em, the product of the effective population size and the rate of migration. Alternative strategies for the estimation of gene flow has been developed using different genetic markers, different models of demography and population genetics, and different methods of parameter estimation. But no alternative strategy now available is clearly superior to the standard approach based on Wright's model and allozyme markers. However, this may soon change as methods are developed that fully utilize the genealogical relationships of DNA sequences. At present, alternative strategies do fill important needs. They can provide independent estimates of gene flow, measure different components of gene flow, and detect historical changes in population structure.

There is an obvious temptation to apply estimators based on familiar population genetic models developed for the F_{ST} allozyme approach to other forms of gene flow and other genetic marker systems. In some cases, the fit would not be bad, and the models would be expected to yield reasonable gene flow estimates. In other cases, the assumptions of these models would be severely violated and could result in very inaccurate estimates of gene flow. However, even where the standard models are not inappropriate, new models may provide more effective methods for the estimation of gene flow. But before we go into details we have to explain the nature of these markers: Two forms of nuclear sequence variation are widely used as genetic markers: base substitutions and variable numbers of tandem repeats (VNTRs). Base substitutions are more difficult to survey in populations, but offer a greater possibility of inferring genealogical relationships among sequences. VNTRs are relatively easy to survey in populations. They are often highly polymorphic, and length variation can be detected by simple electrophoretic methods. VNTR sequences are classified by size as microsatellites or minisatellites. Two mechanisms are expected to generate variation in the number of tandem repeats. Recombination is expected to generate a broad distribution of length changes, at rates that may be as high as 5×10^{-2} (Jeffreys *et al.* 1988, Brudford *et al.* 1992). Thus, in sequence comparisons, little correlation is expected between the magnitude of accumulated length differences and the number of unequal crossing over events that produced them. Replication slippage that occurs during DNA replication appears to favor small stepwise changes in the number of tandem repeats (Shriver *et al.* 1993,Valdes *et al.* 1993). The rates at which variants are generated by this process appears to be between 10^{-4} and 10^{-3} (Edwards *et al.* 1992, Weber & Wong 1993). Although the mechanisms that generate length variation in VNTRs are not well understood, the distribution of length

variation in microsatellite sequences is most consistent with the hypothesis of replication slippage (Tautz & Sclotterer 1994, Valdes *et al.* 1993). Recombination and related gene conversion processes appear to be responsible for variation in minisatellite sequences (Jeffreys *et al.* 1988, Jeffreys *et al.* 1994).

The exact form and magnitude of the amount of error in the estimation of F_{ST}, depends on the mutation process: For an infinite alleles model of mutation and an island model of gene flow: $F_{ST} = 1/(1+4N_e m + 4N\mu)$ (Crow & Aoki 1984) Thus, mutation has the same effect on F_{ST} as gene flow. For a stepwise mutation model, which may be appropriate for microsatellite loci (Schlotter & Tautz 1993, Valdes *et al.* 1993), the bias is greater (Slatkin 1995). This dependence of estimates of F_{ST} on mutation rates should not be important for allozyme markers, which have very low mutation rates. However, for DNA sequences with much higher mutation rates, this problem could be quite severe. If DNA sequence data are used, the problem of mutation saturation can be avoided by using any of several measures that effectively weight pairwise comparisons of sequences by the number of mutations that differentiate them (Nei 1982, Takahata & Palumbi 1985). More generalized methods for the use of weighted pairwise data in analysis of population structure have also been developed (Pons & Petit 1996). However, for mtDNA data, a cladistic measure of gene flow (Slatkin & Maddison 1989, Slatkin & Maddison 1990) may be preferable to an F_{ST} based estimate (Hudson *et al.* 1992). It is less obvious how to correct estimates of F_{ST} for the high mutation rates of microsatellite and minisatellite loci. A weighted pairwise distance measure (Goldstein *et al.* 1995) and a measure of population structure that is analogous to F_{ST} (Slatkin 1995) have been proposed for microsatellites that undergo a stepwise mutation process. These measures perform well in simulations, but further testing of the stepwise mutation model is needed before their reliability can be assumed. For minisatellites, an infinite alleles model may provide a reasonable approximation of random unequal crossover events and a large number of potential length states.

Much still remains to be learned both about how DNA sequences evolve and about how the patterns of variability being measured relate to higher level of characters such as fitness and population dynamics. Therefore, a lot of work still has to be done, before the conclusions obtained with our investigations can be reinforced or negated. However, there seems to be no question that the field of conservation genetics could profit from an influx of QG thinking, and that one has to accept that several markers have to be employed simultaneously when trying to estimate the genetic parameters.

Recommendations for future directions

1) Estimates of census size with genetic tagging

The attempt to estimate the census size with excrement tagging of the populations could be one of the investigations which could produce interesting and usefull results (Palsbøll 1999). In fact, as we have speculated before: for several species and populations the demographic stochasticity is a more immediate threat than genetic erosion, therefore the possibility of making a genetic tagging of excremental DNA can also allow us to quantify the effective number of individuals present in for example a patch, also if sometimes too low genetic variability could complicate the investigation (Palsbøll 1999). More detailed studies at a microgeographical level have also to be undertaken as it will allow us to obtain some more reliable estimates of the genetic differentiation and distance among populations. In fact, the classical models describe how genetic diversity can be partitioned into components that reflect genetic population structure in terms of inbreeding within subpopulations and differentiation between them (Wright 1978). However "random mating within subpopulations" is one of the fundamental assumptions when dealing with the classical model, which considers the species distribution as a set of semi-discrete subpopulations, each comprising males and females mating at random. It has long been appreciated that social structure which prevents random mating exists in many species. A good example could be the badgers we have studied.

2) Ancient DNA investigations

The possibility of amplifing ancient DNA from old museum specimens (Wayne *et al.* 1999) should also be utilised. As discussed before the need of knowing the past history of a declining or extinct population will sometimes help us to understand the reasons, the modality and the entity of the decline or extinction.

One extremely interesting experiment could be the genetic screening of all the European otter population skulls which have been measured in one of our investigations where we have detected a strong reduction of the skull size with time in the endangered populations. With the ancient DNA technique it could be possible to determine if the skull reduction was due to change in allele frequency due to selection within populations or to gene flows or just simply the result of phenotypic plasticity as we have previously suggested. This re-

search could be among the first to analyse genetic change in populations over a significant period of time. The museum material dates from the last century and during this time many avian and mammal populations have suffered substantial decline and fragmentation. The extensive collections of these taxa provide the opportunity to analyse large numbers of samples from throughout this period and directly document changes in genetic diversity. The results will improve our understanding of the historical dimension of population change, and provide important data for the interpretation of genetic diversity studies in conservation and evolutionary studies. By directly dating and quantifying changes in genetic diversity, the present work will allow examination of postulated causes of population decline, such as the intensification of agricultural practices and habitat loss. We could study microsatellite variation in species known from census data to be undergoing, or to have undergone, population bottlenecks at different dates in the past. Surveys of museum collections have revealed that suitable numbers of specimens exist for all the proposed study species, and preliminary studies have determined that microsatellite loci can be amplified from specimens throughout this time frame. Comparative data from modern populations are available for several of the species.

Furthermore, major questions remain about Late Pleistocene paleoecology, such as what were the genetic effects of the population fragmentation caused by climate change? Do the rapid morphological changes in isolated populations represent *in situ* evolution, or genetic replacement? How does the genetic diversity of modern populations compare to that in the late Pleistocenic environment? How does Late Pleistocene genetic diversity relate to palaeoecology? Until now it has not been possible to address these issues, but ancient DNA techniques could help us to obtain some answer to these questions studying mtDNA sequence variability in vegetation fossiles and museum specimens.

3) Laboratory investigations

The field investigation can however, only have a monitoring function because of the limitation in terms of sample size (at least when dealing with endangered populations), therefore we have to understand that the major part of the hypothesis we want to investigate has to be conducted under laboratory conditions. Toxicological tests employing clones could for example be important investigation tools in order to detect the effect of abiotic factors and their interaction with the biotic component. The quantification of V_{env} in presence of different concentrations of contaminants and at different individual density would also be interesting. In fact, as we have previously discussed, V_{env} can have strong influence on the population h^2.

4) Outbreeding depression investigations

Several efforts should also be devoted to investigations on outbreeding effects, but because of the difficulty of detecting in the field hybridisation, we would again suggest laboratory studies with for example *Drosophila* as a model organism. The need for knowledge about the effects of outbreeding is a matter of the most urgency, as translocations among populations and captive reared animal introduction plans in the immediate future will be one of the few conservation strategies possible. By breaking up coadapted gene complexes, hybridisation can lead to the production of individuals that have lower fitness than either parental type, and can even occur between populations that appear to be adapted to identical extrinsic environments. Evidence of outbreeding depression comes from observation of reduced fitness in inbreed lines of flies (Templeton *et al.* 1986), and plants (Parker 1992). It is common for the F1 progeny of interpopulation crosses to exhibit enhanced fitness relative to their parents (Lerner 1954), only to have a dramatic reduction in fitness to occur in the F2 generation (Wu & Palopoli 1994). In some situations the development of some negative consequences of mixing coadapted gene complexes can be more subtle, taking several generations to emerge fully (Lynch 1996). Two distinct approaches could be conducted: 1) Laboratory investigations (with *Drosophila* as study organism) making life-history tables and using DS as a surrogate of fitness indicators, which could be measured in several generations including F2 and F3 where we should have the maximal disruption of the coadapted gene complexes. A morphological study in order to detect changes in size or shape of the hybrids could also give useful information 2) Molecular investigations using a microsatellite based variable (d^2) which gives an estimate of H (which focuses on events deeper in the pedigree of an individual), could also be done. This new estimating method is expected to be more correlated with fitness than the rough H estimate is (Coulson *et al.* 1998), of course d^2 could also be used in inbreeding experiments.

5) h^2 changes in nature and in laboratory conditions and the influences of maternal effect.

The easiest but least accurate way to estimate h^2, is to compare individuals with themselves, that is to take repeated measures of the same trait on individuals. In this way only V_P due to temporary circumstances (the so-called special environmental

variance) can be separated from other causes of variation (genetic, that is additive and non-additive genetic, and general environmental causes arising from permanent circumstances). The resulting measure is thus a very crude estimate of h^2, it merely sets an upper limit to it. The most frequently used comparisons that give reliable h^2 estimates are parent offspring, full sib and half sib analyses. As previously said, h^2 changes with changes in V_{env}, but changes also with inbreeding. During inbreeding the V_a in populations declines and the phenotypic variation (V_p) between individuals is expected to loose its genetic basis. Variation between phenotypes becomes more determined by V_{env} (Falconer & Mackay 1996). This results in a loss of h^2 ($h^2 = V_a / V_p$), and potentially reduces the selective response of populations (Houle 1992; Lynch 1996). Assuming no non-additivity, and that inbreeding does not influence V_{env}, the expected h^2 is given by $h_t^2 = \{(h_0^2 (1-F)\}/(1 - h_0^2 F)$, where h_0^2 and h_t^2 represent the h^2 of the base population and the inbred population, respectively, and F is the inbreeding coefficient (Falconer & Mackay 1996). Because the inheritance and expression of the polygenic traits are controlled by a large number of genes (Brakefield *et al.* 1996), inbreeding is expected to reduce the h^2 of these traits. Hence, it is clear that h^2 is an important index which should be studied further. To this end, I will employ both lab and field studies using a variety of tools. Two goals of my work could be 1) to document the range of genetically based morphological variation within and among populations of a given species estimating the potential for future evolution, and 2) the study of adaptive phenotypic plasticity, especially maternal effects. One significant criticism of many quantitative genetic studies is that they are usually conducted in the laboratory and ignore the effects of natural environmental variation and breeding patterns in the population of interest. What is detected as heritable genetic variation in the lab may be obscured by natural environmental variation. Thus, under real world conditions, many important characters may have low or zero h^2, and may not be easily molded by natural selection. My future research programmes should therefore be focused on the attempt to develop methods to examine quantitative genetic variation in the wild. Statistical methods that use hypervariable molecular markers to infer a degree of genetic relatedness among individuals in wild populations, which are then in turn used to estimate the h^2 of quantitative traits, have been developed. This approach allows one to examine long-lived species in a wild setting without the need for any form of manipulation (other than to collect tissue for DNA fingerprinting). Also the role and the evolution of adaptive maternal effects, could be a future research. Empirical studies of life history variation (Dingle & Mousseau 1994) made it obvious that many traits expressed by offspring were the result of environmental influences experienced by mothers, suggesting that this special form of plasticity has evolved as a mechanism for adaptation to local environmental conditions. This special form of plasticity due to maternal effect can be very important for the survival of populations under changing selective pressures. Especially now that together with the fragmentation effect another threat is becoming increasingly important, the global climate change and its consequence on animal and plant morphology and phenology. These two phenomena have clearly a synergetic negative effect on populations as fragmentation reduces the possibility of migration, which is the only survival strategy possible if a population cannot adapt to the changing environment and global climatic change is too fast for the population, which does not have time to respond in an evolutionary manner to this change. Hence, phenotypic plasticity will play an important role in the future for the populations, and it is therefore essential to understand how spread this phenomenon is, with field and laboratory investigations using sexual reproducing and clonal organism.

In conclusion, we should like to stress that the arguments discussed in this PhD dissertation and the results presented are only the tip of an iceberg, the bottom of which represents a large amount of frustrating accumulated nill-results which have yet to be published. Furthermore, we should also stress that several works are in progress at the moment, but they have not been discussed in this PhD dissertation due to space limitation. What dolphins and banana flies have in common has opened our eyes to the world of possibilities available when applying modelling principles to the present knowledge of DNA and population genetics.

References

Atchley WR, Rutledge JJ, Cowley DE. 1981. Genetic components of size and shape. II. Multivariate covariance patterns in the rat and mouse skull. Evolution 35: 1037-1055

Avise JC, Lansman RA. 1983. Polymorphism of mitchondrial DNA in populations of higher animals. In: Evolution of Genes and Proteins. ed. M.

Nei, RK. Koehn. pp. 147–64. Sunderland, MA: Sinauer

Avise JC. 1994. Molecular Markers, Natural History and Evolution. New York: Chapman & Hall

Avise JC, Arnold J, Ball RM, Bermingham E, Lamb T, *et al.* 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Annu. Rev. Ecol. Syst. 18: 489–522

Brakefield PM, Gates J, Keys D, Kesbeke F, Wijngaarden PJ, Monteiro A, French V, Carrol SB. 1996. Development, plasticity and evolution of butterfly eyespot patterns. Nature 384: 236-242

Bruford MW, Hanotte O, Brookfield JFY, Burke T. 1992. Multi- and single-locus DNA fingerprinting. In: Molecular Analysis of Populations: A Practical Approach, ed. AR Hoelzel. pp. 225–69. Oxford: IRL Press

Brudford MW, Wayne RK. 1993. Microsatellites and their application to population genetic studies. Curr. Opin. Genet. Develop. 3: 939-943

Bryant EH, McCommas SA, Combs LM. 1986. The effect of an experimental bottleneck upon quantitative genetic variation in the housefly. Genetics 114: 1191-1211

Carson HL, Templeton AR. 1984. Genetic revolutions in relation to speciation phenomena: the founding of new populations. Ann. Rev. Ecol. Syst. 15: 97-131

Cheeseman CL, Wilesmith JW, Ryan I, Mallinson PJ. 1987. Badger population dynamics in a high density area. Symp. Zool. Soc. Lond. 58: 279-294

Clarke GM. 1993. Patterns of developmental stability in *Chrysopa perla* in response to environmental pollution. Environ. Entom. 22: 1362-1366

Coope GR. 1979. Late Cenozoic Fossil Coleoptera. Evolution, Biogeography and Ecology. Ann. Rev. Ecol. Syst. 10: 247-267

Cornuet JM, Luikart G. 1996. Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics 144: 2001-2014

Coulson TN, Pemberton JM, Albon SD, Beaumont M, Marshall TC, Slate J, Guinnes FE, Clutton-Brock TH. 1998. Microsatellites reveal heterosis in red deer. Proc. Royal Soc. B. 265: 489-495

Crow JF, Kimura M. 1970 Introduction to population genetics theory. New York: Harper & Row

Crow JF, Aoki K. 1984. Group selection for a polygenic behavioral trait: estimating the degree of population subdivision. Proc. Natl. Acad. Sci. USA 81: 6073–77

Dingle H, Mousseau TA. 1994. Geographic variation in embryonic development time and stage of diapause in a grasshopper. Oecologia 97: 179-185

Edwards A, Hammond HA, Caskey CT, Chakraborty R. 1992. Genetic variation at five trimeric and tetrameric tandem repeat loci in four human population groups. Genomics 12: 241–253

Endler JA. 1977. Geographic Variation, Speciation and Clines. Princeton, NJ: Princeton Univ. Press

Evans PGH, Macdonald DW, Cheeseman CL. 1989. Social structure of the Eurasian badger *Meles meles*: genetic evidence. J. Zool. Lond. 218: 587-595

Falconer DS, Mackay TFC. 1996. Introduction to quantitative genetics. Harlow: Longman

Farris MA, Mitton JB. 1984. Population density, outcrossing rate, and heterozygote superiority in ponderosa pine. Evolution 38: 1151-1154

Fowler K, Whitlock MC. 1999. The distribution of phenotypic variance with inbreeding. Evolution 53: 83-96

Frankel OH, Soulé ME. 1981. Conservation and Evolution. Cambridge: Cambridge University Press

Frankham R. 1995. Effective population size/adult population size ratios in wildlife: a review. Genet. Res. 66: 95-107

Frankham R. 1996. Relationship of genetic variation to population size in wildlife. Cons. Biol. 6: 1500-1508

Franklin IR, Frankham R. 1998. How large must populations be to retain evolutionary potential? Anim. Cons. 1: 69-73

Garton DW. 1984. Relationship between multiple locus heterozygosity and physiological energetics of growth in the estuarine gastropod, *Thais haemastoma*. Physiol. Zool. 57: 520-543

Garton DW, Koehn RK, Scott TM. 1984. Multiple locus heterozygosity and the physiological energetics of growth in the coot clam *Mulina lateralis*, from a natural population. Genetics 108: 445-455

Gilpin ME. 1987. Spatial structure and population vulnerability, In: Viable populations for conservation, ed. ME. Soulé. pp. 125-139. Cambridge; Cambridge University press

Goldstein DB, Ruiz Linares A, Cavalli-Sforza LL, Feldman MW. 1995. An evaluation of genetic distances for use with microsatellite loci. Genetics 139: 463–471

Goodnight CJ. 1987. On the effect of founder events on epistatic genetic variance. Evolution 41: 80-91

Groombridge B. 1992. Global biodiversity: Status of the earth's living resources. London: Chapman & Hall.

Hardy C, Callou C, Vigme JD, Casane D, Dennebouy N, *et al.* 1995. Rabbit mitochondrial DNA diversity from prehistoric to modern times. J. Mol. Evol. 40: 227–237

Hartl GB, Hell P. 1994. Maintenance of high levels of allelic variation in spite of a severe bottleneck in population size: the brown bear *Ursus arctos* in the western Carpathians. Biodiv. Conserv. 3: 546-554

Hedrick PW. 1996. Bottleneck(s) or Metapopulation in Cheetahs. Cons. Biol. 3:897-899

Holsinger KE. Vitt P. 1997. The future of Conservation Biology: What's a geneticist to do? In: The Ecological basis of Conservation: Heterogeneity, Ecosystems, and Biodiversity, eds. STA. Pickett, RS. Ostfeld, M. Shachak, GE. Likens. pp. 202-216. New York: Chapmann & Hall

Höss M, Dilling A, Currant A, Pääbo S. 1996. Molecular phylogeny of the extinct ground sloth *Mylodon darwinii*. Proc. Natl. Acad. Sci. USA 93: 181–185

Houle D. 1989. Allozyme-associated heterosis *in Drosophila melanogaster*. Genetics 123: 195-204

Houle D. 1994. Adaptative distance and the genetic basis of heterosis. Evolution 48: 1410-1417

Hudson RR, Slatkin M, Madison WP. 1992. Estimation of levels of gene flow from DNA sequence data. Genetics 132: 583–589

Hughes JB, Daily GC, Ehrlich PR. 1997. Population diversity: Its Extent and Extinction. Science 278: 689-691

Huson LW, Page RJC. 1980. Multivariate geographical variation of the red fox *Vulpes vulpes* in Wales. J. Zool. Lond. 191: 453-459

Hutter CM, Rand DM. 1995. Competition between mitochondrial haplotypes in distinct nuclear genetic environments: *Drosophila pseudoobscura* vs. *Drosophila persimilis*. Genetics 140: 537-548

Imasheva AG, Loeschcke V, Zhivotovsky LA, Lazebny OE. 1997. Effects of extreme temperatures on phenotypic variation and developmental stability in *Drosophila melanogaster* and *Drosophila buzzatii*. Biol. J. Linn. Soc. 61: 117-126

James JW. 1971. The founder effect and response to artificial selection. Genet. Res. 16: 241-250

Jeffreys AJ, Royle NJ, Wilson V, Wong Z. 1988. Spontaneous mutation rates to length alleles at tandem-repetitive hypervariable loci in human DNA. Nature 332: 278–281

Jeffreys AJ, Tamaki K, Macleod A, Monckton DG, Neil DL, *et al.* 1994. Complex gene conversion events in germline mutations at human minisatellites. Nature Genet. 6: 136–145

Jensen S, Kihlstrom M, Ollson C, Lundberg C, Orberg J. 1977: Effects of PCB and DDT on mink *Mustela vison* during the reproductive season. Ambio 6: 239

Jiménez JA, Hughes KA, Alaks G, Graham L, Lacy RC. 1994 An experimental study of inbreeding depression in natural habitat. Science 266: 271-273

Keller LF, Arcese P, Smith JNM, Hochachka WM, Stearns SC. 1994. Selection against inbred song sparrows during a natural population bottleneck. Nature 372: 356-357

Kierstead H, Slobodkin LB. 1953. The sizes of water masses containing plankton bloom. J. Mar. Res. 12: 141-147

Kohn M, Wayne R. 1997. Facts from feces revisited. Trends Ecol. Evol. 6: 223–227

Kruuk H. 1978. Spatial organisation and territorial behaviour of the European badger *Meles meles*. J. Zool. Lond. 184: 1-19

Lande R. 1998. Demographic stochasticity and Alle effect on a scale with isotropic noise. Oikos: 353-358

Lansman RA, Shade RO, Shapira JF, Avise JC. 1981. The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations. III. Techniques and potential applications. J. Mol. Evol. 17: 214–226

Leamy L. 1984 Morphometric studies in inbred and hybrid mice. V. Directional and fluctuating asymmetry. Amer. Nat. 123: 579-593

Leamy L. 1997a. Is developmental stability heritable? J. Evol. Biol. 10: 21-29

Leamy L. 1997b. Genetic analysis of fluctuating asymmetry for skeletal charaters in mice. J. Hered. 88: 85-92

Leary RF, Allendorf FW, Knudsen KL. 1983. Developmental stability and enzyme heterozygosity in rainbow trout. Nature 301: 71-72

Leary R, Allendorf FW. 1989. Fluctuating asymmetry and indicator of stress: implications for conservation biology. Trends Ecol. Evol. 4: 214–217

Leberg PL. 1992. Effects of population bottlenecks on genetic diversity as measured by allozyme electrophoresis. Evolution 46: 447-494

Lerner IM. 1954. Genetic Homeostasis. London.: Oliver & Boyd

Lewontin RC. 1974. The Genetic Basis of Evolutionary Change. New York: Columbia Univ. Press

Livshits G, Kobyliansky E. 1984. Comparative analysis of morphological traits in biochemically homozygous individuals from a single population. J. Hum. Evol. 13: 161-171

Lougheed SC, Handford P. 1992. Vocal dialects and the structure of geographic variation in morphological and allozymic character in the rufous-collared sparrow, *Zonotrichia capensis*. Evolution 46: 1443-1456

Ludwig W. 1932. Das rechts-links problem im tierreich und beim menschen. Berlin: Springer

Lynch M. 1996. A quantitative genetic perspective on conservation issues. In: Conservation Genetics: Case Histories from Nature. eds. JC. Avise, JL. Hamrick. pp. 457-501. New York: Chapmann & Hall

Lynch JM, O'Sullivan WM. 1993. Cranial form and sexual dimorphism in the Irish *otter Lutra lutra*. Biol. and Environ. Proc. R. Ir. Acad. 93: 97-105

Manly BFJ. 1986. Multivariate statistical methods. A primer London: Chapman & Hall.

Manning TH. 1971. Geographical variation in the polar bear *Ursus maritimus* Phipps. Can. Wild. Serv. Rep. Series 13: 1-27

May RM, Beddington JR, Horwood JW, Shepherd JG. 1978. Exploiting natural populations in an uncertain world. Math. Bio. 42: 219-252

Mitton JB. 1995. Enzyme heterozygosity and developmental stability. Acta Theriol., Suppl. 3: 33-54

Mitton JB, Grant MC. 1984. Associations among protein heterozygosity, growth rate and developmental homeostasis. Annu. Rev. Ecol. Syst. 15: 479-499

Murray V. 1989. Improved double-stranded DNA sequencing using the linear polymerase chain reaction. Nucleic Acids Res. 17: 8889

Myers RA, Bridson J, Barrowman NJ. 1995. Summary of world-wide spawner and recruitment data. Can. Techn. Rep. Fish. Aquat. Sciences 2024

Møller AP, Pomiankowski A. 1993a. Why have birds got multiple sexual ornaments? Behav.Ecol. Sociobiology 32: 167–176

Møller AP, Pomiankowski A. 1993b. Fluctuating asymmetry and sexual selection. Genetica 89: 267–279

Møller AP, Swaddle JP. 1997. Asymmetry, developmental stability and evolution. Oxford: Oxford University Press

Nei M. 1982. Evolution of human races at the gene level. In: Human Genetics, Part A: The Unfolding Genome, eds. B. Bohhe-Tamir, P. Cohen, RN Goodman. pp. 167–181. New York: Alan R. Liss

Nevo E. 1983. Adaptative significance of protein variation. In: Protein polymorphism: adaptative and taxonomic significance eds. GS. Oxford, D. Rollinson. pp. 239-282. London: Academic press

O'Brien SJ. 1994. A role for molecular genetics in biological conservation. Proc. Natl. Acad. Sci. USA 91: 5748-5755

Palsbøll PJ. 1999. Genetic tagging: contemporary molecular ecology. Biol. J. Linn. Soc. 68: 3-22

Parker MA. 1992. Outbreeding depression in a selfing annual. Evolution 46: 837-841

Parsons PA. 1990. Fluctuating asymmetry: an epigenetic measure of stress. Biol. Rev. 65: 131–145

Pimm SL. 1991. The balance of nature? Chicago: University of Chicago Press

Pomiankowski A, Møller AP. 1995. A resolution of the lek paradox. Proc. Royal Soc. B 260: 21–29

Pons O, Petit RJ. 1996. Measuring and testing genetic differentiation with ordered *versus* unordered alleles. Genetics 144: 1237

Porter AH. 1999. Refugees from lost habitat and reorganization of genetic population structure. Cons. Biol. 13: 850-859

Raven PH. 1987. The scope of the plant conservation problem world-wide. In: Botanic Gardens and the world Conservation Strategy. eds. D. Bramwell, O. Hamann, V. Heywood, H. Synge. pp.19-29. London: Academic Press

Roy MS, Geffen E, Smith D, Wayne RK. 1996. Molecular genetics of pre-1940 red wolves. Conserv. Biol. 10: 1413–1424

Rutherford SL, Lindquist S. 1998. HSP90 as a capacitor for morphological evolution. Nature 396: 336-342

Saccheri IJ, Brakefield PM, Nichols RA. 1996. Severe inbreeding depression and rapid fitness rebound in the butterfly *Bicyclus anynana*. Evolution 50: 2000-2013

Schlotter C, Tautz D. 1993. Slippage synthesis of microsatellites. Nucleic Acids Res. 20: 211–215

Schwartz MK, Tallmon DA, Luikart G. 1999. Using genetics to estimate the size of wild populations: many methods, much potential, uncertain utility. Anim. Cons. 2: 321-323

Sciulli PW, Doyle WJ, Kelley C, Siegel P, Siegel MI. 1979. The interaction of stressors in the induction of increased levels of fluctuating asymmetry in the laboratory rat. Am. J. Phys. Anthropol. 50: 279-284

Shiskin MA. 1992. Evolution as a maintenance of ontogenetic stability. Acta Zool. Fenn. 191: 37-42

Shriver MD, Jin L, Chakraborty R, Boerwinkle E. 1993. VNTR allele frequency distributions under the stepwise mutation model: a computer simulation approach. Genetics 134: 983–993

Siegel MI, Doyle WJ. 1975. The differential effect of prenatal and postnatal audiogenic stress on fluctuating dental asymmetry. J. Exp. Zool. 191: 211-214

Slatkin M. 1995. A measure of population subdivision based on microsatellite allele frequencies. Genetics 139: 457–462

Slatkin M, Maddison WP. 1989. A cladistic measure of gene flow inferred from the phylogenies of alleles. Genetics 123:603–613

Slatkin M, Maddison WP. 1990. Detecting isolation by distance using phylogenies of genes. Genetics 126: 249–260

Soulé ME. 1980. Thresholds for survival: Maintaning fitness and evolutionary potential. In: Conservation Biology: An evolutionary-ecological perspective. eds. ME. Soulé BA. Wilcox. pp. 151-169. Sunderland, MA: Sinauer

Stearns SC. 1992. The evolution of life histories. Oxford: Oxford University Press

Swofford DL, Olsen GJ, Waddell PJ, Hillis DM. 1996. Phylogenetic inference. In: Molecular

Systematics. eds. DM Hillis, C Moritz, BK Mable. pp. 407–514. Sunderland, MA: Sinauer

Takahata N, Palumbi SR. 1985. Extranuclear differentiation and gene flow in the finite island model. Genetics 109: 441–457

Tautz D, Schlotterer C. 1994. Simple sequences. Curr. Opin. Genet. Dev. 4: 832–837

Templeton AR. 1986. Coadaptation and outbreeding depression. In: Conservation Biology: The Science of Scarcity and Diversity. ed. ME. Soulé. pp. 105-16. Sunderland, MA: Sinauer

Templeton AR, Routman E, Phillips CA. 1995. Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the Tiger salamander, *Ambystoma tigrinum*. Genetics 140: 619–633

Thoday JM. 1958. Homeostasis in selection experiment. Hereditas 12: 401-415

Thomas WK, Pääbo S, Villablanca FX, Wilson AC. 1990. Spatial and temporal continuity of kangaroo rat populations shown by sequencing mitochondrial DNA from museum specimens. J. Mol. Evol. 31: 101–112

Turelli M. 1988. Phenotypic evolution, constant covariances, and the maintenance of additive variance. Evolution 42: 1342-1347

Valdes AM, Slatkin M, Friemer NB. 1993. Allele frequencies at microsatellite loci: the stepwise mutation model revisited. Genetics 133: 737–749

Valentine DW, Soulé ME. 1973. Effect of p,p' DDT on developmental stability of pectoral fin rays in the grunion *Leuresthes tenius*. Fishery Bull. 71: 920-921

Van Valen L. 1962. A study of fluctuating asymmetry. Evolution 16: 125–142

Vawter L, Brown WM. 1986. Nuclear and mitochondrial DNA comparisons reveal extreme rate variation in the molecular clock. Science 234: 194–196

Waddington CH. 1957. The strategy of the genes. New York: Macmillan

Wagner GP, Booth G, Bagheri-Chaichian H. 1997. A population genetic theory of canalization. Evolution 51: 329-347

Wayne RK, Leonard JA, Cooper A. 1999. Full of sound and fury: History of ancient DNA. Annu. Rev. Ecol. Syst. 28: 391-435

Weber JL, Wong C. 1993. Mutation of human short tandem repeats. Hum. Mol. Genet. 2: 1123–1128

Weiner JG, Rago PJ. 1987. A test of fluctuating asymmetry in bluegills *Lepomis macrochirus* as a measure of PH related stress. Environ. Pollut. 44: 27-36

Wright S. 1978. Evolution and the Genetics of Populations. Vol. 4: Variability Within and Among Natural Populations. Chicago: Univ. Chicago Press

Wu CI, Palopoli MF. 1994 Genetics of postmating reproductive isolation in animals. Annu. Rev. Genet. 28: 283-308.

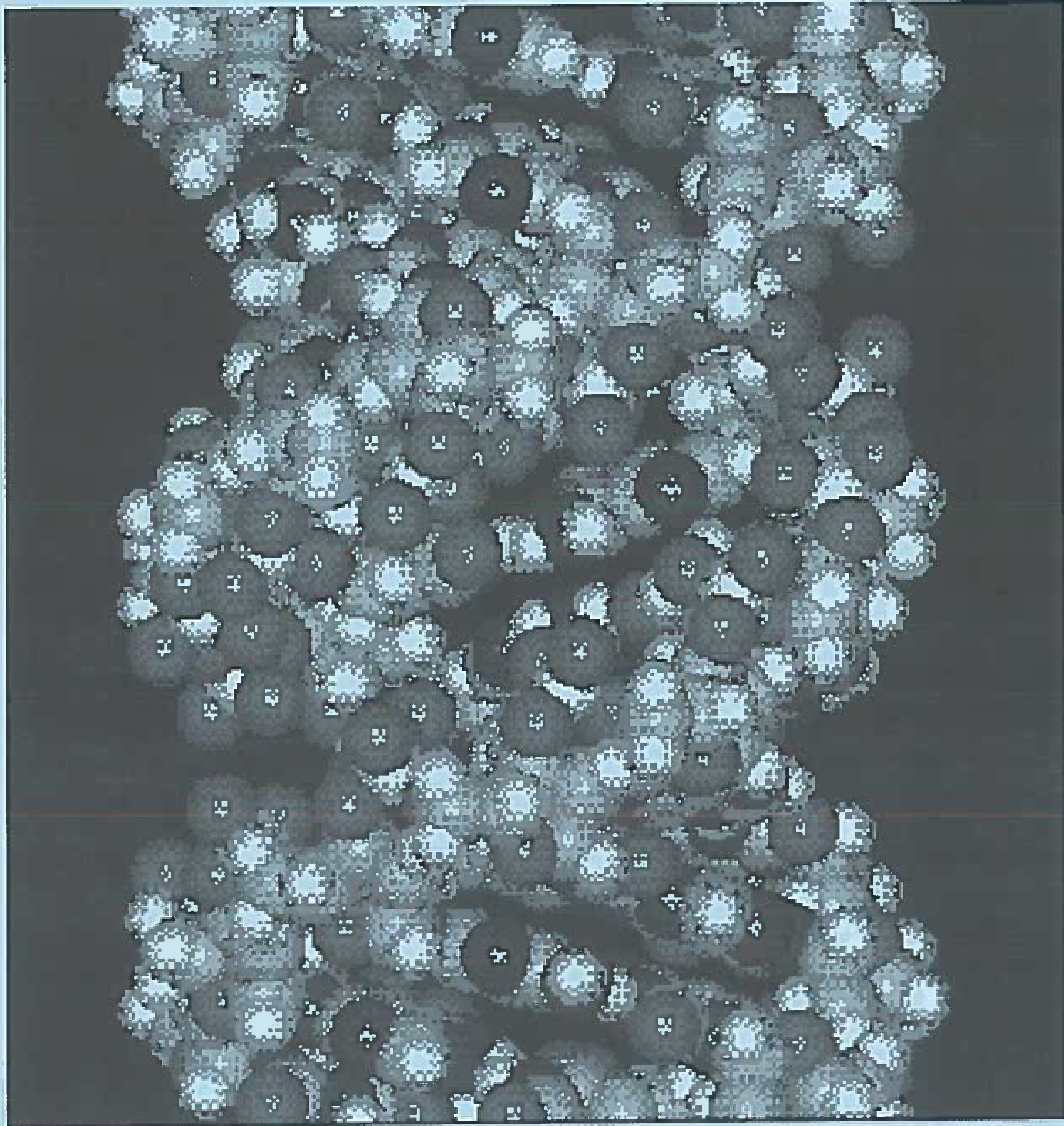
Zakharov VM. 1992. Population phenogenetics: Analysis of developmental stability in natural populations. Acta Zool. Fenn. 191: 7–30

Zouros E, Singh M, Miles H. 1980. Growth rate in oysters: an overdominant phenotype and its possible explanation. Evolution 34: 856-867

Zouros E, Folts DW. 1987. The use of allelic isozyme variation for the study of heterosis. Isozymes: Curr. Topics Biol. Med. Res. 13: 1-59

Åris Sørensen JA. 1995. Road-kills of badgers *Meles meles* in Denmark. Ann. Zool. Fennici 32: 31-36

Published articles



Hereditas 130: 331-336 (1999)

Brief Report
Extremely low mitochondrial DNA control-region sequence variation in the otter *Lutra lutra* population of Denmark

N. MUCCI[1], C. PERTOLDI[2], A. B. MADSEN[3], V. LOESCHCKE[2] and E. RANDI[1]

[1] *Istituto Nazionale per la Fauna Selvatica, via Cà Fornacetta 9, 40064 Ozzano dell'Emilia (BO), Italy and* [2] *Department of Ecology and Genetics, Aarhus University, Ny Munkegade, Build. 540, DK-8000 Aarhus C, Denmark* [3] *National Environmental Research Institute, and Department of Landscape Ecology, Grenåvej 12, Kalø, DK-8410 Rønde, Denmark* E-mail: met0217@iperbole.bo.it

(Received November 23, 1998. Accepted March 14, 1999)

The Eurasian otter *Lutra lutra*, once a widespread species in Denmark, seriously declined in the last three decades. In Denmark, river regulation, wetland destruction, drowning in fish traps and traffic mortality have been responsible for the decline to the remaining otter population. Over much of lowland Europe, toxic chemicals are likely to have been responsible for the widespread decline. Current concentration are unlikely to pose a threat to the Danish otter populations (MADSEN 1996).

In spite of a total protection of the species in Denmark since 1967, an investigation in 1980 showed that otters were still scattered all over the country, but only about 200 animals were left. National field surveys were carried out in 1984–86 and 1991 in Denmark and 1975, 1980 and 1986/87 in Schleswig/Holsten. The result showed that otters were mainly restricted to the northern part of Jutland separated from Schleswig/Holsten (Fig. 1). A positive population trend was found in Denmark in 1996 and otters are now present in the middle and northern part of Jutland and isolated population fragments are present in the western part of the island of Zealand (MADSEN 1996 and references therein).

The expected effects of a population decline and bottlenecks on genetic variability are well known: if the population decline is sharp, the bottleneck extreme, and population recovery after the bottleneck slow, the loss of genetic variability can be high (NEI et al. 1975). Loss of genetic variability can be harmful to the population because it may reduce individual fitness over medium or long time periods (ALLENDORF and LEARY 1986). Significant losses of genetic variability can occur also when the observed population size (N) is relatively large, if the genetically effective population size (N_e) is low, and experimental and field studies have suggested that N_e/N can be as low as 0.1–0.2 in animal populations (FRANKHAM 1995). Bottlenecks and random drift have exacerbated effects on the population dynamics of the mitochondrial DNA (mtDNA), a haploid genome which is maternally transmitted. For mtDNA, N_e = 1/4 the effective population size of the nuclear genome. The expected loss of mtDNA haplotype diversity in isolated populations of small effective size is fast. Only a single mtDNA haplotype will be retained after $4N_ef$ (female effective population size) generations, while declining populations will loose mtDNA diversity even faster (AVISE et al. 1984).

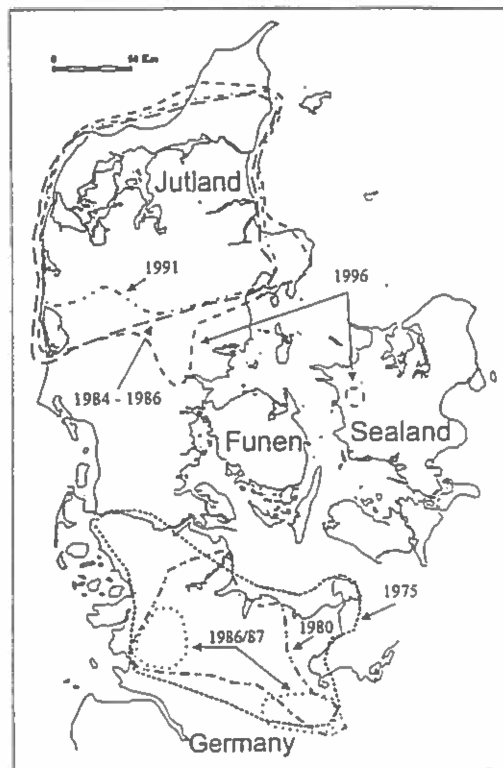


Fig. 1. Distribution ranges of otter populations in Denmark, as resulting from national field surveys in 1975–1996.

In this study we aimed to estimate the genetic effects of isolation and a population bottleneck of otters in Denmark by determining haplotype diversity of the mtDNA control region. The low effective population size of the mitochondrial genome, and the fast rate of mutational change of the peripheral domains of the control-region (CR), make in principle the mtDNA a suitable molecule to study and assess the effects of population decline and bottlenecks (HOELZEL et al. 1993). Further, the mtDNA control region of four captive reared otters of presumed English origin, were determined.

MATERIALS AND METHODS

Tissues were obtained from 30 found-dead otters collected in the period 1990–1994, and stored refrigerated at the Natural History Museum, Aarhus, Denmark. The tissues from the captive reared otters were provided from the Zoological Society la Torbiera, Agrate Conturbia, Italy. Total DNA was extracted using standard proteinase-k digestion and phenol/chloroform/isoamyl alcohol purification (HILLIS et al. 1990: 339). The entire mtDNA CR was amplified using the conserved primers L15926 (5'-TCA AAG CTT ACA CCA GTC TTG TAA ACC-3') and H00651 (5'-TAA CTG CAG AAG GCT AGG ACC AAA CCT-3') (KOCHER et al. 1989), or primers L Pro (5'-CGT CAG TCT CAC CAT CAA CCC CCA AAG C-3') and H-Phe (5'GGG AGA CTC ATC TAG GCA TTT TCA GTG-3'), which bind to the flanking tRNA-Pro (L primer) and 12SrRNA (H primer) genes, respectively, with the following program: 94°C for 2 min, 30 cycles of amplification at 94°C for 15 sec, 50–55°C for 15 sec, 72°C for 15 sec, and the final extension of the PCR products at 72°C for 5 min. The PCR products were purified and directly cycle-sequenced using the Perkin Elmer PRISM dye dideoxy terminator protocol and an ABI 373A automatic sequencer. After the first sequences, we have designed the following specific sequencing primers: OttL-D2 (5'-CCT ATA TTG TCC TGC CAA ACC C-3'), and OttH-D1 (5'-TAA CAA GTG GTG GGA GAG AGA AGC-3'), which bind to positions nos. 691 and 901 (Fig. 2), and that were used for sequencing the 3' half of the CR. The 5' half of the CR was sequenced with primer L-Pro. Nucleotide sequences were compared with published CRs of other carnivores (HOELZEL et al. 1993, 1994; LOPEZ et al. 1996) with the aim to identify the presence of structural conserved motifs which support the authenticity of PCR products.

RESULTS

We have sequenced 894 nucleotides of the CR in 30 otters collected at different localities in Denmark (Fig. 1), and in four captive reared otters of presumed English origin. Moreover, we have obtained sequences of 58 nucleotides of the flanking tRNA genes, for a total of 952 nucleotides. The PCR fragments always showed the expected size (about 1 kb).

Nucleotide sequence and organisation of the CR of otters are shown in Fig. 2. Flanking tRNA-Pro ends with TG nucleotides, a conserved motif found in other vertebrate species (LOPEZ et al. 1996). The central conserved sequence block (CCSB) begins at position no. 210 (Fig. 2), and shows high sequence homology with CRs of other carnivores, like the domestic cat (LOPEZ et al. 1996), dog (GenBank accession no. X97343; Rothuizen et al. unpubl.), brown bear and wolf (unpubl. obs.), and several species of seals (HOELZEL et al. 1993). At positions 154–180 maps a sequence with homology to the termination associated sequences (TAS-A) of mammals, and, a few nucleotides upstream, there is the motif GCCCCAT (positions nos. 134–140), which is associated with the d-loop strand termination in other mammalian species (DODA et al. 1981). Our sequences have a gap of unknown length (probably about 100 nucleotides), from the end of the CCSB to just before a region with eight tandem repeats. These repeats map between the putative CSB-1 and CSB-2, in a region referred as RS3 by HOELZEL et al. (1994). The first three repeats (R1, R2, and R3) are perfectly conserved. R4 has a T-C transition at post no. 16, which is present in R5, R6, and R7. The captive reared otters conserve nucleotide T at position no. 16 of R4, but show the T-C transition at the other repeats. It is therefore probable that T in captive reared otters represents a reversal to the ancestral condition. R6 has a new C-G transversion at position no. 7, which is present also in RS7 and RS8. Therefore, R1, R2, and R3 have identical sequences, R4 and R5 have one transversion, while R6 and R7 have one transition and one transversion. The terminal portion of repeat 8 was difficult to sequence, due to unresolvable ambiguities at positions nos. 14 and 15. All the otter samples we have sequenced had at least eight tandem repeats. These tandem repeats in RS3 of otters diverge progressively from the CCSB towards the tRNA-Phe periphery of the CR. About 20 nucleotides after R8 there is a sequence highly homologous to CSB-2 (nucleotides 651–667), and then, after 33 nucleotides, there is the putative CSB-3 (nucleotides 702–718). The putative TATA promoters of light (LSP) and heavy (HSP) strand transcription (CLAYTON 1992) map at positions 739–742 and 850–853 of the CR of otters, respectively (Fig. 2).

All the sequenced Danish otters showed the same CR haplotype, except specimen DK-911/94, which showed an A-C transversion at position no. 868 (Fig.

```
                                                                                                           1
               1 1111111112 2222222223 3333333334 4444444445 5555555556 6666666667 7777777778 8888888889 9999999990
         123 4567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
  <--tRNA-Pro control-region-->
DK       GTG ACATTCT AACTAAACTA TTCCCTGATT CTCTCACCCC ACATTTCAAT TCATATATTC AACAACATTT ACTGTGCCTG CCCAGTATGT ATTCCGCGCA
DK 911/94 ... ....... .......... .......... .......... .......... .......... .......... .......... .......... ..........
UK       ... ....... .......... .......... .......... .......... .......... .......... .......... .......... ..........

         1111111111 1111111111 1111111111 1111111111 1111111111 1111111111 1111111111 1111111111 1111111111 1111111112
         0000000001 1111111112 2222222223 3333333334 4444444445 5555555556 6666666667 7777777778 8888888889 9999999990
         1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
                                    d-loop-termination         -->        TAS-A      <--
DK       CCGCCCCCTA TGTATATCGT GCATTAATGG TTTGCCCCAT GCATATAAGC ATGTACATAC TATGGTTGAT TTTACATGTA TCCACCTCAC CTAGATCACG
DK 911/94 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
UK       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

         2222222222 2222222222 2222222222 2222222222 2222222222 2222222222 2222222222 2222222222 2222222222 2222222223
         0000000001 1111111112 2222222223 3333333334 4444444445 5555555556 6666666667 7777777778 8888888889 9999999990
         1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
                   --> central CSB
DK       AGCTTGATCA CCATGCCTCG AGAAACCATC AATCCTTGCG CGATGTGTAC CTCTTCTCGC TCCGGGCCCA TCACATGTGG GGGTTTCTAC CGTGAAACTA
DK 911/94 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
UK       .......... .......... .......... .......... ...C...... .......... .......... .......... .......... ..........

         3333333333 3333333333 3333333333 3333333333 3333333333 3333333333 3333333333 3333333333 3333333333 3333333334
         0000000001 1111111112 2222222223 3333333334 4444444445 5555555556 6666666667 7777777778 8888888889 9999999990
         1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890

DK       TACCTGGCAT CTGGTTCTTA CTTCAGGGCC ATAACAATCC TCAATCCAAT CCTACTAACC TCTCAAATGG GACATCTCGA ATGGACTAGT GACTAATCAG
DK 911/94 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
UK       .......... .......... .C........ .......... .......... .......... .......... .......... .......... ..........

         4444444444 4444444444 4444444444 4444444444 444444444 [4] 4444444444 4444444444 4444444444 4444444444 4444444445
         0000000001 1111111112 2222222223 3333333334 444444444 [5] 5555555556 6666666667 7777777778 8888888889 9999999990
         1234567890 1234567890 1234567890 1234567890 123456789 [0] 1234567890 1234567890 1234567890 1234567890 1234567890
                                                                    --> tandem repeat region RS3
                                                     [gap in sequences]     repeat unit 1           2
DK       CCCATGATCA CACATAACTG TGGTGTCATG CATTTGGTAT CTTTAATTT [-] AAGGCACACC CACGTACGTA TACACGCACA CCCACGTACG TATACACGCA
DK 911/94 .......... .......... .......... .......... ......... [.] .......... .......... .......... .......... ..........
UK       .......... .......... .......... .......... ......... [.] .......... .......... .......... .......... ..........

         5555555555 5555555555 5555555555 5555555555 5555555555 5555555555 5555555555 5555555555 5555555555 5555555556
         0000000001 1111111112 2222222223 3333333334 4444444445 5555555556 6666666667 7777777778 8888888889 9999999990
         1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
                 3                        4                5                      6                      7
DK       CACCCACGTA CGTATACACG CACACCCACG TACGCATACA CGCACACCCA CGTACGCATA CACGCACACG CACGTACGCA TACACGCACA CGCACGTACG
DK 911/94 .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
UK       .......... .......... .......... ....T..... .......... .......... .......... .......... .......... ..........
```

Fig. 2.

```
              6666666666 6666666666 6666666666 6666666666 6666666666 6666666666 6666666666 6666666666 6666666666 6666666667
              0000000001 1111111112 2222222223 3333333334 4444444445 5555555556 6666666667 7777777778 8888888889 9999999990
              1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
                               8                                     -->      CSB-2    <--
DK            CATACACGCA CACGCACGTA ??ATCACAGA TATGAAACTA GGTTAATCAA ACCCCCCTTA CCCCCCGTAA CTTCAAAAGT ATACAAATAC CTATATTGTC
DK 911/94     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
UK            .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

              7777777777 7777777777 7777777777 7777777777 7777777777 7777777777 7777777777 7777777777 7777777777 7777777778
              0000000001 1111111112 2222222223 3333333334 4444444445 5555555556 6666666667 7777777778 8888888889 9999999990
              1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
               -->    CSB-3    <--                    <-- LSP
DK            CTGCCAAACC CCCAAAACAG AACTAGGCAC ATGCAACGTA TATGAGAAGT CACTTACACT GGCGCCACGC ATGCTAATCT CATTCACTGA TTCATTAAAA
DK 911/94     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
UK            .......... .......... .......... .......... .......... .......... A......... .......... .......... ..........

              8888888888 8888888888 8888888888 8888888888 8888888888 8888888888 8888888888 8888888888 8888888888 8888888889
              0000000001 1111111112 2222222223 3333333334 4444444445 5555555556 6666666667 7777777778 8888888889 9999999990
              1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
                                                                 HSP -->                                             -->
DK            TAATTCATTA GAAATTCCTA TCCAAAAGAA GCTATCTATA GATGTTATTT ATATCTCCTA CTACCCCAGT CAAAAACGCT TCTCTCTCCC ACCACTTGTT
DK 911/94     .......... .......... .......... .......... .......... .......... .......C.. .......... .......... ..........
UK            .......... .......... .......... .......... .......... .......... .......C.. .......... .......... ..........

              9999999999 9999999999 9999999999 9999999999 9999999999 999
              0000000001 1111111112 2222222223 3333333334 4444444445 555
              1234567890 1234567890 1234567890 1234567890 1234567890 123
              tRNA-Phe
DK            AATGTAGCTT ATTAAATAAA GCAAGGCACT GAAAATGCCT AGATGAGTCT TCC
DK 911/94     .......... .......... .......... .......... .......... ...
UK            .......... .......... .......... .......... .......... ...
```

Fig. 2 continued.

Fig. 2. Nucleotide sequences of the mtDNA control-region of otters. Conserved sequence motifs (from left to right) are underlined: termination of tRNA-Pro, D-loop termination, TAS-A, CSB-2, CSB-3, putative LSP and HSP, tRNA-Phe. Repeat units in tandem repeat region RS3 are numbered 1–8. DK is the common control-region haplotype of otters in Denmark, DK 911/94 is the variant haplotype and UK is the haplotype found in captive-reared otters originated in England.

2). Therefore, mtDNA CR sequence variability was very low in the studied otter sample, and sequence divergence between the two haplotypes was 0.001 only. Captive reared otters of presumed English origin, differ from the predominant haplotype of wild Danish otters at position nos. 244 and 322 (T-C transitions), 535 (C-T transition), 761 (G-A transition), and 868 (A-C transversion). Captive reared otters shared the A-C transversion at post no. 868 with Danish otter DK911 94.

DISCUSSION

Sequence variability at the mtDNA CR was very low in 30 otters from the Danish population: 29 otters shared an identical haplotype, and a single specimen showed a variant haplotype with only one substitution over 952 sequenced nucleotides. Thus, sequence divergence between the two haplotypes was 0.001. The same mtDNA region had 0.005 sequence divergence compared to a different CR haplotype which was found in captive reared otters of UK origin.

Very low levels of mtDNA sequence variability were observed in other populations of carnivore that underwent recent drastic declines in population size and bottlenecks (e.g., HOELZEL et al. 1993; RANDI et al. 1994; GOTTELLI et al. 1994). In fact, AVISE et al. (1984) showed that the probability of survival of two or more mtDNA haplotypes in isolated populations of stable female effective size (N_{ef}) reduces to $p < 0.1$ over $4N_{ef}$ generations. In other words, such kind of populations have a high probability to become monomorphic after $4N_{ef}$ generations since isolation. Moreover, computer simulations have shown higher probability of mtDNA monomorphism in declining populations in less than 100 generations, even if N_{ef} is over 257 (AVISE et al. 1984). Therefore, low mtDNA sequence variability could be due to the bottleneck effect of the last glaciation. During the last glaciation, since late Weichselian (about 13,000 years ago), Denmark was at least partially covered by ice and most of central lowland Europe was permafrost (MAARLEVELD 1976). Colonization of Denmark by otters should have been a recent event, probably not before 10,000 years ago, following Holocene climate warming. It can be postulated that otters which colonized northern temperate zones by a presumed rather quick dispersal from southern refuges had low amounts of genetic variability due to recurrent population decline and bottlenecks during the Pleistocene climatic changes. Assuming that mtDNA control regions of carnivore evolve at a rate of 5–10% sequence divergence per 1 million year (HOELZEL et al. 1993), the observed 0.1% nucleotide substitutions between the two otter haplotypes, could have originated just during the 10,000 years since colonization of Denmark.

However, the extremely low mtDNA CR variability found within the Danish otter population and among the four captive reared otters, should be interpreted with extreme caution. A survey made on other populations in Europe from Sweden, England, Ireland, Spain and Germany (RANDI et al. in prep.), also showed extremely low CR sequence variability. However, some of these otter populations were also investigated for genetic variability using microsatellite loci (DALLAS and PIERTNEY 1998), which showed levels of polymorphism and heterozygosity within the range found in other mammalian species. Therefore, the observed low CR sequence variability could result from a conservative pattern of molecular evolution of the mtDNA CR in otter (see HANSEN and LOESCHCKE 1996, for a case of conserved CR sequences in fish populations), and not from population bottlenecks or postglacial biogeographic history of the species in northern Europe.

ACKNOWLEDGEMENTS

We wish to thank Bo Gaardmand, National Environmental Research Institute, for suggestions and help, and Francesco Rocca for captive-reared otter samples. N. Mucci was supported by the Istituto Nazionale per la Fauna Selvatica, C. Pertoldi by a grant from the Danish Rektorkollegiet. Otter samples were obtained under the appropriate CITES permits.

REFERENCES

Allendorf FW, Leary RF, (1986). Heterozygosity and fitness in natural populations of animals. In: Conservation Biology: The Science of Scarcity and Diversity (ed M Soulé). Sinauer Associates, Sunderland, Massachusetts, p. 57–76.

Avise JC, Neigel JE and Arnold J. (1984). Demographic influences on mitochondrial DNA lineage survivorship in animal populations. J Mol Evol 20: 99–105.

Clayton DA, (1992). Transcription and replication of animal mitochondrial DNAs. In: Mitochondrial genomes (eds DR Wolstenholme and KW Jeon), International Review of Cytology, Vol. 141. Academic Press, San Diego, California, p. 217–232.

Dallas JF and Piertney SB, (1998). Microsatellite primers for the Eurasian otter. Mol Ecol. 9: 1247–1251.

Doda JN, Wright CT and Clayton DA, (1981). Elongation of displacement-loop strands in human and mouse mitochondrial DNA is arrested near specific template sequences. Proc Natl Acad Sci, USA 78: 6116–6120.

Frankham R, (1995). Effective population size/adult population size ratios in wildlife: A review. Genet Res 66: 95–107.

Gottelli D, Sillero-Zubiri C, Applebaum GD, Roy MS, Girman DJ, Garcia-Moreno J, Ostrander EA and Wayne RK, (1994). Molecular genetics of the most endangered canid: the Ethiopian wolf (Canis simensis) Mol Ecol 3: 301–312.

Hansen MM and Loeschcke V. (1996). Genetic differentiation among Danish brown trout populations, as detected by RFLP analysis of PCR amplified mitochondrial DNA sequences. J Fish Biol 48: 422–436.

Hillis DM, Larson A, Davis SK and Zimmer EA. (1990). Nucleic acids III: Sequencing. In: Molecular Systematics (eds DM Hillis and C Moritz). Sinauer Associates, Sunderland, Masschutes, p. 318–370.

Hoelzel AR, Halley J and O'Brien SJ et al., (1993). Elephant seal genetic variation and the use of simulation models to investigate historical population bottlenecks. J Heredity 84: 443–449.

Hoelzel AR, Lopez JV, Dover GA and O'Brien SJ, (1994). Rapid evolution of a heteroplasmic repetitive sequence in the mitochondrial DNA control region of carnivores. J Mol Evol 39: 191–199.

Kocher TD, Thomas WK and Meyer A et al., (1989). Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. Proc Natl Acad Sci, USA 86: 6196–6200.

Lopez JV, Cevario S and O'Brien SJ. (1996). Complete nucleotide sequences of the domestic cat (Felis catus) mitochondrial genome and a transposed mtDNA tandem repeat (Numt) in the nuclear genome. Genomics 33: 229–246.

Maarleveld GC. (1976). Periglacial phenomena and the mean annual temperature during the last glacial time in the Netherlands. Biuletyn Peryglacjalny 26: 57–78.

Madsen AB. (1996). Odderens Lutra lutra okologi og forvaltning i Danmark. The Ecology and Conservation of the Otter (Lutra lutra) in Denmark. PhD Thesis. Danish National Environmental Research Institute, Denmark.

Nei M, Maruyama T and Chakraborty R. (1975). The bottleneck effect and genetic variability in populations. Evolution 29: 1–10.

Randi E, Gentile L, Boscagli G, Huber D and Roth HU. (1994). Mitochondrial DNA sequence divergence among some west European brown bear (Ursus arctos) populations. Lessons for conservation. Heredity 73: 480–489.

Ann. Zool. Fennici 34: 187–196 ISSN 0003-455X
Helsinki 18 September 1997 © Finnish Zoological and Botanical Publishing Board 1997

Developmental stability in the Eurasian Otter (*Lutra lutra*) in Denmark

Cino Pertoldi, Volker Loeschcke, Aksel Bo Madsen & Ettore Randi

Pertoldi, C. & Loeschcke, V., Department of Ecology and Genetics, University of Aarhus, Building 540, Ny Munkegade, DK-8000 Aarhus C, Denmark
Madsen, A. B., Department of Landscape Ecology, National Environmental Research Institute, Kalø Grenåvej 14, DK-8410 Rønde, Denmark
Randi, E., Istituto Nazionale per la Fauna Selvatica, via Ca' Fornacetta 9, I-40064 Ozzano Emilia (Bo), Italy

Received 6 February 1997, accepted 11 April 1997

Fluctuating asymmetry (FA) as expressed in metric and meristic skull traits was analysed in a sample of 172 otters collected in Denmark between 1861 and 1994. Tissue levels of organochlorine pesticide residues and PCBs were determined and the correlation between contaminant concentration and FA was tested. A significant correlation was found between FA in different traits and the year of collection, but there was no significant correlation between FA and the concentration of contaminants. These results suggest that factors other than pesticides have affected the developmental stability of skulls in the Danish otter population. Among these, a population bottleneck following habitat fragmentation is discussed as a possible cause.

1. Introduction

The otter (*Lutra lutra*), once a widespread species in Denmark, is today largely restricted to the Limfjord area in the northern part of Jutland (Fig. 1), with isolated population fragments in the surrounding region (Hammershøj *et al.* 1996). Toxic chemicals are believed to be responsible for the decline of otter populations both in Denmark and over much of lowland Europe (Mason & Macdonald 1986, Mason 1989). Additional threats to the remaining otter population in Denmark are traffic mortality, wetland destruction and human disturbance (see Madsen 1996 for a review).

Fluctuating asymmetry (FA) occurs when an individual is unable to undergo identical development on both sides of a bilaterally symmetrical trait (Van Valen 1962, Palmer & Strobeck 1986). FA tends to become elevated under stress (Leary & Allendorf 1989). Stress factors known to raise FA include various chemicals, including pesticides (Valentine & Soulé 1973), polluted habitats (Weiner & Rago 1987), extreme temperatures (Parsons 1962, Siegel & Doyle 1975, Sciulli *et al.* 1979), audiogenic stress (Sciulli *et al.* 1979), and food deficiency either in terms of quality or quantity (Parsons 1990). Also severe restrictions in the availability of nutrients to females during pregnancy causes asymmetry in the skeletal traits

of offspring (Sciulli *et al.* 1979). Furthermore, directional selection, homozygosity, inbreeding and mutations, are also thought to be associated with elevated levels of FA (see Parsons 1992 for review). For example, Clarke and McKenzie (1987) demonstrated that homozygous individuals were often developmentally less stable than their heterozygous counterparts (but see also Britten 1996 for a different view).

Three groups of stressing factors could have affected the dynamics of otter populations in Denmark, and have influenced their degree of developmental stability: (1) contamination of habitats by pesticides, (2) population decline with subsequent erosion of genetic diversity and increasing rate of inbreeding, (3) strong sexual selection which can differentially affect developmental stability in both sexes (males have a larger body size than females, Moors 1980), can result in sexual dimorphism.

Persistent pollutants, which accumulate in living tissues, are a particular problem in fresh water habitats of otters because there are many sources of contamination. Rainfall washes atmospheric pollutants and chemicals applied to land into waterbodies. Many industries discharge effluents into rivers, directly or indirectly. Small amounts of persistent pollutants in effluents may become quickly concentrated in the biota. PCBs were not identified as environmental contaminants until 1966 (Jensen 1972), though they had been in industrial use for at least 35 years before that, and have since been proved to be widespread in the ecosystem. Of pesticides, the chlorinated hydrocarbons dissolve readily in animal fats and hence accumulate in tissues. When these fats are mobilised during periods of stress, such as food shortage or reproduction, large amounts of pesticides may be released into the blood stream.

The otter population in Denmark declined sharply starting from an estimated size of more than 1 500 individuals in 1961 to only 200 in 1980 (Schimmer 1981), and increased thereafter again to 400 animals censused in 1991. This decline was probably an effect of pesticide contamination (Mason & Madsen 1993). The reproductive biology of otters suggests that the ratio of the effective population size N_e to the observed population size N is on average 0.11 (see Frankham 1995 for review). Therefore, the population bottleneck during the 80s should have been N_e about 20 individuals for at least 5 generations. During this period, the Danish otter population may have lost rare alleles and possibly became less heterozygous. The estimates of low effective population sizes are also supported by very low genetic variability found in the mitochondrial DNA control region (D-loop) (N. Mucci, C. Pertoldi, A. B. Madsen, V. Loeschcke and E. Randi unpubl.).

FA tends to be higher in sexually selected characters than non-sexually selected traits (Møller 1992, Manning & Chamberlain 1993). However, it is not known whether nutrient deficiency, or any of the other factors listed above, cause any differential effect on FA in characters subjected to sexual selection as compared with ordinary morphologically traits.

In this paper, we aim to estimate FA in metric and meristic skull traits in Eurasian otters collected in Denmark from 1861 until 1994. FA will be correlated with morphometric variability, the period of sample collection, demographic trends of the Danish otter population, and levels of pesticide and PCB contaminants in tissues.

2. Material and methods

We used skulls of Eurasian otters (*Lutra lutra*) from the Zoological Museum, Copenhagen (skulls from 1861 until 1959), the Natural History Museum, Aarhus (1959–1961) and the National Environmental Research Institute, Kalø (DMU) (1979–1993). Animals from DMU (only animals from DMU were age-determined) were considered as juveniles (less than about 5 months old) if tooth replacement was incomplete, subadults (5–18 months) if the epiphyseal closure of humerus and femur at their proximal and distal ends was not finished, or as adults (older than about 18 months). In males, the length of the baculum was also used in age determination (Van Bree *et al.* 1966). Skull growth in otters is complete by the third year of life (Chanin 1991), and further changes, except senility changes in the skull will not affect the skull measurements.

All skulls, until 1961 originated from different parts of Denmark (Jutland, and the isles of Zealand and Funen). After that date, no more otters were found on the isles (Fig. 1).

A total of 172 skulls were examined. Four characters of the skulls A, B, C and M, and the Dental foramen (which are small openings for nerves and blood vessels) of the lower jaws were counted (Fig. 2). Trait A is the distance between the opistokranion and zygomatic process of frontal bone, trait B is the shortest distance between the zygomatic process of frontal bone and frontal process of zygomatic bone, trait C is the shortest distance between the jugular foramen

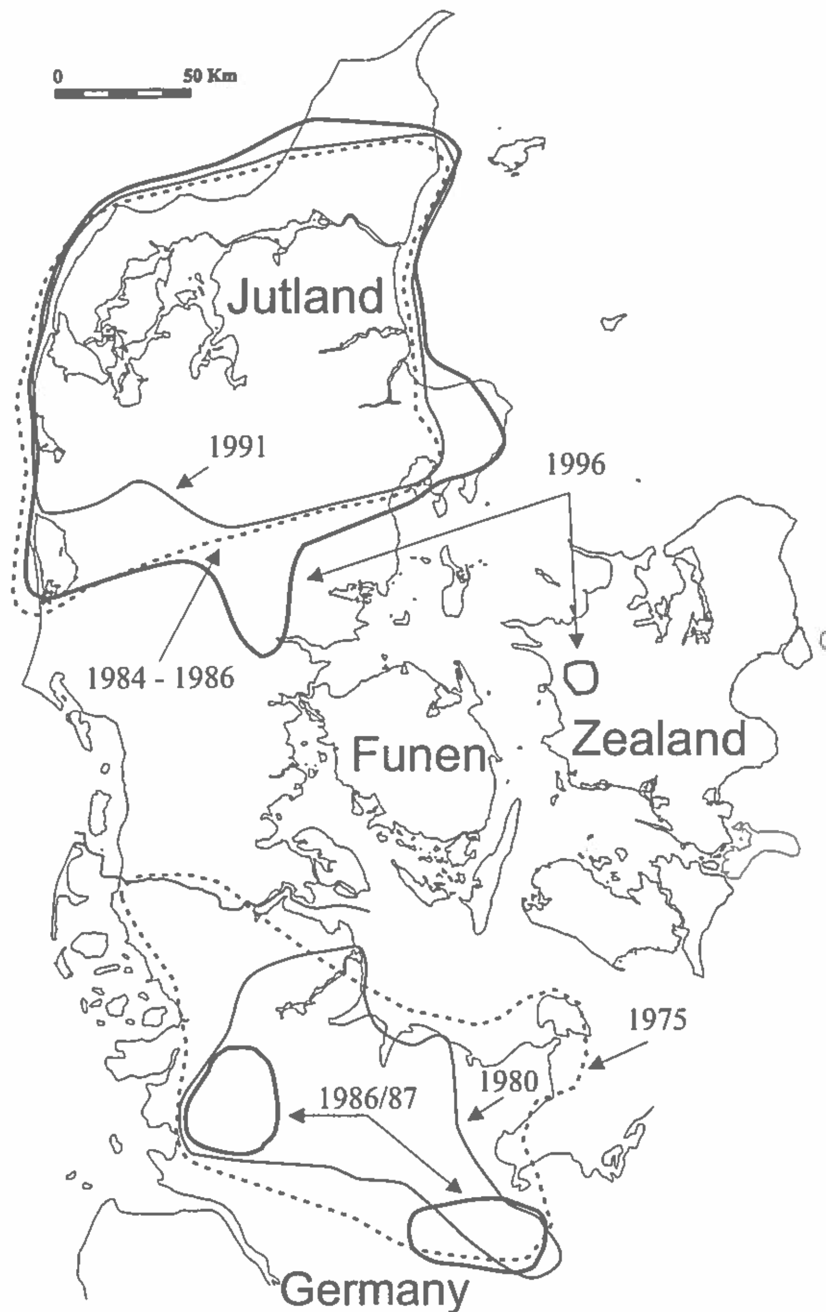


Fig. 1. The distribution of the otter in Denmark in 1996, 1991, between 1984 and 1986, and between 1960 and 1980.

and staphylion and trait M is the total length of the jaw: infradentale-goniocaudale. The traits chosen (Fig. 2a–c) appeared to show a high level of fluctuating asymmetry.

The traits A, B, C and M were measured with a digital calliper to the nearest 0.1 mm. The magnitude of asymmetry was estimated from the difference in length between each bilateral pair as right minus left ($r - l$). FA was calculated as the absolute value of asymmetry and as the variance of ($r - l$) (Palmer & Strobeck 1986). To reduce the measurement error, all measurements were replicated 3 times (at an interval of 3 hours), and the median of the 3 measures was chosen. The overall repeatability (r_l; see Zar 1984) of the size measurement was estimated to be around 97% ($0.968 < r_l < 0.982$, $p < 0.001$), so that the measurement error should have accounted for no more than 3% of the total variation in even the least repeatable character. No measurements were attempted on broken or worn parts of the skulls, therefore, for some skulls we have missing values. The difference in number between sides of the bilateral pair of foramina was used to estimate the magnitude of

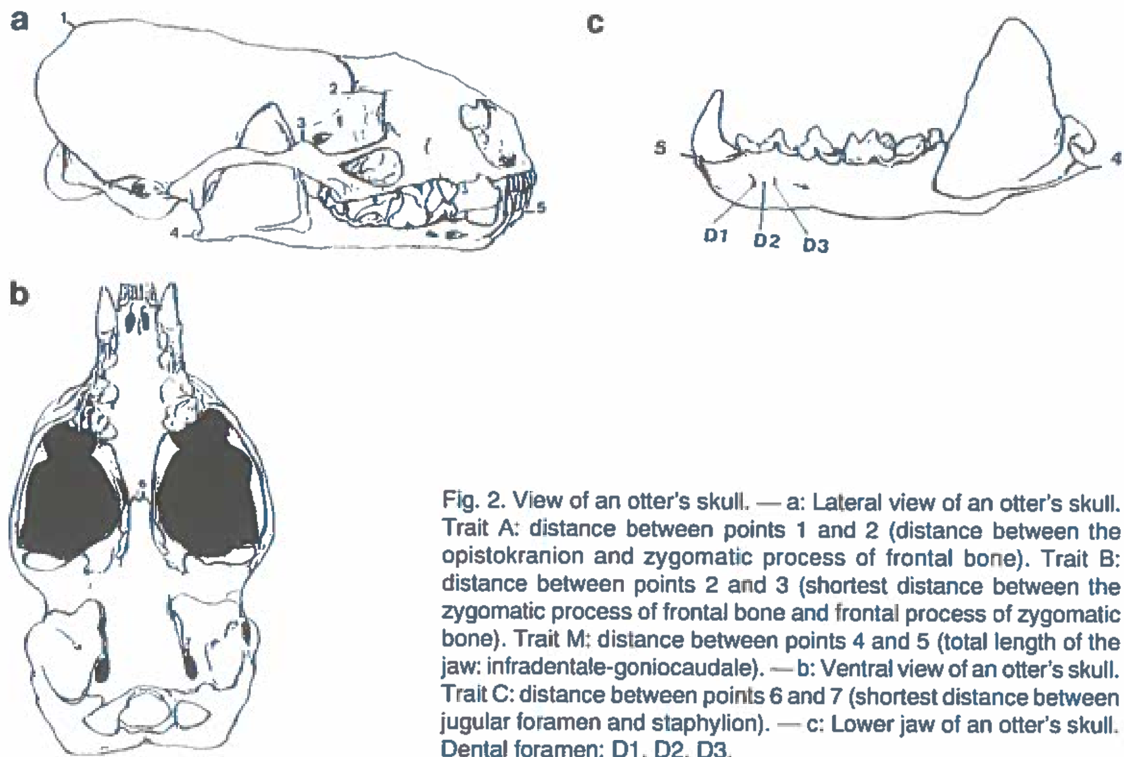


Fig. 2. View of an otter's skull. — a: Lateral view of an otter's skull. Trait A: distance between points 1 and 2 (distance between the opistokranion and zygomatic process of frontal bone). Trait B: distance between points 2 and 3 (shortest distance between the zygomatic process of frontal bone and frontal process of zygomatic bone). Trait M: distance between points 4 and 5 (total length of the jaw: infradentale-goniocaudale). — b: Ventral view of an otter's skull. Trait C: distance between points 6 and 7 (shortest distance between jugular foramen and staphylion). — c: Lower jaw of an otter's skull. Dental foramen: D1, D2, D3.

FA. The numbers were counted macroscopically, each pair of foramina was counted 3 times and when we found incongruence we excluded the measurement. However, it is worth noting that meristic characters will only become asymmetric once stress reaches a threshold level (Swain 1987).

Departures from normality of $(l-r)$ for each trait, were tested with a Kolmogorov-Smirnov test (Sokal & Rohlf 1981). The hypothesis that the mean of left minus right character values equals zero was tested in a one sample *t*-test.

Differences between age groups and periods of skull collection were compared with an *F*-test (Fowler & Cohen 1990). For this purpose, we considered all adult and subadult female otters between 1980 and 1993 and male otters between 1980 and 1994.

A linear and polynomial regression analysis (year of collection versus absolute value of asymmetry) were conducted for each trait. The periods of collection were divided in 3 periods (1, 2 and 3): for males, the three periods were 1926 to 1939, 1960 to 1961 and 1980 to 1994; for females, they were 1932 to 1942, 1959 to 1962 and 1980 to 1993; the variance of $(r-l)$ and the median of FA for each trait A, B and C were calculated.

A Mann-Whitney *U*-test was used to test if the median of FA for traits A, B and C increased through the three periods, and if the median for these traits and dental foramen was different between the two sexes (only periods 2 and 3) and an *F*-test was conducted to see if the variance of $(r-l)$ increased during the 3 periods of collection.

Tissues of otters found dead in Denmark between 1980 and 1990 were analysed for organochlorine pesticide residues and PCBs (Lindane, Dieldrin, pp-DDE, op-DDD, op-DDT, PCBs and pp-DDE/op-DDT). In this period (1980–1990), an average annual decline of 7% per year for PCBs and of 6% for DDE were found (Mason & Madsen 1993).

A Spearman correlation coefficient was used for correlations between the concentration of contaminants in the otter's tissues and the absolute FA of its skull. A Spearman correlation coefficient was performed to test a possible correlation between the condylobasal length (skull length and weight, and length from nose to tail) of the otter before necropsy.

3. Results

There was no evidence of directional asymmetry or antisymmetry and all deviations from normal distributions were not significant (Kolmogorov Smirnov test: $0.089 < p < 0.47$, $125 < n < 150$), and no significant deviations from zero as the mean of the trait $(l-r)$ distributions were found (one sample *t*-test: $0.062 < p < 0.312$, $67 < d.f. < 75$).

All the traits were tested for independence of overall traits with a Spearman rank correlation test,

and no significant correlation was found for males, although for females the trait A was correlated with traits B and C (A and B: $r_s = 0.29, n = 49, p = 0.044$, between traits A and C: $r_s = 0.31, n = 52, p = 0.026$).

No significant differences of FA between the two age groups (adults and subadults) of the same sex were found (Table 1) and, therefore, the two age groups were pooled in the following regression analyses (Table 1).

No correlation was found at the 0.01 level between overall length of the traits (A, B, C and M) and the degree of FA in these traits (males: $-0.02 < r_s < 0.17$, $d.f. = 61–73$, $0.09 < p < 0.96$, females: $-0.05 < r_s < 0.24$, $d.f. = 50–65$, $0.06 < p < 0.74$).

A strong positive correlation (Spearman test) was found in both sexes between the condylobasal length of the skull, and its weight and length before necropsy, indicating that skull length is a good indicator of the body mass of an otter: females $r_s = 0.41$, $n = 25$, $p = 0.046$, males $r_s = 0.75$, $n = 35$, $p = 0.0001$; "body length–condylobasal length": females $r_s = 0.75$, $n = 25$, $p = 0.0002$, males $r_s = 0.84$, $n = 38$, $p = 0.0001$.

No significant correlation (Spearman test) was found between the degree of asymmetry and contaminant concentration at the level of $p < 0.01$, indicating that factors other than contaminants have contributed to the levels of FA (Table 2).

Table 1. Comparison of fluctuating asymmetry variance of left–right-hand size measurements of skull characters (traits: A, B, C and M) in adults and subadults.

Traits	Adults (81–94) Variance	*n*	Subadults (80–93) Variance	*n*	*F*	*p*
Males						
A	0.67	23	0.50	22	1.34	*n.s.*
B	0.50	20	0.35	23	1.43	*n.s.*
C	0.36	27	0.30	28	1.20	*n.s.*
M	0.30	23	0.26	31	1.15	*n.s.*
Dental foramen	0.90	7	1.72	17	1.90	*n.s.*
Females						
A	0.70	21	0.42	10	1.70	*n.s.*
B	0.29	14	0.15	8	1.90	*n.s.*
C	0.29	18	0.28	19	1.10	*n.s.*
M	0.16	17	0.12	19	1.30	*n.s.*
Dental foramen	2.17	6	1.61	10	1.40	*n.s.*

Table 2. Spearman rank correlation coefficients and corresponding test values p and r_s for testing correlations between the absolute value of FA in the traits: A, B, C and M, and the concentration of 6 contaminants. (Contaminants: Lindane, Dieldrin, pp-DDE, op-DDD, op-DDT, PCBs and the ratio of pp-DDE/DDT).

Traits			
Males			
A	$15 < d.f. < 18$	$(0.21 < r_s < 0.20)$	$0.38 < p < 0.83$
B	$15 < d.f. < 17$	$(-0.12 < r_s < 0.27)$	$0.26 < p < 0.88$
C	$19 < d.f. < 22$	$(-0.49 < r_s < 0.18)$	0.02[1] $< p < 0.42$
M	$15 < d.f. < 19$	$(-0.03 < r_s < 0.43)$	$0.06 < p < 0.93$
Females			
A	$8 < d.f. < 14$	$(-0.53 < r_s < 0.23)$	$0.13 < p < 0.84$
B	$6 < d.f. < 10$	$(-0.62 < r_s < -0.05)$	0.05[1] $< p < 0.91$
C	$10 < d.f. < 20$	$(-0.57 < r_s < 0.09)$	$0.07 < p < 0.97$
M	$6 < d.f. < 16$	$(-0.57 < r_s < 0.01)$	0.02[1] $< p < 0.97$

[1] Significance disappears if one extreme value is removed.

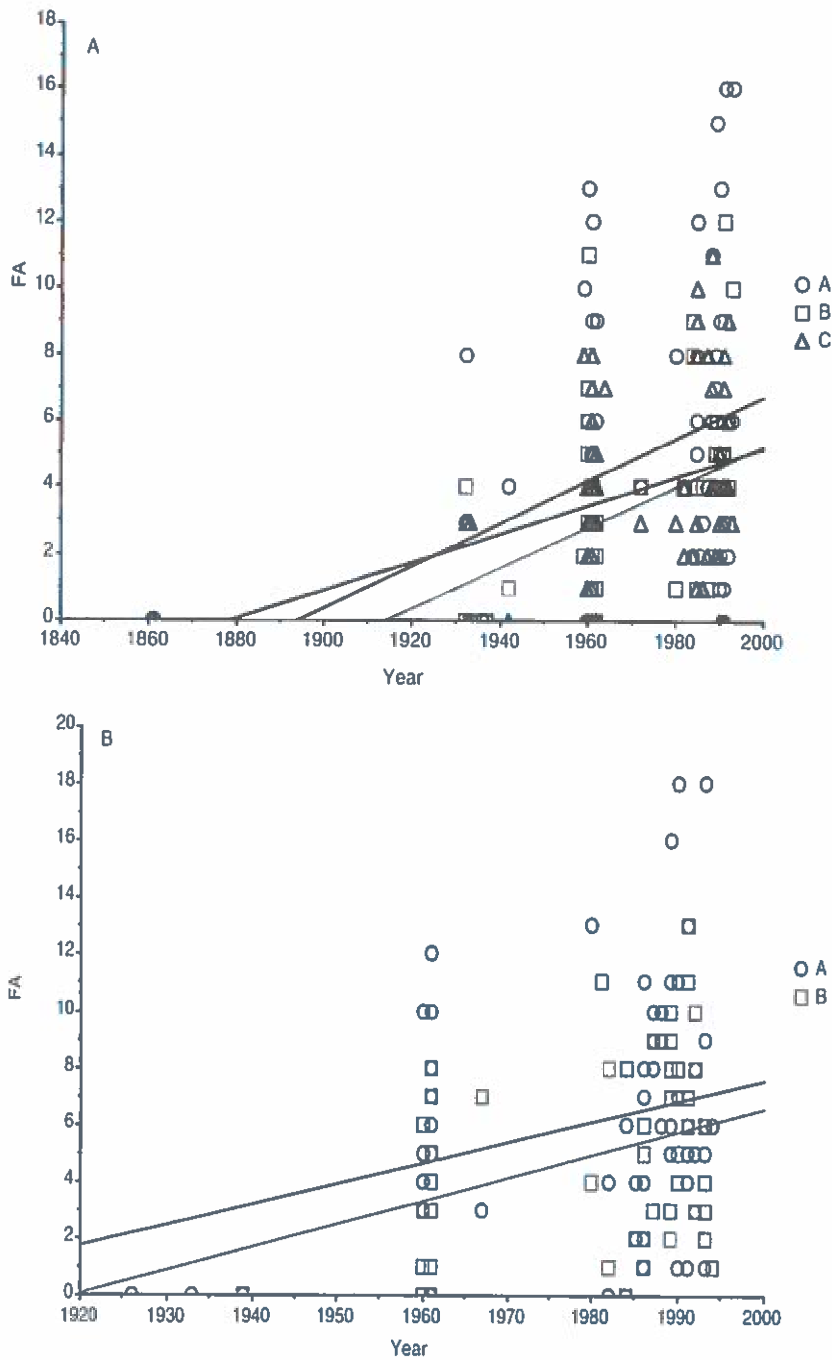


Fig. 3. Linear regression (females) of the absolute values of FA (1/10 mm) for traits A, B and C versus the year of skull collection. — A: Females. — B: Males (the trait C is not significant in the regression).

Significant correlations were found between the year of skull collection and the absolute values of FA (Fig. 3AB, Table 3). The relationship and its significance did not disappear when we removed the two skulls collected in 1861 from the regression analysis for females, but the significance of the correlation disappeared for trait A in males when we removed the 3 skulls collected before 1950 from the regression analysis. Significant differences in variance (*F*-test) of FA between the three different periods were found (Table 4).

Significant differences of the median of FA in the three periods were found (Mann-Whitney *U*-test, Table 5).

Significant differences were found between sexes in the median of FA for traits A and C in the periods two and three (males showed higher FA, see Mann-Whitney *U*-test, Table 6).

4. Discussion

PCBs dissolve readily in animal fats and hence accumulate in tissues. When these fats are mobilised during periods of stress, such as food shortage or periods of reproduction, large amounts of pesticide may be released into the blood stream (see Mason 1989 for review).

There was, however, no evidence for a relationship between asymmetry and pesticide concentration. The lack of correlation between contaminant concentration and FA could be due to different factors that could have confused relations. First, in mammals, females detoxicate

Table 3. Linear, 2nd and 3rd order polynomial regressions between the absolute value of FA in traits A, B and C and the year of skull collection. The numbers in parentheses are the sample sizes and the standard error for the regression coefficient, respectively.

Traits	Linear regression	2nd order	3rd order
Males			
A	0.26 (n = 65, *S.E.* = 0.003)*	*n.s.*	*n.s.*
B	0.34 (n = 62, *S.E.* = 0.003)**	0.35 (n = 62, *S.E.* = 2.682×10^{-4})*	*n.s.*
C	*n.s.*	0.31 (n = 75, *S.E.* = 1.672×10^{-4})*	*n.s.*
Females			
A	0.38 (n = 66, *S.E.* = 0.002)**	0.41 (n = 66, *S.E.* = 3.914×10^{-5})**	0.41 (n = 66, *S.E.* = 1.843×10^{-6})**
B	0.35 (n = 51, *S.E.* = 0.002)*	0.35 (n = 51, *S.E.* = 1.158×10^{-4})*	*n.s.*
C	0.32 (n = 65, *S.E.* = 0.002)**	0.32 (n = 65, *S.E.* = 3.202×10^{-5})*	*n.s.*

$p < 0.05$ = *, $p < 0.01$ = **

Table 4. *F*-test. Comparison of variance of the skull traits (A, B and C and dental foramen, named F) in the 3 periods (1, 2 and 3). The sign (+) or (–) indicate an increase or decrease in variance of the traits ($r - l$) with respect to the previous period. The numbers in parentheses are degrees of freedom and X indicates the absence of sufficent data.

	Period 1				Period 2			
	A	B	C	F	A	B	C	F
Males								
Period 2								
A	X							
B		X						
C			(+)*(2,17)					
F				(+) *n.s.* (2,13)				
Period 3								
A	X				(+) *n.s.* (16,45)			
B		X				(+)*(17,43)		
C			(+)*(3,55)				(–)*(17,55)[1]	
F				(+) *n.s.* (2,24)				(+) *n.s.* (13,24)
Females								
Period 2								
A	(+) *n.s.* (7,25)							
B		(+)*(6,22)						
C			(+) *n.s.* (6,19)					
F				(+) *n.s.* (6,16)				
Period 3								
A	(+)*(7,31)				(+) *n.s.* (25,31)			
B		(+)*(6,22)				(+) *n.s.* (22,22)		
C			(+)*(6,37)				(+)*(19,37)	
F				(+) *n.s.* (6,16)				(+)*(16,16)

[1] Significance disappears if one extreme value is removed, $p < 0.05$ = *

through nursing, where the organochlorines are transferred from mother to cub in the lipids of the milk (Tanabe *et al.* 1982). Thus, a female otter, after the nursing period, will have a lower concentration of PCBs, and developmental stability may thus not be related to pesticide concentrations. Hence, male and subadult otters may be more appropriate for this comparison, however, no significant correlations were found for these groups alone either.

The high correlation between the year of collection and FA may be explained by the increase in disturbing factors due to landscape fragmentation, pollution, human activity and agricultural practice. All these factors began to increase sharply at the beginning of the 1940s. A declining trend in population size prevailed throughout the 1970s. Populations of small size, for a number of generations, may lose a substantial proportion of genetic variation, and the genetic structure of the population will change, homozygosity will increase and inbreeding depression may become significant. The bottleneck around 1970–1980 may have resulted in increased FA in otter skulls. The N_e of about 20 individuals hypothesised in the 1970s, was probably even lower due to the

Table 5. Mann-Whitney *U*-test for testing if FAs of traits A, B and C are significantly higher in the later of the two periods compared. n_1 and n_2 are the sample sizes and *U* the test values. The values in parentheses are the mean and the standard error of FA of the earlier of the two periods compared.

	Traits A	Traits B	Traits C
Males			
Periods 1–2	$U = 4.5^*$ (0, 0) $n_1 = 3$, $n_2 = 16$	n.s.	n.s.
Periods 2–3	n.s.	$U = 220.5^*$ (0.339, 0.061) $n_1 = 17$ $n_2 = 43$	$U = 283.5^*$ (0.659, 0.101) $n_1 = 17$ $n_2 = 55$
Periods 1–3	$U = 3^{**}$ (0.659, 0.069) $n_1 = 3$, $n_2 = 45$	n.s.	n.s.
Females			
Periods 1–2	n.s.	$U = 29^*$ (0.083, 0.065) $n_1 = 6$, $n_2 = 22$	n.s.
Periods 2–3	$U = 238^*$ (0.376, 0.089) $n_1 = 25$, $n_2 = 31$	n.s.	n.s.
Periods 1–3	$U = 40.5^*$ (0.639, 0.079) $n_1 = 7$, $n_2 = 31$	$U = 15^{**}$ (0.44, 0.069) $n_1 = 6$, $n_2 = 22$	$U = 49.5^*$ (0.46, 0.053) $n_1 = 6$, $n_2 = 37$

$p < 0.05$ = *, $p < 0.01$ = **

Table 6. Mann-Whitney *U*-test for testing the differences of FA in traits A, B and C and dental foramen between the two sexes. For this test, only two periods are considered (periods 1 and 2). n_1 and n_2 are the sample sizes and *U* the test values. An asterisk means a significantly higher degree of FA in males.

	Traits A	Traits B	Traits C	Dental foramen
Period 2	$U = 95^*$ $n_1 = 22$, $n_2 = 16$	n.s.	$U = 118^*$ $n_1 = 24$, $n_2 = 17$	n.s.
Period 3	$U = 231^*$ $n_1 = 22$, $n_2 = 45$	n.s.	n.s.	n.s.

$p < 0.05$ = *

effect that PCBs have on female fertility. Experiments with the closely related mink (*Mustela vison*) showed that pup mortality was severe when tissue concentrations of PCBs in their mother exceeded 50 mg kg^{-1} fat (Jensen *et al.* 1977). 18% of Danish otters had tissue concentrations exceeding 50 mg kg^{-1} and 21% had tissue PCB concentrations greater than 30 mg kg^{-1} (Mason & Madsen 1993).

Strong FAs, as in the skulls of otters, that are visible macroscopically, have been suggested to be the result of strong selective forces (Møller 1992). Probably, male and female otters are subject to different selective forces. In the Eurasian otter (like all mustelids), males are always the larger sex and until now there are two explanations for this size dimorphism (Moors 1980). The first hypothesis suggests that the dimorphism reduces intersexual competition for food by enabling each sex to exploit different prey. The second hypothesis takes into account the polygynous breeding systems of mustelids and that females alone raise their litters. It proposes that small females are favoured because they need less energy for daily maintenance. Because of this, they can channel more energy into reproduction than larger females. Larger males are favoured by sexual selection and the ability to exploit a wide range of prey, and for the enhanced dominance and mobility. Under these circumstances, dissimilar evolutionary forces would be acting on each sex and consequently the optimum size of each sex results from different selective pressures.

The only evidence for intrasexual selection favouring larger male mustelids comes from observations of fighting during the breeding season, and this is a common phenomenon among otters and other mustelids (Moors 1980). One factor that may help to promote fight avoidance is that resident and probably larger otters know their place in the social hierarchy. Nothing is known about the extent of epigamic selection in otters or its possible influence on the size of males. In mammals, male hierarchy positions appear to be strongly correlated with body size (Schaller 1967, Grant 1970, Erlinge 1977). If a larger body size gives a reproductive advantage to males and a smaller one gives an energetic advantage to females, body sizes are subject to selection with opposite directions between sexes. The intensity of selection may have increased with increased landscape fragmentation, because the decreasing number of suitable sites will increase the territorial behaviour due to the increased density of individuals in the suitable sites. Only the dominant male, with a large body size, may be able to defend a good territory. The length of skulls (condylobasal-length) was strongly correlated to weight. So probably the size of the skull reflects dominance of an individual, and the selective forces acting on this character may explain the relatively high level of FA found on the more recent otter skulls. Another interesting observation is the higher degree of FA found in males than in females, suggesting the potentially stronger effect of stress acting on males.

Acknowledgements. Part of the work has been supported by a grant from the Danish Rektorkollegiet. We thank Prof. Anders Pape Møller for his constructive critique of the manuscript, Henrik Rønnow for his drawing of the otter skull, Mogens Andersen (Zoological Museum, Copenhagen) and to Erling Mørch and Bjørn Jacobsen (Natural History Museum, Aarhus) for providing access to collections. Furthermore we thank Anabela Jensen and Luigi Cagnolaro for suggestions.

References

Britten, B. H. 1996: Meta-analyses of the association between multilocus heterozygosity and fitness. — Evolution 50: 2158–2164.

Chanin, P. 1991: Otter. — In: Corbet, G. B. & Harris, S. H. (eds.), The handbook of British mammals: 423–431. Blackwell Scientific Publications, Oxford.

Clarke, G. M. & McKenzie, J. A. 1987: Developmental stability of insecticide resistant phenotypes in blowfly; a result of canalizing natural selection. — Nature, Lond. 325: 345–346.

Erlinge, S. 1977: Agonistic behaviour and dominance in stoat (Mustela erminea). — L. Z. Tierpsychol. 44: 375–388.

Fowler, J. & Cohen L. 1990: Practical statistics for field biology. — Open University Press, Philadelphia.

Frankham, R. 1995: Effective population size/adult population size ratios in wildlife: a review. — Genet. Res. 66: 95–107.

Grant, T. R. 1970: Experimental studies of competitive interaction in a two species system: II, the behaviour of (Microtus), (Peromyscus) and (Clethrionomys) species. — Animal Behaviour 18: 411–426.

Hammershøj, M., Madsen, A. B., Bruun-Schmidt, I. Ø., Gaardmand, B., Jensen, A., Jensen, B., Jeppesen, J. L. & Laursen, J. T. 1996: Overvågning af odder (Lutra lutra) i Danmark 1996. Danish Nat. Env. Res. Inst.

Report N° 172.
Jensen, S. 1972: The PCB story. — Ambio 1: 123–131.
Jensen, S. & Kihlstrom, M., Olsson, C., Lundberg, C. & Orberg, J. 1977: Effects of PCB and DDT on mink (Mustela vison) during the reproductive season. — Ambio 6: 239.
Leary, R. F. & Allendorf, F. W. 1989: Fluctuating asymmetry as an indicator of stress: Implications for conservation biology. — Trends Ecol. Evol. 4: 214–217.
Madsen, A. B. 1996: Odderens (Lutra lutra) økologi og forvaltning i Danmark. The ecology and conservation of the Otter (Lutra lutra) in Denmark. — Ph.D.-thesis. Danish Nat. Env. Res. Inst. 84 pp.
Manning, J. T. & Chamberlain, A. T. 1993: Fluctuating asymmetry, sexual selection and canine teeth in primates. — Proc. R. Soc. Lond. B251: 83–87.
Mason, C. F. 1989: Water pollution and otter distribution : a review. — Lutra 32: 97–131.
Mason, C. F. & Macdonald, S. M. 1986: Otters: Ecology and Conservation. — Cambridge University Press, Cambridge.
Mason, C. F. & Madsen, A. B. 1993: Organochlorine pesticide residues and PCBs in Danish otters (Lutra lutra). — The Science of the Total Environment 133: 73–81.
Møller, A. P. 1992: Patterns of fluctuating asymmetry in weapons: evidence for reliable signalling of quality in beetle horns and bird spurs. — Proc. R. Soc. Lond. B248: 199–206.
Moors, P. J. 1980: Sexual dimorphism in the body size of mustelids (Carnivora): the roles of food habits and breeding systems. — Oikos 34: 147–158.
Palmer, A. R. & Strobeck, C. 1986: Fluctuating asymmetry: measurement, analysis, patterns. — Annu. Rev. Ecol. Syst. 17: 391–421.
Parsons, P. A. 1962: Maternal age and developmental variability. — J. Exp. Biol. 39: 251–260.
Parsons, P. A. 1990: Fluctuating asymmetry: An epigenetic measure of stress. — Biol. Rev. 65: 131–145.
Parsons, P. A. 1992: Fluctuating asymmetry: a biological monitor of environmental stress. — Heredity 68: 361–364.
Schaller, G. B. 1967: The deer and the tiger: a study of wildlife in India. — University of Chicago Press, Chicago.
Schimmer, A. 1981: Odderen i Danmark 1950–1980. — M.Sc.-thesis. University of Copenhagen. 108 + 149 pp.
Sciulli, P. W., Doyle, W. J., Kelley, C., Siegel, P. & Siegel, M. I. 1979: The interaction of stressors in the induction of increased levels of fluctuating asymmetry in the laboratory rat. — Am. J. Phys. Anthropol. 50: 279–284.
Siegel, M. I. & Doyle, W. J. 1975: The differential effect of prenatal and postnatal audiogenic stress on fluctuating dental asymmetry. — J. Exp. Zool. 191: 211–214.
Sokal, R. R. & Rohlf, F. J. 1981: Biometry. — W. H. Freeman, San Francisco.
Swain, D. P. 1987: A problem with the use of meristic characters to estimate developmental stability. — Am. Natur. 129: 761–768.
Tanabe, S., Tatsukawa, R., Maruyama, K. & Miyazoki, N. 1982: Transplacental transfer of PCBs and Chlorinated hydrocarbon pesticides from pregnant striped dolphin (Stenella coeruleoalba) to her foetus. — Agric. Biol. Chem. 46: 1249–1254.
Valentine, D. W. & Soulé, M. E. 1973: Effect of p,p' DDT on developmental stability of pectoral fin rays in the grunion (Leuresthes tenius). — Fishery Bull. 71: 920–921.
Van Bree, P. J. H., Jensen, B. & Kleijn, L. J. K. 1966: Skull dimensions and the length/weight relation of the baculum as age indications in the common otter, (Lutra lutra) (Linnaeus, 1758). — Dan. Rev. Game Biol. 4: 97–104.
Van Valen, L. 1962: A study of fluctuating asymmetry. — Evolution 16: 125–142.
Weiner, J. G. & Rago, P. J. 1987: A test of fluctuating asymmetry in bluegills (Lepomis macrochirus) as a measure of PH related stress. — Environ. Pollut. 44: 27–36.
Zar, J. H. 1984: Biostatistical Analysis. — Prentice Hall, New Jersey.

Ann. Zool. Fennici 35: 87–94 ISSN 0003-455X
Helsinki 30 September 1998 © Finnish Zoological and Botanical Publishing Board 1998

Variation of skull morphometry of Eurasian otters (*Lutra lutra*) in Denmark and Germany

Cino Pertoldi, Aksel Bo Madsen, Ettore Randi, Anja Braun & Volker Loeschcke

Pertoldi, C & Loeschcke, V., Department of Ecology and Genetics, University of Aarhus, Building 540, Ny Munkegade, DK-8000 Aarhus C, Denmark.
Madsen, A. B., Department of Landscape Ecology, National Environmental Research Institute, Kalø Grenåvej 14, DK-8410 Rønde, Denmark.
Randi, E., Istituto Nazionale per la Fauna Selvatica, via Ca' Fornacetta 9, I-40064 Ozzano Emilia (Bo), Italy.
Braun, A., Institut für Zoologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, Staudtstrasse 5, D-91058 Erlangen, Germany.

Received 19 January 1998, accepted 30 June 1998

The distance between opistokranion and zygomatic process of frontal bone (A), the condylobasal length (X), and the total length of the mandible (M) were measured in skulls of the Eurasian otter collected in Denmark and in the east of Germany (Lausitz). The Danish population showed a strong reduction in size of the traits in males, and an increase with time in the variances of the traits in females, whereas in the Lausitzian population, no significant changes in the same traits over the same time period were observed. The skulls were also investigated for fluctuating asymmetry (FA) and a negative correlation was found between the size of a skull and FA in the males from both populations, whereas no correlations were found in the females.

1. Introduction

Spatial and temporal variations in habitat quality and population density can affect adult body size and skull traits in species (Klein 1964, Lowe 1972, Holbrook 1982). Morphometrical differences between local populations distributed over the species range may be of a genetic origin, and affect growth rates and development stability (Manning 1971, Huson & Page 1980, Atchley *et al.* 1981).

There are several hypotheses which can explain the relation between ecological factors and size variability. Among them, the "niche variation hypothesis" (Van Valen 1965) suggests that morphological variation is related to the number of competing species, as a niche width can increase in absence of competitors, determining an increase in morphometrical variance (Grant 1967, Rothstein 1973). Moreover, in species with sexual dimorphism, different evolutionary forces are acting on each sex, and the optimum size of males and females may result from different selective pressures (Erlinge 1979, Moors 1980). Finally, variation in fluctuating asymmetry (FA) of traits may be related to the individual's body size. If larger body signals generally good health, body size will be negatively correlated with the FA (see reviews in Møller & Pomiankowski 1993, Møller & Swad-

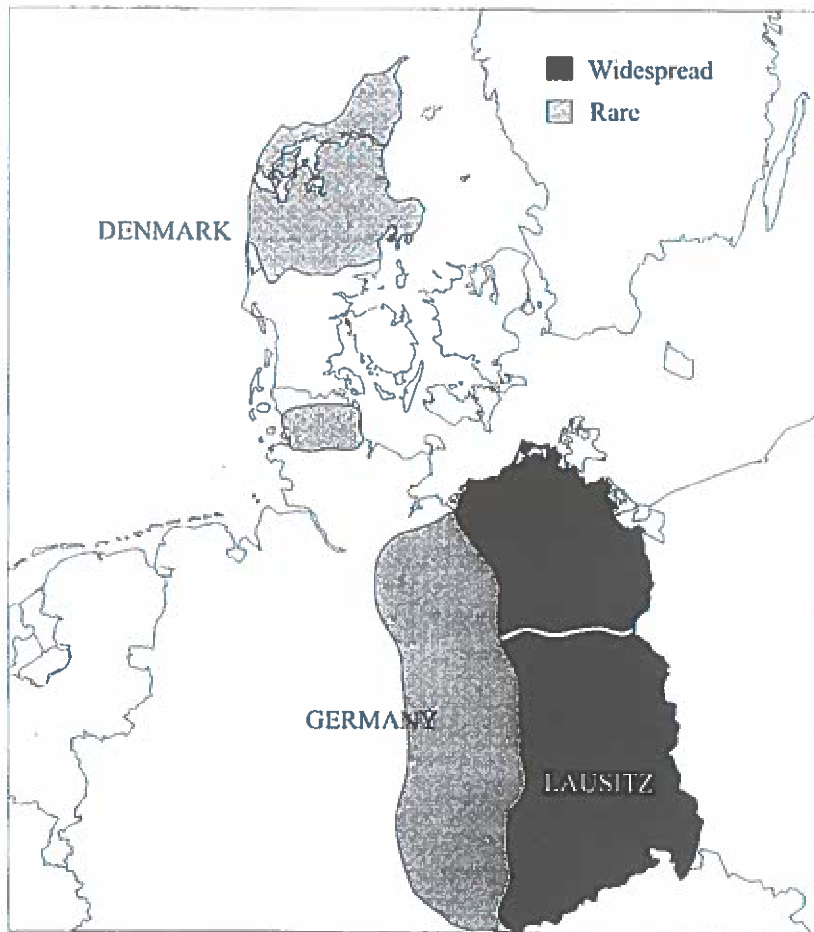


Fig. 1. The distribution of the otter in Denmark and in Germany (according to Macdonald & Mason 1994, and Hammershøj *et al.* 1996).

dle 1997). The above hypotheses could be tested by analysing temporal series of morphometric measurements in species which have experienced environmental or genetic stress.

The Eurasian otter provides an interesting example. The species has declined in many European countries (Macdonald & Mason 1994), and in optimal habitat conditions it has virtually no ecological overlap or potential interspecific competition with other species (Chanin 1991), and therefore, the degree of sexual dimorphism should not depend on interspecific interactions. However, in suboptimal habitats, with a possibility of food shortage, we can suspect a dietary overlap with the smaller American mink (*Mustela vison*), which has been accidentally released from Danish mink farms since the beginning of the 1940s (Andersen 1981).

In Denmark, the otter was widespread, but now it is largely restricted to the Limfjord area in the northern part of Jutland (Fig. 1), with isolated population fragments in the surrounding region (Hammershøj *et al.* 1996). Toxic chemicals are likely to have been responsible for the decline of the otter population in Denmark, and large parts of lowland Europe (Mason & Macdonald 1986, Mason 1989). Today, the additional threat is mortality caused by traffic, wetland destruction, and human disturbance (see Madsen 1996 for review). The otter population in Denmark declined sharply from more than 1 500 animals in 1961 to only 200 individuals in 1980 (Schimmer 1981), and increased thereafter again to 400 animals censused in 1991. The reproductive biology of polygynous mammals like the Eurasian otter suggests that the ratio of the genetically effective to the observed population sizes is low (see Frankham 1995 for review). Assumptions of a small effective population size are also supported by a very low genetic variability found in the mitochondrial DNA control region (N. Mucci, C. Pertoldi, A. B. Madsen, V.

Loeschcke & E. Randi unpubl.), and by the increasing with time levels of fluctuating asymmetry in skulls (Pertoldi *et al.* 1997). Therefore, we can consider the Danish otter population to be endangered as compared to, for example, the population living in Lausitz (eastern Germany).

In this paper, we aim to estimate the variation in size of different skull traits of two populations of the European otter. We test for variation in length and its variance in different periods of collection, to see whether the presumed bottleneck in the Danish population, and the concomitant increased habitat fragmentation in the Danish territory, have influenced the otters' developmental stability. The same test was made for the Lausitzian population (Fig. 1) which presumably has not experienced bottlenecks and does not live in a fragmented habitat (Macdonald & Mason 1994).

2. Material and methods

2.1. Measurements

We measured three traits in 94 Danish and 44 Lausitzian skulls of adult Eurasian otters *Lutra lutra* collected in 1861–1959 (13 specimens at the Zoological Museum in Copenhagen, Denmark), in 1959–1961 (34 specimens at the Natural History Museum in Aarhus, Denmark), in 1979–1993 (47 specimens at the National Environmental Research Institute in Kalø, Denmark), and in 1955–1996 (44 specimens at the Staatliches Museum für Naturkunde in Görlitz, Germany). The traits, measured with a digital calliper to the nearest 0.1 mm, were the distance between opistokranion and zygomatic process of a frontal bone (A), the condylobasal length (X), and the total length of a mandible (M; Infradentale-Goniocaudale), (illustration given in Pertoldi *et al.* 1997). Because traits A and M are bilateral, and in a previous investigation (Pertoldi *et al.* 1997) they showed a significant fluctuating asymmetry, they were measured on both sides of the skulls. Unfortunately, trait A was not measured in the Lausitzian skulls.

Asymmetry was estimated as the difference in length between each bilateral pair of traits (right side [r] – left side [l]). Fluctuating asymetry (FA) was calculated as the absolute value of asymmetry (Palmer & Strobeck 1986).

$$\mathrm{FA} = |r - l| \quad (1)$$

In a previous investigation of the skulls of the Danish otters, the overall repeatability of the FA was estimated to be 97% or greater, so that the measurement error should have accounted for no more than 3% of the total variation (for details see Pertoldi *et al.* 1997). To estimate possible errors in measuring the condylobasal length, we had chosen ten skulls at random, and measured each of them ten times. Coefficient of variation (CV, %) with Haldane's (1955) correction for small sample size, was calculated for the mean condylobasal length (X) of each skull. The average CV was 0.1%.

We estimated the age of the animals whose skulls were used in our research using the methods described by Pertoldi *et al.* (1997).

2.2. Statistical analysis

In all the analysis, sexes were treated separately. Spearman's rank correlation coefficients (r_s) were calculated for the condylobasal (X), and mandible lengths (M) versus total body length (from nose to tail), and versus body weight of the Danish adult otters. These correlations were computed to test if the skull traits are good indicators of the adult body mass. A linear regression analysis and a Spearman test for traits X and M versus the year of collection of the otters' skull were used. The years of the skulls' collection were grouped in two periods: 1960–1961 and 1981–1994 for the Danish males, 1959–1964 and 1982–1993 for the Danish females, 1955–1985 and 1986–1996 for the Lausitzian males, 1968–1986 and 1987–1996 for the Lausitzian females. Means and variances of traits X and M were calculated, and an F-test (Fowler & Cohen 1990) was employed to compare the variances between both periods of collection. For traits which variances did not differ significantly, we conducted a two way t-test to test for significant differences between the means.

A Mann-Whitney U-test was used to test if the median of the distribution of traits X and M increased or decreased, and consequently, if the degree of dimorphism varied in the two periods. The average degree of sexual dimorphism was measured for the skull length (X) and calculated as follows: $\mathrm{SD} = [\mathrm{mean}_{males} - \mathrm{mean}_{females}]/\mathrm{mean}_{females} \times 100\,(\%)$ (Rossolimo & Pavlinov 1974).

A linear regression analysis and a Spearman rank correlation test were used to test if the fluctuating asymmetry of traits A and M was correlated with X, A and M. When calculating these relations we used largest size per pair to avoid autocorrelation effects (Sullivan *et al.* 1993).

3. Results

In a population of the Danish male otters, positive correlations (Spearman test) between the condylobasal length (X) and mandible length (M) of an otter's skull versus the weight and length of an animal before necropsy were found (Table 1). However, the correlations in females were weaker than those found in males, and the correlation between the skull mandible length and an animal body weight in females was not significant (Table 1). Significant negative correlations (linear

regression analysis and Spearman test) between the year of collection of the skulls and traits *X* and *M* in the Danish males were found. No such correlations were detected in Danish females and in Lausitzian males and females (Table 2).

In the Danish males and Lausitzian males and females, no significant differences in variances (*F*-test) of traits *X* and *M* between the two periods of collection were found (Table 3). A significant increase in variance of the Danish female skull traits was recorded in the second period of collection (Table 3). In the Danish males, we found a significant reduction in the means of the traits in the second period. In the Lausitzian males and females, we did not find a significant change in the means of the traits in the second period of collection (Table 3). The average degree of sexual dimorphism in trait *X* of the Danish population was 9.5% in the first period and 6.1% in the second period, hence, a reduction of 3.4%.

In the Danish males, significant differences ($p < 0.05$) in the medians of the traits between the two periods were found (Mann-Whitney *U*-test, trait *X*: $U = 112.5$*, $n_1 = 15$, $n_2 = 25$, trait *M*: $U = 76.5$*, $n_1 = 13$, $n_2 = 22$), (lower value of the median in the second period), which was in concordance with the result obtained with the parametric *t*-test (Table 3). No significant differences in the medians of the Danish female traits or in the Lausitzian male and female traits between the collection periods were found.

A negative correlation (linear regression analysis and Spearman test) between traits *A* and *M* and the degree of FA in the males, and a general positive correlation between some traits and the degree of FA in the females were found (Table 4).

Table 1. The Spearman rank correlation coefficient (r_s) calculated for traits *X* and *M* versus the body weight, and length of the Danish otters; *n* = sample size

Traits	Body weight	Body length
X (♂)	0.75 (*n* = 35)***	0.84 (*n* = 38)***
M (♂)	0.47 (*n* = 23)*	0.69 (*n* = 24)**
X (♀)	0.41 (*n* = 25)*	0.75 (*n* = 25)***
M (♀)	n.s. (*n* = 23)	0.49 (*n* = 31)**

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

4. Discussion

The strong correlation between traits *X* and *M* (condylobasal length and mandible length) and the body weight and length found in the males otters, indicates that the size of a head is a good estimator of the body size of a male. Hence, we can suspect that the head is used for interspecific and intraspecific signals in threat behaviour as was demonstrated in amphibians (Shine 1979), snakes (Shine 1994), and salmon (Quinn and Foote 1994). A large head may enhance male's mating success, either because males with large heads will more often win a male-male combat for access to females or because they have a possibility to maintain the best territory (Moors 1980, Lynch & Hayden 1995). Some authors (Dayan & Simberloff 1994) suggested that selection acts directly on the trophic apparati, while skull and body sizes may also be affected by other, perhaps autecological, factors. The reasons for the lower degree of correlation or lack of correlation found between body weight of a female and the mandible length (*M*), could have different explanations. The first one, which supports the hypothesis that the head is involved in signalling the dominance status in males, is that there is no intrasexual competition between females and that the males usually tolerate the presence of females within their home range. Therefore, the dominance status should not

Table 2. The linear regressions (*r*) and the Spearman test (r_s) between trait *X*, *M* and the year of the skulls' collection; *n* = sample size.

Country	*r*	r_s
Denmark		
X (♂)	– 0.41 (*n* = 44)**	– 0.36 (*n* = 44)*
M (♂)	– 0.38 (*n* = 37)*	– 0.33 (*n* = 37)*
X (♀)	n.s. (*n* = 49)	n.s. (*n* = 49)
M (♀)	n.s. (*n* = 36)	n.s. (*n* = 36)
Germany (Lausitz)		
X (♂)	n.s. (n = 27)	n.s. (*n* = 27)
M (♂)	n.s. (*n* = 26)	n.s. (*n* = 26)
X (♀)	n.s. (*n* = 16)	n.s. (*n* = 16)
M (♀)	n.s. (*n* = 17)	n.s. (*n* = 17)

* = $p < 0.05$, ** = $p < 0.01$

Table 3. Comparison of traits X and M in the skulls collected in two periods. For traits which variances did not differ significantly, we conducted a two ways t-test to test for significant differences between the means.

Traits	Variance	n	Mean (mm)	Variance	n	Mean (mm)	F-test		t-test	
							p	F	p	df
				Denmark males						
	Period (1960–1961)			Period (1981–1994)						
X	13.30	15	119.2	12.4	25	116.5	n.s.	1.07	*	38
M	5.60	13	75.6	5.00	22	73.7	n.s.	1.12	*	33
				Denmark females						
	Period (1959–1964)			Period (1982–1993)						
X	5.50	19	108.9	12.28	22	109.8	*	2.22	–	–
M	1.30	17	69.1	6.89	17	68.5	**	4.99	–	–
				Lausitzian males						
	Period (1955–1985)			Period (1986–1996)						
X	14.71	7	117.3	16.64	20	119.0	n.s.	1.13	n.s.	25
M	4.24	6	75.9	8.52	20	76.2	n.s.	2.02	n.s.	24
				Lausitzian females						
	Period (1968–1986)			Period (1987–1996)						
X	1.44	6	109.4	2.76	10	110.7	n.s.	1.92	n.s.	25
M	0.93	6	69.2	2.43	11	69.5	n.s.	2.61	n.s.	24

* = $p < 0.05$, ** = $p < 0.01$

Table 4. The linear regressions (r) and the Spearman tests (r_s) between traits X, A, M and FA of these traits; n = sample sizes.

Country	Traits	FA	r	r_s
Denmark	A (♂)	FA (A)	n.s. (n = 26)	n.s. (n = 23)
	M (♂)	FA (M)	– 0.37 (n = 30)*	– 0.42 (n = 30)*
	X (♂)	FA (A)	– 0.41 (n = 24)*	– 0.51 (n = 24)*
	X (♂)	FA (M)	– 0.52 (n = 28)**	– 0.50 (n = 28)**
	A (♀)	FA (A)	0.28 (n = 48)*	0.37 (n = 48)*
	M (♀)	FA (M)	n.s. (n = 36)	n.s. (n = 36)
	X (♀)	FA (A)	n.s. (n = 48)	n.s. (n = 48)
	X (♀)	FA (M)	n.s. (n = 35)	n.s. (n = 35)
Germany (Lausitz)	M (♂)	FA (M)	– 0.57 (n = 26)**	n.s. (n = 26)
	X (♂)	FA (M)	– 0.54 (n = 26)**	n.s. (n = 26)
	M (♀)	FA (M)	0.60 (n = 15)*	0.65 (n =15)*
	X (♀)	FA (M)	0.66 (n = 14)*	0.75 (n = 14)**

* = $p < 0.05$, ** = $p < 0.01$

be important for females. The second explanation could be that the females used in our research were collected in different seasons, and that they represented different breeding status and therefore, that weight fluctuations could have confused the correlation.

The strong negative correlation found between the year of collection of the Danish male otters and the length of traits *X* and *M* (which means a shortening of the skull), may be explained by the general increase of stressing factors like pollution, human activity, food shortage in terms of quantity and quality, agricultural practice, habitat fragmentation, increased intrasexual competition, and their impact on the genetic structure of the population. The progressive reduction of suitable habitat and carrying capacity could have a particularly intense effect on otters which have a linear home range (Erlinge 1968, Kruuk 1995). All these factors will increase fragmentation effects that can be particularly intense if the costs associated with dispersal from one patch to another are high. That is the case in the Danish otter population, in which the number of otters killed by trafic increased with the increasing population and road network (see Madsen 1996 for review). The non-significant reduction in size of the skulls of the Lausitzian male otters indicates a more healthy and viable population, living in a more suitable habitat.

The reasons why we did not find a significant reduction in female skull traits in both the Lausitzian and Danish populations are different. Principally, the lower daily energy requirement of females, because of their smaller body size, smaller home range and the lack of intrasexual interaction (competition for mating, territory defending) (Moors 1980, Durbin 1996), will reduce the risk of starvation in periods of food shortage. These characteristics should lower the sensitivity to environmental fluctuations, particularly with regard to changes in food abundance. Another explanation could be the possibility that intermale competition (which is thought to have increased with the reduction of suitable habitats) may also affect female characteristics: if a large body confers major advantages on males and large mothers produce larger male offspring, intense competition between males may select for increased body size in females (Maynard Smith 1978). The increased morphological variance of skull traits *X* and *M* in the second period of collection with respect to the first one detected only in the Danish females, is probably due to some distinct causes. Some empirical studies indicate that higher phenotypic variability may be related to the reduction of the heterozygosity level (Eanes 1978, Mitton 1978, Leamy 1982, Mitton & Grant 1984). The low genetic variability in female otters could be the reason for the significant increase in phenotypical variance found in the second period (post bottleneck) as compared to the first period of collection. Also increased levels of stress may result in an increase of the phenotypic variance (e.g., Imasheva *et al.* 1997). The second reason could be the otters' increased niche width and diversification, as a degraded environment has a less structured ecosystem.

The discovered reduced sexual dimorphism in the skull length (*X*) (3.4%) between the Danish males and females (no significant reduction of the sexual size dimorphism were detected for the Lausitzian population) can influence the otter's diet. We should expect a significant increase in dietary overlap between sexes (intersexual competition) because of the reduction in size of the males which results in their reduced capacity to hunt bigger prey (Lynch & O'Sullivan, 1993). At the same time we should also expect an increase in an interspecific competition with smaller sympatric mustelid species like american mink, *Mustela vison*, especially in periods of food shortage as may happen during severe winters (Erlinge 1972).

The negative correlations between the skull traits (*A*, *M* and *X*) versus the level of FA found in males from both populations may be interpreted in the way that the big body size of a male otter is a good indicator of its general condition and its fitness (see reviews in Møller & Pomiankowski 1993, Møller & Swaddle 1997). The positive correlations between body size and FA found in females could then indicate the non-adaptive function of a big body size in females and the increased energetic cost associated with a bigger body size.

Acknowledgements: We thank Hermann Ansorge (Staatliches Museum für Naturkunde, Görlitz), Mogens Andersen (Zoological Museum, Copenhagen), Erling Mørch and Bjørn Jacobsen (Natural History Museum, Aarhus), for providing access to collections and Kirsten Zaluski for linguistic suggestions. Furthermore, we thank Bo Gaardmand for invaluable suggestions and help.

References

Andersen, J. 1981: Minken (Mustela vison) og minkjagten i Danmark 1970/71 og 1972/73. — Danske Vildtundersøgelser 34: 1–24.

Atchley, W. R., Rutledge, J. J. & Cowley, D. E. 1981: Genetic components of size and shape. II. Multivariate covariance patterns in the rat and mouse skull. — Evolution 35: 1037–1055.

Britten, B. H. 1996: Meta-analyses of the association between multilocus heterozygosity and fitness. — Evolution 50: 2158–2164.

Chanin, P. 1991: Otter. — In: Corbet, G. B. & Harris, S. H. (ed.), The handbook of British mammals: 423–431. Blackwell Scientific Publications, Oxford.

Dayan, T. & Simberloff, D. 1994: Character displacement, sexual dimorphism, and morphological variation among British and Irish mustelids. — Ecology 75: 1063–1073.

Durbin, L. S. 1996: Individual differences in spatial utilization of a river-system by otters (Lutra lutra). — Acta Theriologica 41: 137–147.

Eanes, W. F. 1978: Morphological variance and enzyme heterozygosity in the monarch butterfly. — Nature 276: 263–264.

Erlinge, S. 1968: Territoriality of the otter (Lutra lutra). — Oikos 19: 81–98.

Erlinge, S. 1972: Interspecific relations between otter (Lutra lutra) and mink (Mustela vison) in Sweden. — Oikos 23: 327–335.

Erlinge, S. 1979: Adaptative significance of sexual dimorphism in weasels. — Oikos 33: 233–245.

Fowler, J. & Cohen L. 1990: Practical statistics for field biology. — Open University Press, Philadelphia.

Frankham, R. 1995: Effective population size/adult population size ratios in wildlife: a review. — Genet. Res. 66: 95–107.

Grant, P. R. 1967: Bill length variability in birds of the Tres Marias Islands, Mexico. — Can. J. Zool. 45: 805–815.

Haldane, J. B. S. 1955: The measurement of variation. — Evolution 9: 484.

Hammershøj, M., Madsen, A. B., Bruun-Schmidt, I. Ø., Gaardmand, B., Jensen, A., Jensen, B., Jeppesen, J. L. & Laursen, J. T. 1996: Overvågning af odder (Lutra lutra) i Danmark 1996. Otter (Lutra lutra) Survey of Denmark 1996. — Danish Nat. Env. Res. Inst. Report N° 172.

Holbrook, S. 1982: Ecological inferences from mandibular morphology of (Peromyscus maniculatus). — J. Mammal. 61: 436–448.

Huson, L. W. & Page, R. J. C. 1980: Multivariate geographical variation of the red fox (Vulpes vulpes) in Wales. — J. Zool., Lond. 191: 453–459.

Imasheva, A. G., Loeschcke, V., Zhivotovsky, L. A. & Lazebny, O. E. 1997: Effects of extreme temperatures on phenotypic variation and developmental stability in (Drosophila melanogaster) and (Drosophila buzzatii) — Biol. J. Linn. Soc. 61: 117–126.

Klein, D. R. 1964: Range-related differences in growth of deer reflected in skeletal ratios. — J. Mammal. 45: 226–235.

Kruuk, H. 1995: Wild Otters: predation and populations. — Oxford University Press, Oxford. 290 pp.

Leamy, L. 1982. Morphometric studies in inbreed and hybrid house mice. II. Patterns in the variances. — J. Hered. 73: 267–272.

Lowe, V. P. W. 1972: variation in mandible length and body weight of Red deer (Cervus elaphus). — J. Zool., Lond. 166: 303–311.

Lynch, J. M. & Hayden, T. J. 1995 Genetic influences on cranial form: variation among ranch and feral American mink (Mustela vison). — Biol. J. Lin. Soc. 55: 293-307.

Lynch, J. M. & O'Sullivan, W. M. 1993 Cranial form and sexual dimorphism in the Irish otter (Lutra lutra). — Biol. and Environ. Proc. R. Ir. Acad. 93: 97–105.

Macdonald, S. M. & Mason, C. F. 1994: Status and conservation needs of the otter (Lutra lutra) in the western Palaearctic. — Nature and Environment 67. 53 pp.

Madsen, A. B. 1996: Odderens (Lutra lutra) økologi og forvaltning i Danmark [The ecology and conservation of the Otter (Lutra lutra) in Denmark]. — PhD Thesis, Aarhus University. Danish Nat. Env. Res. Inst. 84 pp. [In Danish with English abstract].

Manning, T. H. 1971: Geographical variation in the polar bear (Ursus maritimus) Phipps. — Canadian Wildlife Service Report Series 13: 1–27.

Mason, C. F. 1989: Water pollution and otter distribution : a review. — Lutra 32: 97–131.

Mason, C. F. & Macdonald, S. M. 1986: Otters: Ecology and Conservation. — Cambridge University Press, Cambridge.

Maynard Smith, J. 1978: The evolution of sex. — Cambridge University Press, Cambridge.

Mitton, J. B. 1978: Relationship between heterozygosity for enzyme loci and variation of morphological characters in natural populations. — Nature 273: 661–662.

Mitton, J. B., & Grant, M. C. 1984: Associations among protein heterozygosity, growth rate and developmental homeostasis. — Annu. Rev. Ecol. Syst. 15: 479–499.

Møller, A. P. & Pomiankowski, A. 1993: Fluctuating asymmetry and sexual selection. — Genetica 89: 267–279.

Møller, A. P. & Swaddle, J. P. 1997: Asymmetry, developmental stability and evolution. — Oxford University Press, Oxford.

Moors, P. J. 1980: Sexual dimorphism in the body size of mustelids (Carnivora): the roles of food habits and breeding systems. — Oikos 34: 147–158.

Palmer, A. R. & Strobeck, C. 1986: Fluctuating asymmetry: measurement, analysis, patterns. — Annu. Rev. Ecol. Syst. 17: 391–421.

Pertoldi, C., Loeschcke, V., Madsen, A. B. & Randi E. 1997: Developmental stability in the Eurasian Otter (Lutra lutra) in Denmark. — Ann. Zool. Fennici 34: 187–196.

Quinn, T. P. & Foote, C. J. 1994: The effects of body size

and sexual dimorphism on the reproductive behaviour of sockeye salmon (Onchorhyncus nerka). — Anim. Behav. 48: 751-761.
Rossolimo, O. L. & Pavlinov, I. J. 1974: Sexual dimorphism in the development, size proportions of the skull in the pine marten (Martes martes). In: Biology of Mustelids: some Soviet research. — M. King, Dept. of Scientific and Industrial Research, New Zealand.
Rothstein, S. I. 1973: The niche-variation model. Is it valid? — Amer. Nat. 107: 598–620.
Schimmer, A. 1981: Odderen i Danmark 1950–1980. — MSc Thesis, University of Copenhagen. 108 + 149 pp.
Shine, R. 1979: Sexual selection and sexual dimorphism in the Amphibia. — Copeia 1979: 297-306.
Shine, R. 1994: Sexual size dimorphism in snakes revisited. — Copeia 1994: 326-346.
Sullivan, M. A., Robertson, P. A. & Aebischer, N. A. 1993: Fluctuating asymmetry measurement. — Nature 361: 409–410.
Van Valen, L. 1965: Morphological variation and the width of the ecological niche. — American Naturalist 99: 377–390.

Biological Journal of the Linnean Society (2000) *70*: 309–323. With 1 figure

doi:10.1006/bijl.1999.0379, available online at http://www.idealibrary.com on IDEAL

Craniometrical variability and developmental stability. Two useful tools for assessing the population viability of Eurasian otter (*Lutra lutra*) populations in Europe.

CINO PERTOLDI* AND VOLKER LOESCHCKE

Department of Ecology and Genetics, University of Aarhus, Building 540, Ny Munkegade, DK-8000 Aarhus C, Denmark

ANJA BRAUN

Institut für Zoologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, Staudtstrasse 5, D-91058 Erlangen, Germany

AKSEL BO MADSEN

Department of Landscape Ecology, National Environmental Research Institute, Kalø Grenåvej 14, DK-8410 Rønde, Denmark

ETTORE RANDI

Istituto Nazionale per la Fauna Selvatica, via Ca'Fornacetta 9, I-40064 Ozzano Emilia (Bo), Italy

Received 21 December 1998; accepted for publication 5 July 1999

Morphometrical univariate analyses of otter skulls collected over the past hundred years in European countries from presumed healthy populations were compared with skulls from presumed endangered populations. The average degree of sexual dimorphism of the European populations was found to be directly correlated to the skull size of the male otters. Fluctuating asymmetry (FA) in metric skull traits was analysed as an estimator of developmental stability. There was evidence for increased FA in different traits over time in some of the presumed endangered populations, and for a reduction in size of skull traits. In contrast, the healthy populations did not show any significant changes in the same traits during the same period. The reduced sexual dimorphism of the endangered populations is suggested to be a product

* Corresponding author: E-mail: Cino.Pertoldi@biology.aau.dk

0024–4066/00/060309 + 15 $35.00/0

© 2000 The Linnean Society of London

of relaxed sexual selection and deteriorated habitat conditions. Environmental and genetic forces that may have shaped these patterns are discussed.

© 2000 The Linnean Society of London

ADDITIONAL KEY WORDS:—Developmental stability – phenotypic plasticity – reaction norm – canalization – fluctuating asymmetry – otter.

CONTENTS

Introduction 310
Status of the otter in Europe 310
Fluctuating asymmetry and stressing factors 311
Variation in sexual dimorphism 311
Material and methods 311
Choice of traits 311
Fluctuating asymmetry and indices used 313
Measurement errors 314
Statistical analysis 314
Sexual dimorphism 315
Results 315
Measurement errors 315
Statistical analysis of fluctuating asymmetry 315
Sexual dimorphism and fluctuating asymmetry 316
Discussion 319
Correlation between skull size and fluctuating asymmetry 319
Fluctuating asymmetry 320
Environmental stresses 320
Genetic stresses 320
Sexual dimorphism and sexual selection 321
Phenotypic plasticity 321
Conservation strategies 322
Acknowledgements 322
References 322

INTRODUCTION

Status of the otter in Europe

The otter *Lutra lutra* has declined rapidly in numbers throughout much of the western Palearctic in the last forty years. In Scandinavia, major losses have occurred in Sweden (MacDonald & Mason, 1994 and references therein) and Denmark (Madsen, 1996). In western Europe, the species has disappeared from large areas. However, in France, along the Atlantic seaboard and in the Massif Central, healthy and viable populations have been detected, and this zone can be considered the last stronghold for the otter in western Europe. Another healthy and viable population is found in eastern Germany, while in the north of Germany (in Schleswig Holstein) there is a declining population that is probably completely isolated from the population living in eastern Germany (MacDonald & Mason, 1994). In Belgium, the species is extinct. In Spain, there is recent evidence that shows a declining trend of the population (MacDonald & Mason, 1994). In undisturbed areas in the north of Finland and in Hungary the otter populations seem healthy and viable. In southern Finland, the otter distribution is scattered and declining, which has been ascribed to increased road traffic (MacDonald & Mason, 1994). Causes of decline

are different in different countries, although water pollution (Mason, 1989), road casualties, poaching and drowning in fish nets (Madsen, 1996) could be the most relevant. Water pollution by PCBs does not seem to be one of the major threats. Kruuk (1995) found that otters in Scotland were thriving in spite of high PCB levels.

Fluctuating asymmetry and stressing factors

All the populations were investigated for fluctuating asymmetry (FA). Measures of developmental instability such as FA are often much more sensitive indicators of current environmental conditions than traditional measures used for monitoring various life history components such as growth, fecundity and survival (Clarke, 1995). Their potential for use in conservation biology was first reviewed by Leary & Allendorf (1989), in which they focused on the relationship between developmental stability and stress. However, developmental stability is influenced by many factors, e.g. pollution, stressful conditions during development and genetic stress (Møller & Swaddle, 1997). Furthermore, several authors have suggested that FA tends to be higher in sexually selected characters than in non-sexually selected traits because of their recent history of intense directional selection (Møller, 1992; Manning & Chamberlain, 1993; see Møller & Swaddle, 1997, for review).

In this investigation, we tested populations which are presumed endangered (on basis of the demographic data) to ascertain whether they had a lowered level of developmental stability compared to that of the populations presumed to be healthy and viable. We also took into account the limits of the dichotomy (endangered versus viable), as it is difficult in otters to quantify population densities.

Variation in sexual dimorphism

The degree of sexual dimorphism has often been used as a measure of the intensity of sexual selection in both interspecific (Barraclough *et al.*, 1995) and population-level studies (Price, 1984). Using variation in sexual dimorphism among the European populations of otters as an indirect measure of the intensity of sexual selection, we examined how FA varies with varying mean degree of sexual dimorphism among populations.

The populations were also investigated for changes of skull trait size that may reveal an improved or deteriorated habitat condition and/or a change of the genetic structure of a population (Frankel & Soulé, 1981; Pertoldi *et al.*, 1998). Hence, we tested if populations which were presumed to be endangered had a reduced mean sexual size dimorphism when compared to populations presumed to be healthy and viable. The otter skull shows a high degree of phenotypic plasticity (Pertoldi *et al.*, in prep) despite the low genetic variability which was found within and among populations (Randi, pers. comm.).

MATERIAL AND METHODS

Choice of traits

A total of 234 adult otter skulls taken from nine populations in seven different countries was analysed. The skulls were considered to be from adults on basis of the closure of the sutures in the nasal region (Van Bree *et al.*, 1966).

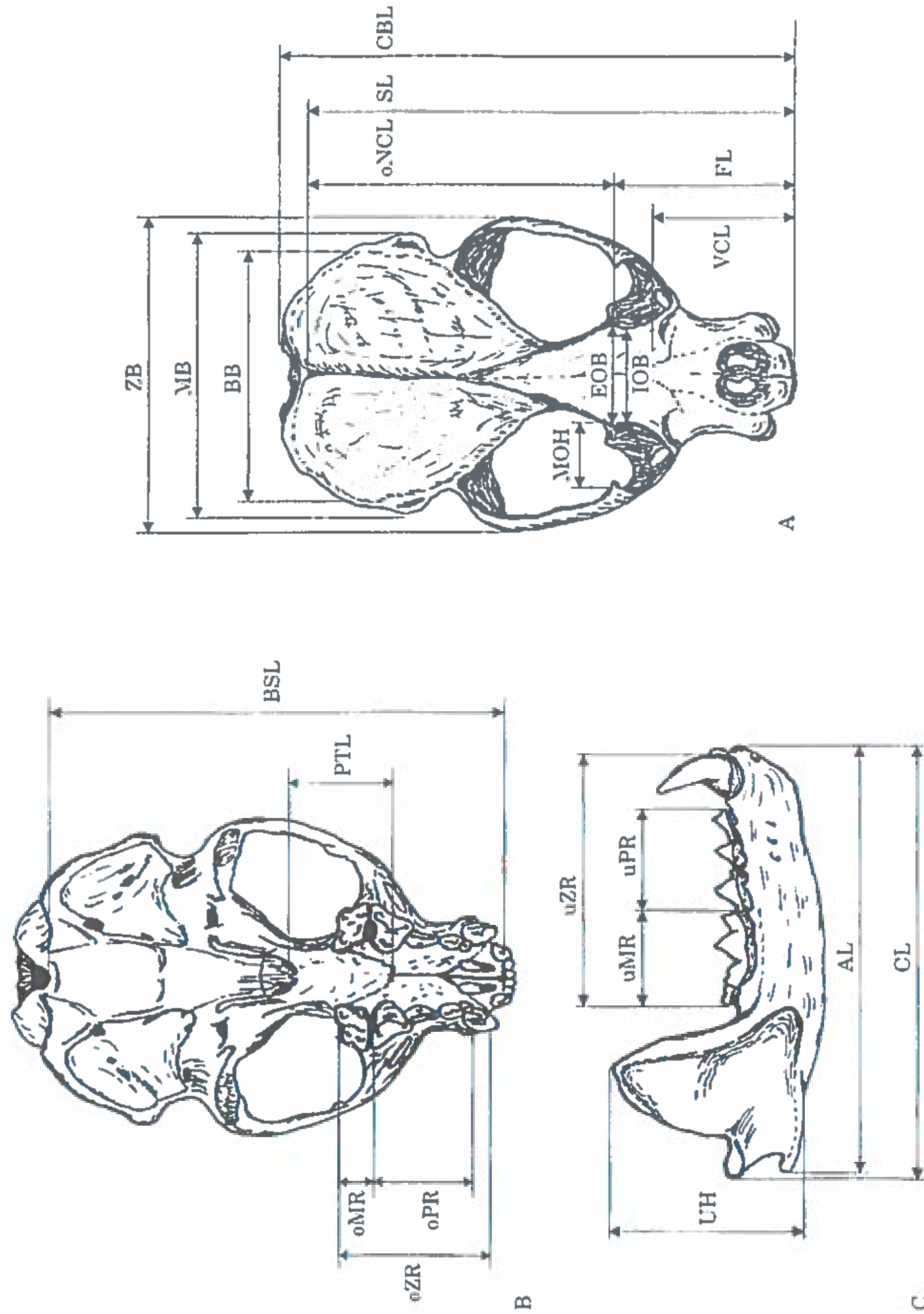


Figure 1. Caption on facing page

Condylobasal length (CBL) was taken as a measure of the skull size; this has been shown to be tightly correlated with body mass (body weight and body length) (Pertoldi *et al.*, 1997, 1998) in both male and female otters. Furthermore, CBL has been suggested to be a trait under directional selection in the Danish otter population (Pertoldi *et al.*, 1997, 1998). Selection may be for small females and large males as in other mustelids (Ralls & Harvey, 1985) and the full potential size of males is probably determined by long-term sexual selection. The operation of sexual selection is governed mainly by the relative investment of the sexes in their offspring, with individuals of the sex investing less (males in the case of mustelids) competing among themselves to breed with members of the sex investing more (Trivers, 1972). This is likely to result in morphological or behavioural adaptations that maximize dominance and mobility. Both characteristics are, in general, directly proportional to body size. Therefore, variation in nutritional status during the period of growth should have different consequences for males and females. A linear regression analysis is used in order to determine which traits were correlated with condylobasal length. All the skull and mandible traits (Fig. 1A–C) were measured with a digital calliper to the nearest 0.1 mm.

Fluctuating asymmetry and indices used

Bilateral traits were measured on the right side. Two mandible traits, mandible length (CL) and mandible height (UH) were measured on both sides. They were found to be strongly correlated both to each other and to condylobasal length. The magnitude of asymmetry was estimated from the difference in length between each bilateral pair as right minus left $(r-l)$. FA was calculated using four different indices of Palmer & Strobeck (1986): FA1, FA2, FA4 and FA6. The latter two were modified following Sullivan *et al.* (1993). FA1 is the absolute value of asymmetry (absolute FA); FA2 is the relative value of asymmetry (relative FA); FA4 is the variance of $(r-l)$, and FA6 is the variance of relative $(r-l)$. No measurements were attempted on broken or worn parts of the skulls. Therefore, for some skulls we have missing

Figure 1. View of an otter's skull. A, dorsal view. Abbreviations: CBL: (condylobasal length: condyli occipitales–prosthion), SL: (total length: akrokranion–prosthion), oNCL: (upper neurocranium length: (akrokranion–frontal midpoint), FL: (facial length: frontal midpoint–prosthion), VCL: (viscerocranium length: nasion–prosthion), IOB: (infraorbital breadth), EOB: (ectorbital breadth), ZB: (zygomatic breadth: zygion–zygion), MB: (mastoid breadth: otion–otion), BB: (max. breadth of braincase: euryon–euryon), MOH: (max. inner orbita height).

B, ventral view. BSL: (basal length: basion–prosthion), PTL: (palatinum length: staphylion–palatinoorale), oPR: (length of the upper premolar row: alveolar distance P1–P4), oMR: (length of the upper molar row: alveole M1), oZR: (length of the upper tooth row: alveolar distance C–M1). The following traits were not indicated: PTL*: (palatal length: staphylion–prosthion), BSBL: (Basion), P4B: (breadth of P4 at the cingulum), M1B: (breadth of M1 at the cingulum).

C. mandible. AL: (angular length: infradentale processus–angularis), CL: (total length: infradentale–processus condylis), UH: (coronion–basal point of angular process), uZR: (length of lower tooth row: alveolar distance C–M2), uPR: (length of lower premolar row: alveolar distance P2–P4), uMR: (length of lower molar row: alveolar distance M1–M2). The trait M1L was not indicated: (length of M1 at the cingulum).

values and that is why we chose a univariate approach for the craniometrical statistical analysis (Sokal & Rohlf, 1981).

Measurement errors

In an effort to quantify possible measurement errors, we chose eight individuals at random, and for each of these we measured the different skull traits and the tooth length ten times. The within individual coefficient of variation for each mean was then taken as an estimate of the measurement error, adding Haldane's (1955) correction for small sample size. For the two mandible traits CL and UH (used for assessing FA) we calculated the probability of obtaining identical estimates of subsequent measures by using a subsample of fifty random chosen individuals, and we calculated the contribution of measurement errors (the difference between two independent estimates of FA) in relation to FA. Furthermore, we tested if average FA was significantly larger than the mean measurement error using an unpaired *t*-test.

Statistical analysis

For tests of the independence of FA from trait length and for testing the normal distribution of FA values, we used the Lausitzian population, because of its relatively large sample size (44 individuals) and relatively short period of collection (1968–1996). The hypothesis that the mean of right minus left character values equals zero was tested in a one sample *t*-test and normality was tested with a Lilliefors test and by inspecting the distributions graphically. Because absolute FA has a half normal distribution we employed non-parametric statistics when dealing with absolute FA. A Spearman's rank correlation test was performed to test if there was a significant correlation between FA and the length of mandible traits CL and UH. The sexes were separated in this test because of sexual dimorphism in otter skulls (Wiig, 1986) and different selective forces acting on the two sexes (Moors, 1980).

The statistical analyses described below were conducted after separating the sexes and the populations. The latter were grouped as follows: (1) populations considered healthy and viable (northern Finland, Lausitz, France and Hungary); (2) populations considered threatened, endangered or near the verge of extinction (Denmark, southern Finland, Spain, Belgium and northern Germany). This a priori grouping was carried out following the demographic data from different authors.

A linear regression analysis was conducted for all trait lengths versus the year of collection of the otter skull. We calculated the Pearson product-moment correlation coefficient (Zar, 1984) for CBL lengths (usually the most reliable measure of overall skull size) versus the year of collection. All the coefficients of correlation were z-transformed (Zar, 1984), and the means of the z-values of the viable and the endangered populations were tested for differences with an unpaired *t*-test, the null hypothesis being that the mean of the z-transformed correlation coefficients should not be significantly different in the viable and endangered population.

A Spearman's rank correlation test was conducted for the absolute value of FA of the traits CL and UH versus the year of collection of the otter skull.

For those countries where it was not statistically correct to make a Spearman's

rank correlation test (because of a lack of data in the intermediate period of collection), we grouped the years of skull collection into two periods. The mean and variance of the distributions of the trait lengths and of FA were calculated for every period considered. An *F*-test (Zar, 1984) was conducted to compare the variances of $(r-l)$ (FA4 index) and the variance of relative $(r-l)$ (FA6 index).

Sexual dimorphism

The average degree of sexual dimorphism (DI) was measured for the skull length (CBL) and calculated using a proportional ratio which is properly scaled for species in which the males are always the larger sex: % DI = 100 × [mean (males) − mean (females)]/mean (females) (Rossolimo & Pavlinov, 1974).

Spearman rank correlation tests were conducted to test whether there were significant correlations between: (1) the mean FA and the mean degree of sexual dimorphism, and (2) the mean size of CBL and the mean degree of sexual dimorphism in the populations in the different countries. We used additional published data on mean sexual dimorphism and mean CBL length from Ireland, Scotland, Norway, former Czechoslovakia and the Shetland Isles (Lynch *et al.*, 1996). No correction for multiple comparisons was made, as the high number of correlated tests increased strongly the conservativeness of the sequential Bonferroni test (Rice, 1989). With no correction for multiple comparisons, we reduced the possibility of committing an error of type 2 (not to reject the null hypothesis when it is false) (Zar, 1984).

RESULTS

Measurement errors

Measurement errors were low for the skull measurements, ranging from 0.05% to 0.94% (mean = 0.29%, median = 0.17%). The error for the teeth measurements was higher but still reasonable, ranging from 0.24% to 3.04% (mean 1.01%, median = 0.75%). For the two mandible traits (CL and UH), the probability of obtaining identical estimates of subsequent measures was 89% for CL and 93% for UH, and the contribution of measurement errors (the difference between two independent estimates of FA) had a mean of 0.04 mm for CL 0.06 mm for UH.

The mean FA of CL was 0.40 mm (median = 0.35) and of UH was 0.34 mm (median = 0.3). The average FA was significantly larger than the mean measurement error (*t*-test, CL: $t = 20.71$, df = 736, $P < 0.0001$, UH: $t = 17.686$, df = 734, $P < 0.0001$). The mean measurement error in relation to mean FA was 10% for CL and 17% for UH, within the range of 25% accepted as reasonable by Palmer, 1994, 1996).

Statistical analysis of fluctuating asymmetry

No significant deviations from zero of the mean of the trait $(r-l)$ distributions were found: CL (one sample *t*-test: $P = 0.68$, df = 41), UH: (one sample *t*-test: $P = 0.086$, df = 44). No deviations from the normal distribution of $(r-l)$ for CL and

UH were found: Lilliefors test: $P=0.77$, $n=42$) and $p=0.452$, $n=45$, respectively. Furthermore, the distributions were inspected graphically and there was no sign of antisymmetry.

Most of the analysed skull traits (with the exception of teeth measurements and few others) were significantly correlated with condylobasal length (see Table 1). However, some traits that were significantly correlated with condylobasal length in some populations or in one of the two sexes were not significantly correlated in other populations or in the other sex. Both CL and UH were strongly correlated with CBL in all the populations (see Table 1), with only a few exceptions, that were partly due to small sample size (see Palmer, 1994). CL and UH were also strongly correlated with each other ($r=0.95$, $n=232$, $SE=0.012$, $P=0.0001$), however, FA of the two traits was not significantly correlated, either in males ($r_s=-0.03$, $n=111$, $P=0.780$) or females ($r_s=-0.13$, $n=104$, $P=0.2$).

The Lausitzian males showed a significant negative correlation between FA of traits and length of CL ($r_s=-0.57$, $n=26$, $P=0.025$) and UH ($r_s=-0.44$, $n=27$, $P=0.020$) while the Lausitzian females showed a significantly positive correlation between the former ($r_s=0.60$, $n=15$, $P=0.0167$), and no significant correlation for the latter ($r_s=0.25$, $n=17$, $P=0.330$). We used both the absolute FA index (FA1) and the relative index (FA2) to calculate the correlations versus the year of collection (see Discussion), which gave similar results in this investigation.

Sexual dimorphism and fluctuating asymmetry

There is evidence of a decreased length of the skull traits in almost every country where the otter populations are endangered: Denmark (male), northern Germany (male) and southern Finland (female). The Belgian and the Danish females did not show significant variation of the skull trait size (see Table 2). Of those traits that showed significant negative trends in the regression analysis, more than 82% were correlated with condylobasal length (see Table 1).

There was a significant difference between the mean of the z-transformed correlation coefficient of the male CBL versus the year of collection of the viable and the endangered populations (t-test, $t=4.85$, $df=4$, $P<0.001$), whereas in females no significant differences were found ($t=1.95$, $df=5$, $P=0.11$). The significance of the t-test in males indicates that the mean of the correlation coefficient between CBL and the year of collection is significantly decreasing in the endangered as compared to the viable otter populations.

There is also evidence for increased FA in populations from Denmark, N Germany, Spain and S Finland (see Tables 3 and 4). In those countries where the otter populations are healthy there was no evidence of a reduction in skull size (see Table 2) or increased FA (see Table 3).

There was a significant correlation between the mean male CBL value and the mean degree of sexual dimorphism, whereas no significant correlation was found for females (males: $r_s=0.56$, $n=14$, $P=0.0415$, females: $r_s=-0.05$, $n=14$, $P=0.868$).

No significant correlation was found between the mean degree of sexual dimorphism in the otter populations and the mean absolute FA in the different populations of CL (males: $r_s=0.25$, $n=9$, $P=0.4795$, females: $r_s=-0.083$, $n=9$, $P=0.814$) and UH (males: $r_s=-0.2$, $n=9$, $P=0.572$, females: $r_s=0.033$, $n=9$, $P=0.925$).

TABLE 1. Linear regression analysis of the skull in the presumably viable and endangered populations. Significance of the correlation between condylobasal length and the length of the other trails. See Fig.1 for explanation and abbreviations.

#	Status	Sex	Country	Period	Sample size	BSL	SL	oNCL	FL	VCL	PTL	PTL*	BSBL	IOB	EOB	ZB	MB	BB
1	viable	m	Finland (north)	(1979–1996)	($20<n<21$)	***	***	**	***	n.s.	n.s.	**	***	n.s.	n.s.	n.s.	n.s.	n.s.
2	viable	f	Finland (north)	(1851–1996)	($21<n<23$)	***	***	**	***	n.s.	n.s.	**	***	n.s.	n.s.	n.s.	n.s.	n.s.
3	viable	f	France	(1900–1984)	($9<n<10$)	***	*	n.s.	n.s.	*	n.s.	*	***	n.s.	n.s.	n.s.	n.s.	n.s.
4	viable	m	Hungary	(1950–1982)	($17<n<19$)	***	***	***	***	***	**	***	***	***	**	**	**	**
5	viable	f	Hungary	(1950–1986)	($15<n<17$)	***	**	*	**	*	*	**	**	n.s.	n.s.	n.s.	*	n.s.
6	viable	m	Lausitz	(1955–1996)	($25<n<27$)	***	***	***	***	***	*	***	***	*	n.s.	**	*	n.s.
7	viable	f	Lausitz	(1968–1996)	($n=21$)	***	*	*	n.s.	n.s.	n.s.	**	*	n.s.	n.s.	*	*	n.s.
8	endangered	f	Belgium	(1872–1984)	($20<n<22$)	***	***	**	**	n.s.	*	**	**	*	n.s.	n.s.	*	n.s.
9	endangered	m	Denmark	(1895–1995)	($19<n<22$)	***	***	***	***	n.s.	**	***	***	*	n.s.	*	**	*
10	endangered	f	Denmark	(1851–1995)	($17<n<20$)	***	***	**	*	n.s.	*	*	**	**	**	*	*	n.s.
11	endangered	f	Finland (south)	(1911–1996)	($19<n<20$)	***	***	***	***	**	*	***	***	n.s.	*	*	***	*
12	endangered	m	Germany (north)	(1879–1987)	($19<n<22$)	***	***	***	*	*	**	***	***	n.s.	n.s.	*	**	n.s.
13	endangered	m	Spain	(1972–1995)	($15<n<16$)	**	**	**	*	**	*	**	**	n.s.	n.s.	n.s.	n.s.	n.s.

#	Status	Sex	Country	Period	Sample size	MOH	oZR	oPR	oMR	P4B	M1B	AL	CL	UH	uZR	uPR	uMR	M1L
1	viable	m	Finland (north)	(1979–1996)	($20<n<21$)	n.s.	**	**	n.s.	n.s.	n.s.	**	***	n.s.	**	**	n.s.	n.s.
2	viable	f	Finland (north)	(1851–1996)	($21<n<23$)	n.s.	**	*	n.s.	n.s.	n.s.	***	**	n.s.	**	**	n.s.	n.s.
3	viable	f	France	(1900–1984)	($9<n<10$)	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.
4	viable	m	Hungary	(1950–1982)	($17<n<19$)	**	**	***	**	*	***	**	**	***	**	**	**	*
5	viable	f	Hungary	(1950–1986)	($15<n<17$)	n.s.	**	**	n.s.	n.s.	n.s.	**	**	**	**	*	n.s.	n.s.
6	viable	m	Lausitz	(1955–1996)	($25<n<27$)	**	***	**	n.s.	n.s.	n.s.	***	***	***	*	*	*	n.s.
7	viable	f	Lausitz	(1968–1996)	($n=21$)	*	**	n.s.	n.s.	n.s.	*	**	*	*	n.s.	n.s.	*	n.s.
8	endangered	f	Belgium	(1872–1984)	($20<n<22$)	n.s.	*	n.s.	n.s.	*	n.s.	**	**	**	*	n.s.	*	*
9	endangered	m	Denmark	(1895–1995)	($19<n<22$)	*	**	**	*	**	**	**	***	***	***	**	*	*
10	endangered	f	Denmark	(1851–1995)	($17<n<20$)	*	**	*	*	*	n.s.	**	*	**	**	**	n.s.	n.s.
11	endangered	f	Finland (south)	(1911–1996)	($19<n<20$)	**	***	**	**	**	**	***	***	**	**	n.s.	n.s.	n.s.
12	endangered	m	Germany (north)	(1879–1987)	($19<n<22$)	n.s.	**	n.s.	n.s.	n.s.	n.s.	***	*	**	**	n.s.	n.s.	n.s.
13	endangered	m	Spain	(1972–1995)	($15<n<16$)	n.s.	*	n.s.	n.s.	n.s.	n.s.	*	n.s.	*	n.s.	*	n.s.	n.s.

$P<0.05$, $P<0.01$, $P<0.001$

TABLE 2. Linear regression between the traits' length and the year of skull collection of the presumably viable and endangered otters' populations. The signs (+) or (−) indicate a positive or negative trend

#	Status	Sex	Country	Period	Sample size	CBL	BSL	SL	oNCL	FL	VCL	PTL	PTL*	BSBL	IOB	EOB	ZB	MB	BB
1	viable	m	Finland (north)	(1979–1996)	(n=21)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2	viable	f	Finland (north)	(1851–1996)	(21<n<23)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
3	viable	f	France	(1900–1984)	(9<n<10)	(+)*	(+)*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	(+)*	n.s.	n.s.	n.s.	n.s.	n.s.
4	viable	m	Hungary	(1950–1982)	(17<n<19)	(+)*	(+)*	(+)**	(+)**	(+)*	n.s.	(+)*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	(+)*
5	viable	f	Hungary	(1950–1986)	(11<n<13)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
6	viable	m	Lausitz	(1955–1996)	(25<n<27)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	(+)**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
7	viable	f	Lausitz	(1968–1996)	(15<n<17)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	(+)*	n.s.	n.s.
8	endangered	f	Belgium	(1872–1984)	(13<n<15)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	(+)*	n.s.	n.s.	n.s.	n.s.
9	endangered	m	Denmark	(1895–1995)	(19<n<22)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	(−)*	(−)**	(−)*	n.s.	n.s.	n.s.	n.s.	n.s.
10	endangered	f	Denmark	(1851–1995)	(15<n<16)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
11	endangered	f	Finland (south)	(1911–1996)	(17<n<20)	(−)**	(−)**	(−)**	(−)**	(−)**	(−)*	(−)*	(−)**	(−)***	(−)*[1]	(−)*	(−)*	(−)***	(−)**
12	endangered	m	Germany (north)	(1879–1987)	(18<n<20)	n.s.	(−)*	(−)*	n.s.	(−)*	(−)***	(−)*	(−)**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
13	endangered	m	Spain	(1972–1995)	(9<n<11)	n.s.	n.s.	(−)*	(−)*	n.s.	(−)*	n.s.	n.s.	(−)*	(−)**[1]	(−)*[1]	(−)***[1]	(−)**[1]	n.s.

#	Status	Sex	Country	Period	Sample size	MOH	oZR	oPR	oMR	P4B	M1B	AL	CL	LH	uZR	uPR	uMR	M1L
1	viable	m	Finland (north)	(1979–1996)	(n=21)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2	viable	f	Finland (north)	(1851–1996)	(21<n<23)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	(−)*[1]	n.s.	n.s.	n.s.	n.s.
3	viable	f	France	(1900–1984)	(9<n<10)	n.s.	n.s.	n.s.	n.s.	(+)[1]	n.s.	n.s.	(+)*	n.s.	n.s.	n.s.	n.s.	n.s.
4	viable	m	Hungary	(1950–1982)	(17<n<19)	n.s.	(+)*	(+)*	(+)**	(+)*	(+)**	n.s.	n.s.	n.s.	n.s.	(+)*	(+)**	(+)**
5	viable	f	Hungary	(1950–1986)	(11<n<13)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
6	viable	m	Lausitz	(1955–1996)	(25<n<27)	n.s.	n.s.	n.s.	n.s.	n.s.	(+)*[1]	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
7	viable	f	Lausitz	(1968–1996)	(15<n<17)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
8	endangered	f	Belgium	(1872–1984)	(13<n<15)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
9	endangered	m	Denmark	(1895–1995)	(19<n<22)	(−)**	(−)*	(−)***	n.s.	n.s.	n.s.	(−)*	(−)*	n.s.	n.s.	(−)***	n.s.	n.s.
10	endangered	f	Denmark	(1851–1995)	(15<n<16)	n.s.	n.s.	n.s.	n.s.	(−)*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
11	endangered	f	Finland (south)	(1911–1996)	(17<n<20)	n.s.	(−)**	(−)**	(−)*	(−)**	(−)**	(−)**	(−)**	(−)**	(−)*	n.s.	(−)*[1]	n.s.
12	endangered	m	Germany (north)	(1879–1987)	(18<n<20)	n.s.	(−)**	(−)**[1]	n.s.	n.s.	n.s.	(−)*	(−)*	(−)*	(−)*	(−)***	n.s.	n.s.
13	endangered	m	Spain	(1972–1995)	(9<n<11)	n.s.	n.s.	n.s.	(−)**[1]	n.s.	(−)*[1]	(−)*	(−)*[1]	n.s.	n.s.	n.s.	(−)***[1]	(−)**[1]

P<0.05 = *, P<0.01 = **, P<0.001 = ***

[1] The trait is not significantly correlated with condylobasal length.

TABLE 3. Spearman rank correlation test of the absolute (FA1) and relative values (FA2) for traits CL and UH in the presumably viable and endangered otter population versus the year of skull collection. The numbers in parentheses are the sample sizes. (+) or (−) indicate a positive or negative relationship. *$P<0.05$

Status	Sex	Country and period of collection	Sample size	FA indices	CL	UH
viable	m	Finland (north) (1979–1996)	(n=21)	FA1 FA2	(−) 0.51 (n=21)* (−) 0.52 (n=21)*	*n.s.* *n.s.*
viable	f	Finland (north) (1851–1996)	(21<n<23)	FA1 FA2	*n.s.* *n.s.*	*n.s.* *n.s.*
viable	f	France (1900–1984)	(n=10)	FA1 FA2	*n.s.* *n.s.*	*n.s.* *n.s.*
viable	m	Hungary (1950–1982)	(16<n<19)	FA1 FA2	*n.s.* *n.s.*	*n.s.* *n.s.*
viable	f	Hungary (1950–1986)	(11<n<12)	FA1 FA2	*n.s.* *n.s.*	*n.s.* *n.s.*
viable	m	Lausitz (1955–1996)	(26<n<27)	FA1 FA2	*n.s.* *n.s.*	*n.s.* *n.s.*
viable	f	Lausitz (1968–1996)	(15<n<17)	FA1 FA2	*n.s.* *n.s.*	*n.s.* *n.s.*
endangered	f	Belgium (1872–1984)	(14<n<15)	FA1 FA2	(+) 0.57 (n=14)* (+) 0.56 (n=14)*	*n.s.* *n.s.*
endangered	m	Denmark (1895–1995)	(20<n<22)	FA1 FA2	*n.s.* *n.s.*	(+) 0.451 (n=22)* (+) 0.480 (n=22)*
endangered	f	Denmark (1851–1995)	(n=15)	FA1 FA2	*n.s.* *n.s.*	*n.s.* *n.s.*

TABLE 4. F-test. Comparison of variance (FA4) and relative variance (FA6) of the skull traits (r−1) in the presumably endangered otters' population in the two periods of collection. (+) indicates an increase of variance in the second period of collection. *P=<0.05

Sex	Country and periods of collection	Trait	Period 1 (n), Variance (r−1)	Period 2 (n), Variance (r−1)	FA indices	F-test for FA
Female	Finland (south) (1911–1940)–(1973–1996)	CL	(5), 0.0392	(15), 0.2678	FA4 FA6	6.8316 (+)* 7.8300 (+)*
		UH	(5), 0.0820	(15), 0.0828	FA4 FA6	*n.s.* *n.s.*
Male	Germany (north) (1879–1940)–(1961–1987)	CL	(12), 0.0902	(5), 0.3830	FA4 FA6	4.1630 (+)* 3.7212 (+)*
		UH	(14), 0.0593	(5), 0.0407	FA4 FA6	*n.s.* *n.s.*
Male	Spain (1972–1973)–(1985–1995)	CL	(3), 0.0433	(7), 0.6223	FA4 FA6	36.2837 (+)* *n.s.*
		UH	(3), 1.8033	(6), 0.0497	FA4 FA6	*n.s.* *n.s.*

DISCUSSION

Correlation between skull size and fluctuating asymmetry

Tooth length was not correlated with CBL. Once the teeth are erupted there is no further growth in crown lengths; hence, if some stress occurs after their eruption, the teeth will not be affected in the same way as other skull traits. Less obvious is

why a trait which is significantly correlated with condylobasal length in one population or in one sex, is not correlated with the same trait in another population or in the other sex. A possible explanation could be that the otter populations have been under different selective forces which can, permanently or temporarily, have modified the proportions (ratio) between the traits in the skulls, and therefore the correlation between the traits. However, another explanation could be that some of these correlations were caused by chance.

As the otter populations are probably exposed to different selective forces, we used both the relative FA index (normally used when there is a correlation between FA and trait size) and the absolute FA index (normally used when there is no correlation between FA and trait size). Also, while we found a negative relationship between FA and trait size in the Lausitzian male otters and a positive relationship in the Lausitizian females, suggesting strong directional selection (see Møller & Swaddle, 1997, for review), we cannot confirm that all the other otter populations are under the same selective regime. We suspect that the intensities and the directions of the selective forces change not only from one zone to another, but also between years. Therefore, it is statistically incorrect to try to find correlations between FA and trait length when considering individuals collected in different years or zones.

Fluctuating asymmetry

The results obtained here have not revealed a clear dichotomy between populations considered healthy or endangered, and in some instances they were not in concordance with our predictions, based on the demographic data available. However, in other cases, we found that results coincided with predictions.

Generally, the healthy populations did not show evidence for increased absolute FA (FA1) or relative FA (FA2) and/or for decreased skull size during the collection period. The reasons for the increased (absolute and relative) FA and the concomitant reduction in skull trait size discovered in almost all the endangered populations differ from population to population. However, from our investigation it clearly emerges that developmental stability is linked to general individual fitness.

Environmental stresses

The low level of significance of some correlations between FA versus the year of collection (which implies a linear relationship between two variables; see Table 3) may be explained by the fact that the power of FA as an indicator of developmental stability decreases when the stress becomes prolonged (Palmer & Strobeck 1986). The demographic data of the Eurasian otters suggest that the endangered populations have been under continued stress for several generations. Furthermore, we cannot suppose that environmental stress is increasing linearly with the year of collection. Therefore we suggest that in order to detect a possible environmental change a comparison be made of individuals collected in two distinct periods (see Table 4).

Genetic stresses

All the previously cited environmental factors have in a different way altered the genetic structure of the otter populations. Several authors (Frankel & Soulé, 1981)

have suggested that a 10% increase in inbreeding coefficient may result in a concomitant reduction in traits closely associated with fitness, such as longevity, fecundity and body size. It is also generally acknowledged that reduced parental survival leads to selection for increased fecundity (Michod, 1979) which would imply that parents in a poor condition reproduce more instead of less, producing less fit offspring (which are generally smaller). Furthermore, we cannot exclude the possibility of a genotype–environment interaction; Turelli (1988) has argued that the dynamics of quantitative genetic parameters cannot be predicted only from models of maintenance of heritable variation, because genetic variances, covariances and correlations, for example, can change with environmental conditions if there are genotype–environment interactions.

Sexual dimorphism and sexual selection

The positive relationship between the average degree of sexual dimorphism and male CBL is difficult to explain, as this relationship is absent in females. However, if sexual dimorphism is a function of increasing male body size, this may account for this phenomenon. Secondary sexual characters (including large male body size) are presumed to be costly (Andersson, 1994); hence, if maintenance costs increase in certain areas, we should expect a decrease in sexual size dimorphism, especially, if the declining population trends imply a reduced density. A lowered population size reduces the amount of intrasexual and intersexual interaction. The consequences are an immediate relaxation of sexual selection. However, the previous finding, a decreasing FA with increasing size in males and an increasing FA with increasing size in females, found in two distinct populations, the Danish and the Lausitzian (see Pertoldi *et al.*, 1998), contributes to the expectation that large size is advantageous for males but not for females (Manning, 1995).

The lack of relationship between the degree of mean sexual dimorphism and the degree of FA could have different explanations. First of all we found that skull size is changing over the years. Therefore, the intensity of sexual selection is also probably changing and for this reason the expected relationship (higher FA in the populations with higher degree of mean sexual dimorphism) could be confused. On the other hand, if the prey base available for otters has been reduced in the areas with endangered populations, we will expect a decrease of sexual dimorphism and an increase of FA (higher FA in the populations with lower degree of mean sexual dimorphism. These factors (sexual selection and reduced food availability) are acting in an antagonistic way on the relationship between FA and sexual dimorphism.

Phenotypic plasticity

In this investigation the Eurasian otter has been shown to have a low canalization of the skull traits and therefore to have high phenotypic plasticity. Changes in the reaction norm are considered to be a possible pathway of evolutionary transformation known as the Baldwin effect (Shiskin, 1992). Following this hypothesis we should expect an initial increase of development instability of a trait, followed by a decrease when the development of the trait is switched to another reaction norm. For a skull, for example, we would anticipate high FA in populations that are undergoing a

change of the reaction norm or are living under sub-optimal conditions and low FA in populations that are not undergoing directional changes and are not subject to environmental stress.

Conservation strategies

Despite the high conservativeness of the F-test when dealing with small sample sizes, and the reduced significance of the regression analyses when dealing with scattered data, this investigation confirms that the study of developmental stability and craniometrical variability at the interpopulation level can be a useful tool for detecting stressful environmental situations in otters. The results of this investigation support the view that the Eurasian otter is experiencing environmental stress in the major part of the European populations.

The ideal conservation strategy would include measures to reduce water pollution and artificially increase the availability of otter prey, as the observed reduction of the male skull size in the endangered population could be due to relaxed sexual selection but also to reduced food availability. A breeding programme taking individuals from scattered populations would increase the effective population size (N_e) and also reduce the role of genetic drift. For populations with N_e smaller than a few hundred individuals, the expected amount of variation for a typical quantitative character is nearly independent of the strength of selection and is largely a result of mutation-drift balance (Houle, 1989).

ACKNOWLEDGEMENTS

We wish to thank all the museums and universities for providing access to collections. Furthermore, we thank Kirsten Zaluski, Danish National Environmental Research Institute, for improvements to the text, Vibeke Simonsen for comments on the manuscript and Bo Gaardmand for useful suggestions and help. The research was in part supported by a grant from the Danish National Sciences Research Council.

REFERENCES

Andersson M. 1994. *Sexual Selection.* Princeton: Princeton University Press.

Barraclough TG, Harvey PH, Lee S. 1995. Sexual selection and taxonomic diversity in passerine birds. *Proceedings of the Royal Society of London B* **259:** 211–215.

Clarke GM. 1995. Relationship between developmental stability and fitness: application for conservation biology. *Conservation Biology* **1:** 18–24.

Frankel OH, Soulé ME. 1981. *Conservation and Evolution.* Cambridge: Cambridge University Press.

Haldane JBS. 1955. The measurement of variation. *Evolution* **9:** 484.

Houle D. 1989. The maintenance of polygenic variation in finite populations. *Evolution* **43:** 1767–1780.

Kruuk H. 1995. *Wild Otters: Predation and populations.* Oxford: Oxford University Press.

Leary RF, Allendorf FW. 1989. Fluctuating asymmetry as an indicator of stress: Implications for conservation biology. *Trends in Ecology and Evolution* **4:** 214–217.

Lynch JM, Conroy JWH, Kitchener AC, Jefferies DJ, Hayden TJ. 1996. Variation in cranial form and sexual dimorphism among five European populations of the otter, *Lutra lutra. Journal of Zoology, London* **238:** 81–96.

Macdonald SM, Mason CF. 1994. Status and conservation needs of the otter *Lutra lutra* in the western Palaearctic. *Nature and environment* **67:** 1–54.

Madsen AB. 1996. The ecology and conservation of the otter *Lutra lutra* in Denmark. PhD Thesis. Danish National Environmental Research Institute.

Manning JT. 1995. Fluctuating asymmetry and body weight in men and women: implications for sexual selection. *Ethology and Sociobiology* **16:** 145–153.

Manning JT, Chamberlain AT. 1993. Fluctuating asymmetry, sexual selection and canine teeth in primates. *Proceedings of the Royal Society of London B* **251:** 83–87.

Mason CF. 1989. Water pollution and otter distribution: a review. *Lutra* **32:** 97–131.

Michod RE. 1979. Evolution of life histories in response to age-specific mortality factors. *American Naturalist* **113:** 531–550.

Moors PJ. 1980. Sexual dimorphism in the body size of mustelids (Carnivora): the roles of food habits and breeding systems. *Oikos* **34:** 147–158.

Møller AP. 1992. Patterns of fluctuating asymmetry in weapons: evidence for reliable signalling of quality in beetle horns and bird spurs. *Proceedings of the Royal Society of London B* **248:** 199–206.

Møller AP, Swaddle JP. 1997. *Asymmetry, developmental stability and evolution.* Oxford: Oxford University Press

Palmer AR. 1994. Fluctuating asymmetry analyses: a primer. In: Markov TA, ed. *Developmental instability: its origins and evolutionary implications.* Netherlands: Kluwer Academic, 335–364.

Palmer AR. 1996. Waltzing with asymmetry. *BioScience* **46:** 518–532.

Palmer AR, Strobeck C. 1986. Fluctuating asymmetry: measurement, analysis, patterns. *Annual Review of Ecology and Systematics* **17:** 391–421.

Pertoldi C, Loeschcke V, Madsen AB, Randi E. 1997. Developmental stability in the Eurasian Otter *Lutra lutra* in Denmark. *Annales Zoologici Fennici* **34:** 187–196.

Pertoldi C, Madsen AB, Randi E, Braun A, Loeschcke V. 1998. Temporal variation of skull morphometry of Eurasian otters *Lutra lutra* in Denmark and Germany. *Annales Zoologici Fennici* **35:** 87–94.

Price T. 1984. Sexual selection on body size, territory and plumage variables in a population of Darwin's finches. *Evolution* **38:** 483–494.

Ralls K, Harvey PH. 1985. Geographic variation in size and sexual dimorphism of North American weasels. *Biological Journal of the Linnean Society* **25:** 119–167.

Rice WR. 1989. Analysing tables of statistical tests. *Evolution* **43:** 223–225.

Rossolimo OL, Pavlinov IJ. 1974. Sexual dimorphism in the development, size proportions of the skull in the pine marten *Martes martes.* In: *Biology of Mustelids: some Soviet research.* New Zealand: M. King, Dept. of Scientific and Industrial Research, New Zealand, 34–36.

Shiskin MA. 1992. Evolution as a maintenance of ontogenetic stability. *Acta Zoologica Fennica* **191:** 37–42.

Sokal RR, Rohlf FJ. 1981. *Biometry.* San Francisco: WH Freeman.

Sullivan MA, Robertson PA, Aebischer NA. 1993. Fluctuating asymmetry measurement. *Nature* **361:** 409–410.

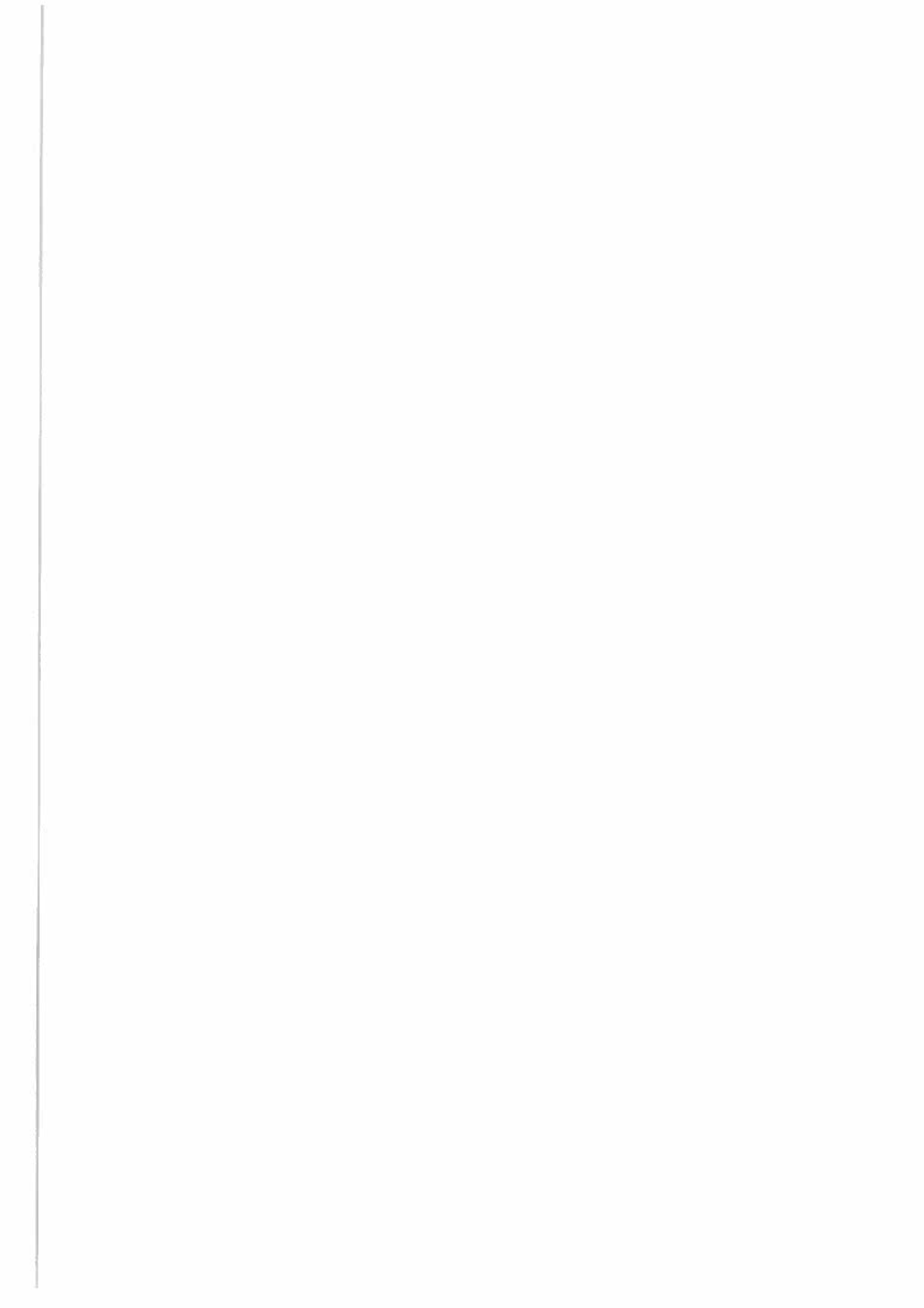
Trivers RL. 1972. Parental investment and sexual selection. In: Campbell B, ed. *Sexual selection and the descent of man.* Chicago: Aldine, 136–179.

Turelli M. 1988. Phenotypic evolution, constant covariances, and the maintenance of additive variance. *Evolution* **42:** 1342–1347.

Van Bree PJH, Jensen B, Kleijn LJK. 1966. Skull dimensions and the length/weight relation of the baculum as age indications in the common otter, *Lutra lutra* (Linnaeus, 1758). *Danish Review of Game Biology* **4:** 97–104.

Wiig O. 1986. Sexual dimorphism in the skull of minks *Mustela vison*, badgers *Meles meles* and otters *Lutra lutra*. *Zoological Journal of the Linnean Society* **87:** 163–179.

Zar JH. 1984. *Biostatistical Analysis.* New Jersey: Prentice Hall.



Biological Journal of the Linnean Society (2000), *71*: 61–70. With 1 figure

doi:10.1006/bijl.1999.0414, available online at http//www.idealibrary.com on IDEAL®

Effect of the 1990 die-off in the northern Italian seas on the developmental stability of the striped dolphin *Stenella coeruleoalba* (Meyen, 1833)

CINO PERTOLDI[1]*, MICHELA PODESTÁ[2], VOLKER LOESCHCKE[1], SØREN SCHANDORFF[3], LETIZIA MARSILI[4], CECILIA MANCUSI[5], PAOLA NICOLOSI[5] AND ETTORE RANDI[6]

[1]*Department of Ecology and Genetics, University of Aarhus, Building 540, Ny Munkegade, DK-8000 Aarhus C, Denmark,* [2]*Museo Civico di Storia Naturale di Milano, corso Venezia 55, I-20121, Milano, Italy;* [3] *Department of Population Biology, Zoological Institute, University of Copenhagen, Universitetsparken 15, DK-2100 Copenhagen Ø, Denmark;* [4]*Dipartimento di Biologia Ambientale, Università di Siena, via delle Cerchia 3, I-53100 Siena, Italy;* [5]*Museo di Storia Naturale del Mediterraneo, via Roma 234, 57100 Livorno, Italy,* [6]*Istituto Nazionale per la Fauna Selvatica, via Cà Fornacetta 9, I-40064 Ozzano Emilia (Bo), Italy*

Received 5 May 1999; accepted for publication 22 September 1999

Developmental stability of the community of striped dolphins, *Stenella coeruleoalba* (Meyen, 1833), that died during the Mediterranean epizootic of 1990 was compared with that of the population prior to and after the epizootic, to assess whether animals that died were the developmentally less stable individuals in the population. Significantly higher levels of fluctuating asymmetry (FA) were found in those individuals that died. Tissue levels of organochlorine pesticide residues and PCBs were determined and the correlation between contaminant concentration and FA was tested. No correlations were found between the contaminant level and FA.

© 2000 The Linnean Society of London

ADDITIONAL KEY WORDS:—fluctuating asymmetry – directional asymmetry – contaminants – epizootic.

CONTENTS

Introduction 62
Material and methods 63
Results 64
Discussion 69
Acknowledgements 69
References 70

* Corresponding author. Present address: Department of Landscape Ecology, National Environmental Research Institute, Kalo Grenåvej 14, DK-8410 Rønde, Denmark.
E-mail: Cino.Pertoldi@biology.aau.dk

0024–4066/00/090061 + 10 $35.00/0 © 2000 The Linnean Society of London

INTRODUCTION

Die-offs of marine mammals

In recent years die-offs have occurred in populations of marine mammals. These include the harbour seal *Phoca vitulina* (Linnaeus, 1758) in the North Sea in 1988 (Dietz, Heide-Jørgensen & Härkönen, 1989), the bottlenose dolphin, *Tursiops truncatus* (Montagu, 1821) in the Mediterranean in 1987 and the striped dolphin, *Stenella coeruleoalba* (Meyen, 1833) in the Mediterranean in 1990. A morbillivirus seems to have been the primary cause, while other environmental factors may have been contributory factors (Aguilar & Raga, 1993). A generally poor state of health might have existed in the striped dolphin population prior to 1990, and made the individuals more vulnerable to infections. When diseases hit populations of animals it is generally expected that less fit individuals are mostly affected. We suspect that the less fit striped dolphins were more vulnerable to the complications of the virus infection because of a higher level of infections prior to the epizootic. It has been suggested that exposure to PCB contaminants may have had an adverse effect on the defence against virus infections (Aguilar & Borrel, 1994). The epidemic appears to have started in the coast off Valencia in July 1990, spreading to southern France and the Italian Riviera in September (Aguilar & Raga, 1993; Podestà *et al.*, 1992).

Pollution and epidemics as stress factors and their impact on developmental stability

Pollution and epidemics are environmental stress factors. Stresses of environmental and genetic origin affect developmental processes, as reflected in reduced developmental stability (Leary & Allendorf, 1989). Developmental stability (DS) reflects the ability of an individual to buffer its development against disturbance. Stress makes the individual less able to develop a predetermined phenotype (Møller & Swaddle, 1997). DS is generally high in individuals of natural populations not subject to considerable genetic or environmental stress (Møller & Swaddle, 1997).

In pinnipeds, xenobiotics are known to cause abnormal skeletal development (Zakharov & Yablokov, 1990). Since many cetaceans have a similar lifestyle and diet to pinnipeds, they should be similarly vulnerable. A common method for estimating the degree of DS is to measure fluctuating asymmetry (FA). FA occurs when an individual fails to develop identical bilateral symmetrical traits (Palmer & Strobeck, 1986).

One of the aims of this paper is to discover whether there is a correlation between FA and the concentrations of PCBs and DDT and their metabolites (DDE, DDD). We measured the concentrations of these contaminants in the tissues of some of the investigated cetaceans. FA of the skulls was measured to check whether the cetaceans that became stranded during the die-off had a lower level of developmental stability (reflected in a higher level of FA) when compared to those individuals found dead before and after the epizootic.

Directional asymmetry

The skull of the striped dolphin is strongly skewed on the right side, i.e. its skull shows directional asymmetry (DA). DA occurs whenever mean left–right character values have a normal distribution, with a mean value deviating from zero. The striped dolphin belongs to the sub-order Odontoceta, in which asymmetrical structures have evolved in a wide variety of taxa (Neville, 1976). The Odontocetes are the only

mammals, living as well as extinct, in which cranial asymmetry is the normal condition (Bourdelle & Grassé, 1955). Debate continues concerning the reliability of DA as a measure of DS (McKenzie & O'Farrell, 1993) but we decided to use it in this investigation.

MATERIAL AND METHODS

Methods of collection and age determination

The striped dolphins were collected between 1980 and 1994. Skulls or complete skeletons are preserved in the Natural History Museums of Milano, Genova, Livorno, Pisa and Firenze. When possible, the individuals were sexed and aged. Individuals were considered young if less than 3 years or old if more than 3 years (when the skull is fully grown). The time of collection was divided into three periods: period 1 (pre-epizootic, 1980–1989), when the major part of the individuals collected had drowned in fish traps (36 specimens, 28 accidentally netted by fishermen and 8 found stranded); period 2 (epizootic, 1990) when all the individuals collected were found stranded on the Italian Riviera (27 specimens), and period 3 (post-epizootic, 1991–1994) where the individuals collected were found stranded on the Italian Riviera and in the northern part of the Tirrenian (57 specimens). There is no evidence for local genetic differentiation of the specimens found stranded in the different localities (Archer, 1996).

Choice of traits

The traits were measured with a digital calliper to the nearest 0.01 mm, with the exception of traits 1, 2 and 6, which were measured to the nearest 0.1 mm. The traits measured, following Perrin's (1975) nomemclature, were: trait 1: postorbital process-junction between the nasals, ventral end of the internasal suture (sutura internasalis); trait 2: temporal fossa; trait 3: (Perrin's #2) hindmost limit of antorbital notch-interior limit of the premaxillary; trait 4: (Perrin's #26) length of antorbital process of left lacrimal; trait 5: (Perrin's #40) length of mandibular fossa, measured to mesial rim of internal surface of condyle; trait 6: (Perrin's #38) greatest length of ramus; trait 7: greatest distance between the 2nd and 11th tooth; trait 8: greatest distance between the 12th and 22nd tooth; trait 9: (Perrin's #39) height of ramus.

Fluctuating asymmetry, statistical analysis and indices used

The magnitude of asymmetry was estimated from the difference in length between each bilateral pair as right minus left ($r-l$). To reduce the measurement error, all measurements were replicated three times, and the median of the three measures was chosen, as this method produces a more representative measure than the arithmetic mean if the distribution of measurements is asymmetric (Zar, 1984). No measurements were attempted on broken or worn parts of the skulls; therefore, some skulls have missing values. A two-way ANOVA was conducted to test for the significance of FA relative to measurement error and for detecting the presence of DA (following Palmer & Strobeck, 1986). All the traits, in which the difference between right and left size deviated significantly from zero, were considered as traits which have a natural component of DA. FA in these traits was calculated in two different ways: as the absolute value of the residuals of a regression of the left and

right traits, and as the variance of (r−l) of each trait. We also calculated the mean values of FA for all the traits (the sum of the absolute values of FA of every traits divided the number of traits considered). FA in the traits which did not show DA was calculated as the variance of (r−l), as the absolute value of FA and the mean values of FA for all the traits was calculated. Departures from normality of (r−l) for each trait were tested on all individuals, with a Kolmogorov–Smirnov test (Zar, 1984). Difference of the degree of FA between sexes (with the age groups pooled) and between age groups (with sexes pooled) were compared with an F-test. For these two tests we considered only the individuals collected in period 2. For testing whether there is a dependence of FA on trait size, and for testing whether FAs and the mean FAs (or the absolute values of the residuals in those traits which showed DA) on different traits were correlated with each other, we used a Spearman's rank test. We again considered only the individuals collected in period 2.

Fluctuating asymmetry in the three periods of collection

A Levene's test was conducted for all traits to test whether absolute FA was significantly different during the three periods of collection. As the absolute values of FA are half-normally distributed we also performed a Kruskal–Wallis one-way non-parametric analysis of variance (Zar, 1984). The homogeneity of variances of the traits' FA in the three periods of collection were checked with a F_{max}-test. Multiple comparison tests were made with a Scheffé F-test, for comparing the differences between the periods of collection. An F-test was conducted for every trait to test if the variance of (r−l) was significantly different during the three periods of collection.

Concentration of contaminants and correlation with fluctuating asymmetry

The muscle, brain, liver and fat tissues of 20 individuals (13 males and 7 females, collected between 1989 and 1994) of the striped dolphin were analysed for the following contaminants: *pp*DDE, *op*DDE, *pp*DDD, *op*DDD, total DDT and HCB. The PCB congeners: 153, 187, 183, 180, 170, 196, 201, 194, 195, 95, 101, 99, 151, 146, 178, 128, 174, 177, 199, 206, 138, the sum of all the congeners. PCBmix1 (144+135), PCBmix2 (149+118), PCBmix3 (PCB 156+171+202) and the ratio: *pp*DDE/*pp*DDT which was used to estimate the exposure time of the individuals to the pesticides (Podestà *et al.*, 1992). A Spearman's rank correlation test was used for measuring the association between the concentration of contaminants in each dolphin's tissues and the absolute FA values of its skull (tests performed both with pooled and separated sexes). A Bonferroni test (Rice, 1989) was applied to avoid significant results arising as a consequence of a large number of related tests.

RESULTS

Measurement errors

The interaction mean square (MS) containing information about FA was tested against error MS (reflecting measurement error) showing that FA was significantly larger than measurement errors in all cases ($2.44 \leq$ interaction MS ≤ 87.52, $0.00 <$ error MS ≤ 0.06, $95 \leq df \leq 301$, $P < 0.001$).

Directional asymmetry

For metric traits the two-way ANOVA used to test for significance of FA relative to measurement error was also used to test for DA. The two-way ANOVA (sides × individuals) showed that there was highly significant DA in traits 1, 2 and 4 ($92 \leq F \leq 379$, $P<0.001$). In all other traits there was no significant directional asymmetry ($0.001 \leq F \leq 6.06$, $0.29 \leq P \leq 1$). As a consequence the analysis of traits 1, 2 and 4 was treated separately from the other traits.

Statistical analysis of fluctuating asymmetry

No traits showed differences in the degree of FA between the two sexes at the 0.05 level ($1.04 \leq F \leq 6.63$, $4 \leq n \leq 16$, $0.06 \leq P \leq 0.74$). The F-test on sex differences could not be performed on traits 5, 6 and 9 because of the absence of sufficient data. No traits showed differences in the degree of FA among the two age groups at the 0.05 level ($1 \leq F \leq 15.35$, $2 \leq n \leq 9$, $0.07 \leq P \leq 0.81$). The F-test on age differences could not be performed on trait 6 because of the absence of sufficient data. Therefore, because there was no evidence of differences in the degree of FA among age and sex groups, both sexes and age classes were pooled together in the following analysis. Individuals of unknown sex and age were included in the analysis.

There was no evidence to prove antisymmetry, and deviations from normal distributions were not significant at the 0.05 level for almost all of the traits (Kolmogorov–Smirnov test: $0.06 \leq P \leq 0.63$, $26 \leq n \leq 80$).

No correlations were found at the 0.05 level between overall length of the traits and the degree of FA in the examined traits (males: $0.22 \leq r_s \leq 0.98$, $16 \leq n \leq 44$, $0.06 \leq P \leq 0.93$, females: $0.77 \leq r_s \leq 0.43$, $4 \leq n \leq 26$, $0.06 \leq P \leq 0.99$). Only trait 3 in females showed a positive relationship between FA and length at the 0.05 level. However, this relationship disappeared when we removed one extreme value. Hence in our investigation we did not use the FA index corrected for size.

FA in different traits did not appear to be correlated with some exceptions: males: trait (5 and 8)*, (7 and 8)*, (3 and 5)*, females: (3 and 8)*. Because these correlations (4 significant correlations at the 0.05 level out of 36 correlations) could be due to chance and because the correlated FAs were different in the two sexes, we decided to calculate the mean FA of the traits. FA of traits 1, 2 and 4 with a natural component of DA was not correlated with FA of the other traits. The mean FA and the mean of the absolute value of the residuals were not correlated (males: $r_s = 0.11$, $n=43$, $P=0.46$, females: $r_s = -0.05$, $n=27$, $P=0.80$).

Fluctuating asymmetry differences in the three periods of collection

The Levene's tests were significant for all the traits with the exception of trait 6 and the traits with a natural component of DA (traits 1, 2 and 4) (see Table 1). If we had excluded all the significant results where the F_{max}-test was significant (indicating that the required condition of the homogeneity of variance for performing an ANOVA analysis was not met), we would obtain only two significant results (traits 3 and 7). However, the one-way non-parametric analysis of variance (Kruskal–Wallis test) was significant for the FA of all traits with the exception of trait 6 (Table 1) in complete concordance with the result obtained performing the test.

Period 3 was characterized by generally increased DS, compared with periods 1 and 2 (Fig. 1, Tables 1, 2). Those traits without a natural component of DA showed significantly higher levels of FA in period 1 than period 2 samples and the individuals

Table 1. Levene's test for comparing the degree of FA of traits 3, 5, 6, 7, 8 and 9 and the absolute values of the traits 1, 2 and 4 (with a natural component of DA), during the three periods of collection. *F* max-test for testing the homogeneity of variances of the traits' FA during the three periods of collection, Kruskal–Wallis one-way non-parametric analysis of variance and Scheffé's *F*-test for multiple comparisons

Traits	Period 1 (*n*), Mean ± SD	Period 2 (*n*), Mean ± SD	Period 3 (*n*), Mean ± SD	Source of variation	df	MS	Levene's test (*F* value)	*P*	*F* max test	*P*	K-W test (H value)	*P*	Scheffé's *F*-test
3	1.1 ± 0.98 (33)	1.95 ± 0.94 (26)	0.63 ± 0.77 (26)	Between	2	12.00	14.23	***	1.63	n.s.	22.94	***	(1<2)**, (2>3)***
				Within	82	0.82							
5	0.76 ± 0.77 (15)	1.72 ± 1.02 (15)	0.39 ± 0.51 (20)	Between	2	7.75	13.08	***	4	**	17.23	***	(1<2)**, (2>3)***
				Within	47	0.59							
6	0.95 ± 0.90 (6)	1.34 ± 1.24 (7)	0.93 ± 0.63 (13)	Between	2	0.41	0.52	n.s.	1.9	n.s.	0.47	n.s.	
				Within	23	0.79							
7	0.96 ± 0.89 (28)	2.35 ± 1.17 (23)	0.89 ± 0.94 (26)	Between	2	16.37	16.37	***	1.75	n.s.	22.68	***	(1<2)***, (2>3)***
				Within	74	0.10							
8	0.95 ± 0.78 (28)	1.83 ± 1.32 (24)	0.67 ± 0.78 (28)	Between	2	9.17	9.69	***	2.84	**	16.57	***	(1<2)**, (2>3)***
				Within	77	0.95							
9	0.89 ± 0.6 (18)	2.35 ± 2.34 (16)	0.58 ± 0.65 (18)	Between	2	14.90	7.62	**	15.28	**	13.64	**	(1<2)*, (2>3)**
				Within	49	1.95							
Mean FA	0.98 ± 0.4 (34)	1.96 ± 0.63 (27)	0.71 ± 0.49 (30)	Between	2	12.00	47.74	***	2.5	**	46.35	***	(1<2)***, (1>3)*, (2>3)***
				Within	88	0.26							
Resid. 1	7.57.33 (26)	5.33 ± 4.44 (21)	4.18 ± 3.77 (26)	Between	2	73.82	2.47	n.s.	5.24	**	0.43	n.s.	
				Within	70	29.94							
Resid. 2	2.07 ± 1.67 (27)	1.81 ± 1.29 (25)	2.04 ± 1.7 (25)	Between	2	0.51	0.21	n.s.	1.75	n.s.	0.23	n.s.	
				Within	74	2.46							
Resid. 4	1.21 ± 0.94 (27)	0.9 ± 0.69 (15)	1.2 ± 0.89 (21)	Between	2	0.55	0.72	n.s.	1.89	n.s.	1.28	n.s.	
				Within	60	0.76							
Mean Resid. FA	2.7 ± 2.21 (34)	2.56 ± 2.29 (25)	2.5 ± 1.3 (27)	Between	2	0.34	0.08	n.s.	3.1	**	0.95	n.s.	
				Within	83	3.99							

$P<0.01$ = **, $P<0.001$ = ***.

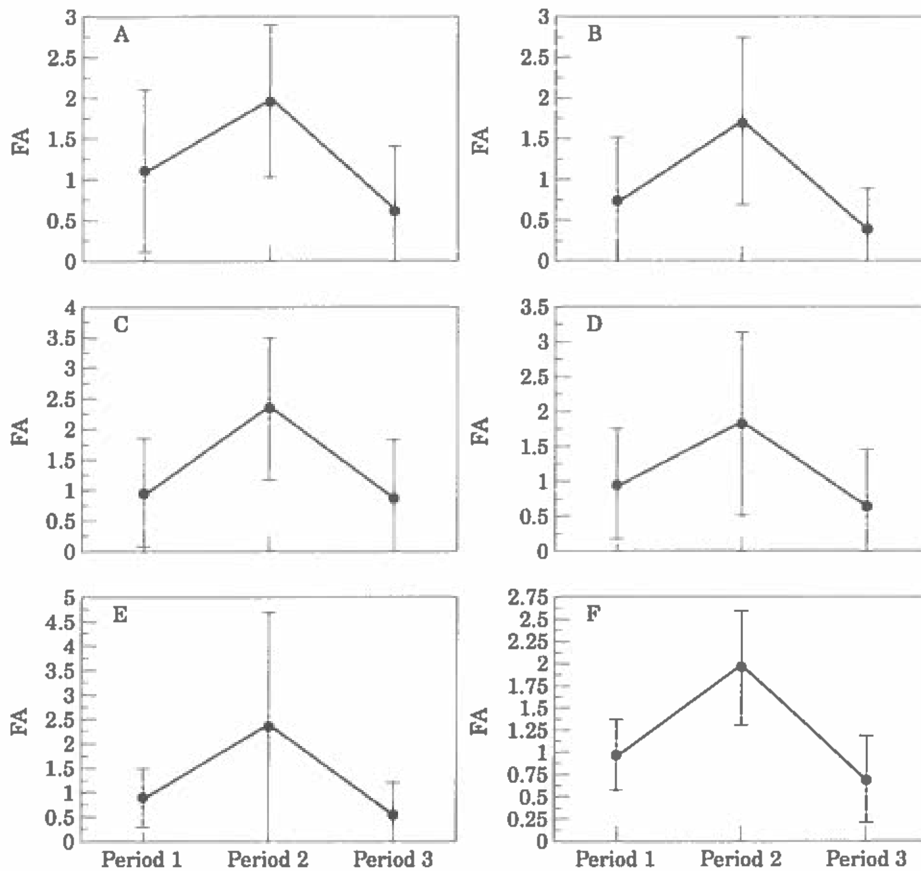


Figure 1. Scheffé's *F*-test of the traits' FA (mean ± SD) in the three periods of collection (*$P<0.05$, **$P<0.01$, **$P<0.001$). A, trait 3 (1<2)**, (2>3)***. B, trait 5 (1<2)**, (2>3)***. C, trait 7 (1<2)***, (2>3)***. D, trait 8 (1<2)**, (2>3)***. E, trait 9 (1<2)*, (2>3)**. F, mean FA of traits 3, 5, 6, 7, 8 and 9 (1<2)***, (1>3)*, (2>3)***.

collected during period 2 showed a significantly higher level of FA with respect to both the other periods. The traits with a natural component of DA did not follow that pattern (Table 2), with only one exception: a significant reduction of the variance in the period 3 compared with the period 2 sample of the variance of trait 1 residuals.

Fluctuating asymmetry and concentrations of contaminants

No significant correlation (Spearman's test) was found between the degree of asymmetry and contaminant concentration at the level of $P<0.01$; 66 out of 13043 = [(13 males + 7 females) × (9 traits + 2 mean values) × 3 tissues × (31 contaminants' values) − missing values] correlations tested were found at the 0.05 level. When we conducted the sequential Bonferroni test all the correlations were no longer significant (data on contaminant level, skull measurements and specimen details are available from the corresponding author on request).

TABLE 2. *F*-test. Comparison of variance of (r−l) of the skull traits 3, 5, 6, 7, 8 and 9 in the three periods (1, 2 and 3) and comparison of variance of (r−l) of the skull traits 1, 2 and 4 with a natural component of DA. The signs (+) or (−) indicate an increase or decrease in variance of the traits (r−l) with respect to the previous period

		Period 1									Period 2								
	Traits	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
Period 2	1	(+)																	
	2		(−)																
	3			(+)*															
	4				(−)														
	5					(+)**													
	6						(+)												
	7							(+)**											
	8								(+)**										
	9									(+)**									
Period 3	1	(−)									(−)*								
	2		(−)									(+)							
	3			(−)*									(−)**						
	4				(+)									(+)					
	5					(−)*									(−)**				
	6						(−)									(−)*			
	7							(−)									(−)**		
	8								(−)									(−)**	
	9									(+)									(−)**

*$P<0.05$; **$P<0.01$.

DISCUSSION

Fluctuating asymmetry and the concentrations of contaminants

We do not report or discuss differences in PCB levels between samples collected in the different periods, as sample sizes were too low to draw general conclusions. However, in two previous investigations (Aguilar & Raga, 1993; Aguilar & Borrel, 1994) it was found that tissues of dolphins stranded during period 2 showed significantly higher levels of all xenobiotic compounds than tissues from dolphins stranded in period 1. The lack of a general correlation between FA of the traits and the contaminant concentrations can have several explanations. Concentrations of PCBs in dolphins are dependent on age, sex and reproductive status. In mammals, organochlorines are transferred from mother to cub in the lipid content of the milk (Tanabe *et al.*, 1982). However, when we excluded females from our investigation we still did not find any correlation. It could also be that different individuals' genotypes have a different susceptibility to the organochlorines. Thus we may not expect a correlation between FA and organochlorines. Additionally, most individuals came from the period 2 sample and therefore had high levels of FA. Another factor which could have obscured the correlation between preservation of the stranded specimens, as significant losses of CHs have been reported in relation to the state of putrefaction of specimens (Borrel & Aguilar, 1990).

Fluctuating asymmetry in the three periods of collection

The sample from period 3 was characterized by increased level of DS (lowered FA), compared with that of period 1, while the sample from period 2 showed a significantly reduced level of DS (increased FA), compared with most of the other periods. The absence of correlations between FA (and mean FA) of traits with a natural component of DA and FA (and mean FA) of all the other traits, could reinforce the hypothesis that traits with DA are unsuitable for monitoring developmental instability. In fact, those traits which in this investigation have shown to have a natural component of DA did not show any pattern or correlations (only one significant result, see Table 2). Hence, this investigation suggests that less developmentally stable individuals are more susceptible to diseases and infections.

In conclusion, it seems that epizooics have a 'purging' effect on natural populations, particularly for species without natural predators or which are top predators. However, thresholds for the outbreak of epidemics may depend on environmental factors such as pollution.

ACKNOWLEDGEMENTS

We wish to thank the Centro Studi Cetacei which has, since 1986, been monitoring the strandings of cetaceans along the Italian coast, and Dr Luigi Cagnolaro for support. This paper would not have been possible without the co-operation of the CSC and represents publication number 70 of this scientific network. We also thank Dr Roberto Poggi, Dirctor of the Natural History Museum of Genova and Dr Paolo Agnelli, curator of the Zoological Museum La Specola di Firenze, for their helpfulness when consulting the collections. Furthermore, we thank Kirsten Zaluski of the National Environmental Research Institute for linguistic improvements and Vibeke

Simonsen, Aksel Bo Madsen, Riccardo Navone and Morten Elmeros for constructive criticisms.

REFERENCES

Aguilar A, Borrel A. 1994. Abnormally high polychlorinated biphenyl levels in striped dolphins *Stenella coeruleoalba* affected by the 1990–1992 Mediterranean epizootic. *Science of the Total Environment* **154:** 237–247.

Aguilar A, Raga JA. 1993. The striped dolphin Epizootic in the Mediterranean Sea. *Ambio* **22:** 524–528.

Archer FI. 1996. Morphological and genetic variation of striped dolphins *Stenella coeruleoalba* (Meyen, 1833). Unpublished PhD Thesis, Univ. of California, San Diego.

Borrel A, Aguilar A. 1990. Loss of organochlorine compounds in the tissues of a decomposing stranded dolphin. *Bulletin Environmental Contamination Toxicology* **45:** 46–53.

Bourdelle E, Grassé PP. 1955. Ordre des Cetaces. In: Grassé PP, ed. *Traitè de Zoologie.* Paris: Masson, 1170.

Brouwer A, Lans MC, de Haan LHJ, Murk AJ, Morse DC. 1994. Formation and toxicological aspects of phenolic metabolites of polychlorobiphenyls (PCBs) and related compounds. *Organohalogen Compounds* **20:** 465–469.

Dietz R, Heide-Jørgensen MP, Härkönen T. 1989. Mass death of harbour seals *Phoca vitulina* in Europe. *Ambio* **18:** 258–264.

Leary RF, Allendorf FW. 1989. Fluctuating asymmetry as an indicator of stress: Implications for conservation biology. *Trends in Ecology and Evolution* **4:** 214–217.

McKenzie JA, O'Farrell K. 1993. Modification of developmental instability and fitness: malathion-resistance in the Australian sheep blowfly *Lucilia cuprina. Genetica* **89:** 67–76.

Møller AP, Swaddle JP. 1997. *Asymmetry, developmental stability and evolution.* Oxford: Oxford University Press.

Neville AC. 1976. *Animal Asymmetry.* London: Edward Arnold.

Palmer AR, Strobeck C. 1986. Fluctuating asymmetry: measurement, analysis, patterns. *Annual Review of Ecology and Systematics* **17:** 391–421.

Perrin WF. 1975. Variation of spotted and spinner porpoise (*genus Stenella*) in the eastern tropical Pacific and Hawaii. *Bulletin of the Scripps Institution of Oceanography* **21:** 1–206.

Podestà M, Marsili L, Focardi S, Manfredi MT, Mignone W, Genchi C. 1992. Ricerche patologiche, parassitologiche e sulla presenza di xenobiotici in *Stenella coeruleoalba* (Meyen, 1833) (Mammalia, Cetacea). *Atti della Società Italiana di Scienze Naturali del Museo Civico di Storia Naturale, Milano* **133:** 101–112.

Rice WR. 1989. Analysing tables of statistical tests. *Evolution* **43:** 223–225.

Tanabe S, Tatsukawa R, Maruyama K and Miyazoki N. 1982. Transplacental transfer of PCBs and chlorinated hydrocarbon pesticides from pregnant striped dolphin (*Stenella coeruleoalba*) to her foetus. *Agriculatural Biological Chemistry* **46:** 1249–1254.

Zakharov VM, Yablokov AV. 1990. Skull asymmetry in the Baltic grey seal: Effects of environmental pollution. *Ambio* **19:** 266–269.

Zar JH. 1984. *Biostatistical Analysis.* New Jersey: Prentice Hall.

J. Zool., Lond. (2000) **252**, 544–547 © 2000 The Zoological Society of London

Allozyme variation in the Eurasian badger *Meles meles* in Denmark

Cino Pertoldi[1]*, Volker Loeschcke[1], Aksel Bo Madsen[2] and Ettore Randi[3]

[1] Department of Ecology and Genetics, University of Aarhus, Building 540, Ny Munkegade, DK-8000 Aarhus C, Denmark
[2] Department of Landscape Ecology, National Environmental Research Institute, Kalø Grenåvej 14, DK-8410 Rønde, Denmark
[3] Istituto Nazionale per la Fauna Selvatica, via Cá Fornacetta 9, I-40064 Ozzano Emilia (Bo), Italy

Abstract

Genetic diversity in four populations of the Eurasian badger *Meles meles* from Denmark was estimated by allozyme electrophoresis. Low genetic variability was found within populations as compared to the variability found in badger populations from other countries, whereas significant genetic differentiation was found between populations. The low genetic variability and the differentiation between populations is explained by the fragmentation of the Danish landscape, which reduces the effective population size of local populations and the gene flow between them. Low genetic variability found for Danish badgers also supports the hypothesis of a rapid recolonization following the last glaciation.

Key words: *Meles meles*, allozyme, genetic drift, Wahlund effect, esterase

INTRODUCTION

Populations of badger *Meles meles* in Denmark, with a few local exceptions, show a relatively low density (Aaris Sørensen, 1995). Badgers supposedly suffer from habitat fragmentation as they need different habitats which encompass water, woodland and pasture. Moreover, human disturbance and outdoor activities have greatly increased in recent decades and road traffic has become a major threat. From 1983 to 1991, road traffic measured by the number of kilometres driven on the roads, increased by 38% in Denmark and the total number of motor vehicles by 15% (Aaris Sørensen, 1995). The number of badgers killed by traffic has sharply increased in the last 20 years.

Small and isolated populations have a high risk of local extinction and may suffer from the fixation of deleterious alleles (Loeschcke, Tomiuk & Jain, 1994 and references therein). The possible consequences of isolation and small population size include inbreeding depression and the loss of genetic variation. Inbreeding is usually deleterious in species that normally outbreed, whereas when inbreeding is part of the natural social system of a species, inbreeding depression is far less severe, and the genetic load is usually low. The Eurasian badger has a restricted male juvenile dispersal (Cheeseman, Wilesmith *et al.*, 1987) and forms highly stable social groups (Kruuk, 1978). Therefore, one may expect that the demographic consequences of fragmentation outweigh the genetic consequences of finite population size supporting the contention (Gilpin, 1987) that ecological effects may be more significant than genetic effects in the short term.

*All correspondence to: Cino Pertoldi.
E-mail: Cino.Pertoldi@biology/auu.dk

The aim of this paper is to screen the genetic structure of four subpopulations of Danish badgers (see Fig. 1). Allozyme variation was screened using standard electrophoretic techniques.

MATERIAL AND METHODS

Tissues from 75 (38 males and 37 females) badgers killed on the road were collected during 1995–97 in Denmark. Seventy-five samples of badger kidney, liver and muscle collected from 4 zones (zones 1, 2, 3 and 4; see Fig. 1) were stored at −30 °C, and analysed by horizontal starch gel electrophoresis using procedures described by Simonsen (1982) and Selander *et al.* (1971). Fourteen protein systems representing 19 presumptive structural loci, were surveyed for allozyme variation: alcohol dehydrogenase (ADH, E.C. 1.1.1.1), malic enzyme (MOD, E.C. 1.1.1.40), glucose dehydrogenase (GDH, E.C. 1.1.1.47), peptidases (PEP, E.C. 2.7.5.1), mannosephosphate isomerase (MPI, E.C. 5.3.1.8), sorbitol dehydrogenase (SDH, E.C. 1.1.1.37), isocitrate dehydrogenase (IDH, E.C. 1.1.1.42), glucose phosphate isomerase (GPI, E.C. 5.3.1.9), lactate dehydrogenase (LDH, E.C. 1.1.1.27), esterase (EST, E.C. 3.1.1.1), malate dehydrogenase (MDH, E.C. 1.1.1.37), phosphoglucomutase (PGM, E.C. 2.7.5.1), adenylate kinase (AK, E.C. 2.7.4.3), 6-phosphgluconate dehydrogenase (GPD, E.C. 1.1.1.49). Only EST was polymorphic.

To avoid the possibility of obtaining anomalous levels of genetic similarity within zones, zone was subdivided into 8 rectangles of 10 × 20 km. A subsequent χ^2 analysis of the number of samples within each rectangle indicated a uniform sampling within

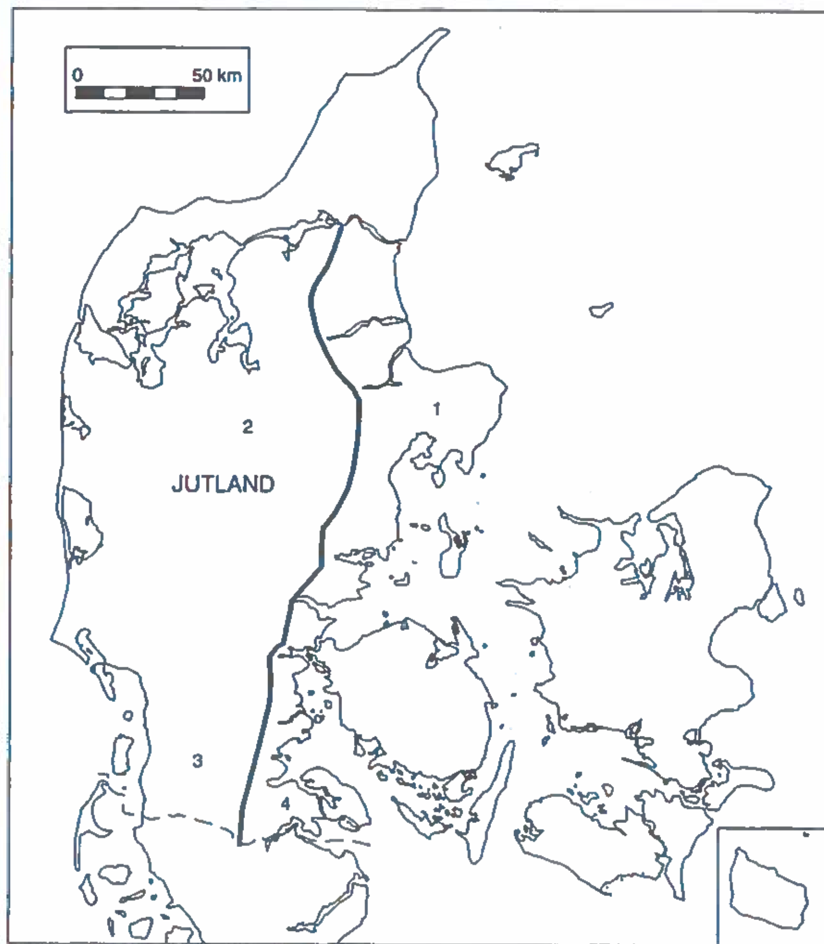


Fig. 1. Map of Denmark with the four collecting zones. Dark solid line = motorway.

zones, as the within-zone variances in all the 4 zones were significantly lower than the mean (Bishop, 1983).

Statistical tests for departure from Hardy–Weinberg expectations and differentiation among zones were performed using GENEPOP 2.0 (Raymond & Rousset, 1995*a*). The average heterozygosity (H_o) was calculated for every subpopulation. The Hardy–Weinberg test was performed using the Markov chain method (see Rousset & Raymond, 1995). An unbiased estimate of the *P*-value for population genetic differentiation (Fisher's exact test) for all populations and for all pairs of populations was performed, as described by Raymond & Rousset (1995*b*). Deviation from panmixia was estimated with F_{IS} coefficients according to Weir & Cockerham (1984). The amount of interpopulation heterogeneity and genetic differentiation between zones was estimated using standardized genetic variance, F_{ST} (Wright, 1965).

RESULTS

Two esterase isozyme systems were found (*EstI* and *EstII*). However, only the *EstI locus* showed detectable variability, whereas the *EstII locus* appeared overstained and smeared. Three alleles *a* (100), *b* (127) and *c* (140) were detected for the *EstI locus*. The combination of these alleles determined six different genotypes, all present in the Danish subpopulations (see Table 1). H_o (considering all the 19 loci investigated), ranged from 0.015 in subpopulation 4 to 0.029 in subpopulation 2, whereas H_o considering only the *Est I locus* ranged from 0.28 to 0.56 (see Table 1).

Deviations from the Hardy–Weinberg equilibrium were significant in the total population ($P = 0.041$, SE = ±0.012). Fisher's exact test for population differentiation was highly significant ($P < 0.001$), as was (with only one exception) the genetic differentiation between subpopulations (see Table 2). The overall genetic differentiation between zones, considering all the zones was

Table 1. Genotype frequencies of the *Est*1 locus, observed heterozygosity (H_o), expected heterozygosity (H_e) and mean heterozyghosity (mean H_o) in the four zones. *n*, number of individuals

Zone	*n*	Males:females	Genotype *aa*	*ab*	*bb*	*ac*	*bc*	*cc*	H_3	H_o
1	18	8:10	5	7	5	0	1	0	0.54	0.44
2	25	13:12	8	8	3	2	4	0	0.6	0.56
3	25	11:14	3	6	2	4	1	9	0.65	0.44
4	7	6:1	0	0	5	0	2	0	0.26	0.28

Table 2. F_{st} values of the pairwise comparisons between the four zones. Values in parentheses are the minimum distance (km) between the two zones compared; asterisks, the level of significance of the genetic differentiation for pairwise comparisons of subpopulations. *** = $P<0.001$, NS = non significant. Zero value means that zone 3 and 4 are separated by the motorway

Zone	F_{st} 1	2	3
2	0 (40) NS		
3	0.104 (140)***	0.171 (100)***	
4	0.306 (140)***	0.249 (120)***	0.32 (0)***

high ($F_{ST}=0.154$), and sometimes higher than the overall genetic differentiation, when considering pairwise comparisons between zones (see Table 2). The average F_{IS} coefficient was positive ($F_{IS}=0.189$), considering all the zones together, and it was positive in three out of four zones (zone 1 $F_{IS}=0.183$; zone 2 $F_{IS}=0.064$; zone 3 $F_{IS}=0.328$; zone 4 $F_{IS}=-0.009$).

DISCUSSION

The significant deviations from Hardy–Weinberg proportions within the entire population and the heterozygote deficiency within the single subpopulations (positive values of F_{IS}) could be the result of deviation from panmixia, differences in N_e, different degrees of isolation of the four subpopulations, or more generally to a Wahlund effect (Wahlund, 1928). That only the *EST*1 locus was polymorphic and the generally low genetic variability found in the Danish badgers, is in accordance with a previous investigation on badgers from Gloucestershire, southern England (Evans, Macdonald & Cheeseman, 1989).

The positive overall F_{ST} coefficient, the highly significant value ($P<0.001$) obtained for Fisher's exact test, and the highly significant genetic differentiation found between subpopulations, indicate substructuring perhaps because of bottlenecking. The high F_{ST} values for pairwise comparisons between subpopulations indicate substantial differentiation, suggesting that genetic drift is acting on the genetic structure of the subpopulations.

The positive F_{IS} values found for the different subpopulations indicate a heterozygote deficit within every single subpopulation. However, F_{IS} values could also be biased by the small sample size investigated, which could have contributed to this result.

The high genetic similarity among individuals within single subpopulations, is consistent with inbreeding and limited dispersal obtained from field studies of ecology and behaviour of badgers in a high-density area (Cheeseman, Cresswell *et al.*, 1988). A balance must be maintained between the proportion of individuals dispersing and the proportion remaining philopatric such that inbreeding and resource competition are minimized. This scenario could be the consequence of a prevalent agricultural landscape that is thought to reduce the migration rate and density of badgers (Cheeseman, Cresswell *et al.*, 1988), with increased differentiation between subpopulations as a consequence.

Habitat fragmentation reduces mating distance increasing the genetic heterogeneity between subpopulations. Close inbreeding in these isolated patches, and the low genetic variability found within subpopulations, confirms this hypothesis. Badgers killed on the road reduce the population size by *c.* 10% (Aaris Sørensen, 1995). Findings in Denmark indicate that the loss of badgers as a result of road traffic is particularly significant, and this source of mortality equals the annual level of cub production (Griffiths, Griffiths & Thomas, 1993).

Colonization of Denmark by badgers began 10 000 years ago, following Holocene climate warming (Hayden, 1993). We suspect that badgers which colonized northern temperate zones by dispersal from southern refuges had low amounts of genetic variability because of recurrent population decline and bottlenecks during Pleistocene climatic changes. The average level of heterozygosity (considering all the loci investigated) found for the four Danish badger subpopulations (from 0.015 to 0.029), was low compared with H_o found in one English badger population (Bristol, $H_o=0.082$, number of loci investigated = 20) and two Irish populations (Offaly, $H_o=0.32$, number of loci investigated = 14, Cork, $H_o=0.103$, number of loci investigated = 14) but was similar to $H_o=0.020$ (number of loci investigated = 23) found for another English population from Gloucestershire (data from Hayden, 1993, and references therein). The data from Hayden (1993) showed that H_o of badger populations can vary widely, from a low genetic variability (as found in the Danish subpopulations) to a high value (as found in one of the English

and in the two Irish populations) when compared to other carnivore populations (e.g. Baccus *et al.*, 1983). The higher mean H_o of one English and both Irish populations compared to that of Danish badgers cannot, however, be explained by post-glacial colonization, as the separation of Britain from the continental landmass happened at least 9000 years ago (Hayden, 1993).

In conclusion, there are indications that the low amount of genetic variation found in the Danish subpopulations might be explained by a combination of ancient bottlenecking and genetic drift. Future work should seek to develop markers which do not require well-preserved tissue, e.g. microsatellites (Tautz, 1989). With this technique, hypervariable polymorphisms at individual loci can be determined with minimal template DNA and the data analysed using conventional population genetic analyses. However, satisfactory primers have yet to be developed (Burke, Hanotte & Van Pijlen, 1996), although work is in progress (K. Bijlsma, pers. comm.). An unpublished study based on minisatellites also showed extremely low levels of variability. But as only a small subset of our samples provided template DNA of sufficient quality, it was difficult to draw conclusions on between-population variability. A parallel study of the mitochondrial DNA control region also found no variabilty (E. Randi, pers. comm.).

Acknowledgements

We thank Jürgen Tomiuk, Vibeke Simonsen for constructive comments of the manuscript and Bo Gaardmand and Kirsten Zaluski for help with figures and linguistic suggestions.

REFERENCES

Aaris Sørensen, J. A. (1995). Road-kills of badgers (*Meles meles*) in Denmark. *Ann. Zool. Fenn.* **32**: 31–36.

Baccus, R., Ryman, N., Smith, M. H., Reuterwall, C. & Cameron, D. (1983). Genetic variability and differentiation of large grazing mammals. *J. Mammal.* **64**: 109–120.

Bishop, O. N. (1983). *Statistics for biology*. New York: Longman.

Burke, T., Hanotte, O. & Van Pijlen, I. A. (1996). Minisatellite analysis in conservation genetics. In *Molecular genetic approaches to conservation*: 251–275. Smith, T. B. & Wayne, R. K. (Eds). New York: Oxford University Press.

Cheeseman, C. L., Cresswell, W. J., Harris, S. & Mallinson, P. J. (1988). Comparisons of dispersal and other movements in two badger (*Meles meles*) populations. *Mammal Rev.* **18**: 51–59.

Cheeseman, C. L., Wilesmith, J. W., Ryan, J. & Mallinson, P. J. (1987). Badger population dynamics in a high density area. *Symp. zool. Soc. Lond.* **58**: 279–294.

Evans, P. G. H., Macdonald, D. W. & Cheeseman, C. L. (1989). Social structure of the Eurasian badger (*Meles meles*): genetic evidence. *J. Zool. (Lond.)* **218**: 587–595.

Gilpin, M. E. (1987). Spatial structure and population vulnerability. In *Viable populations for conservation*: 125–139. Soulé, M. E. (Ed.). Cambridge: Cambridge University Press.

Griffiths, H. I., Griffiths, C. A. & Thomas, D. H. (1993). *The badger (Meles meles). An assessment of the population status, conservation needs and management requirements of the species in the western Palearctic*. Report to the Standing Committee of the Convention on the Conservation of European Wildlife and Natural Habitats, EU Report.

Hayden, T. J. (1993). *The badger*. Dublin: Royal Irish Academy.

Kruuk, H. (1978). Spatial organisation and territorial behaviour of the European badger (*Meles meles*). *J. Zool. (Lond.)* **184**: 1–19.

Loeschcke, V., Tomiuk, J. & Jain, S. K. (1994). *Conservation genetics*. Basel: Birkhäuser Verlag.

Raymond, M. & Rousset, F. (1995*a*). Genepop (version 1.2): population genetics software for exact tests and ecumenicism. *J. Heredity* **86**: 248–249.

Raymond, M. & Rousset, F. (1995*b*). An exact test for population differentiation. *Evolution* **49**: 1280–1283.

Rousset, F. & Raymond, M. (1995). Testing heterozygote excess and deficiency. *Genetics* **140**: 1413–1419.

Selander, R. K., Smith, M. H., Yang, S. Y., Johnson, W. E. & Gentry, J. B. (1971). Biochemical polymorphism and systematics in the genus (*Peromyscus. i.*). Variation in the old-field mouse (*Peromyscus poten*). *Univ. Texas Publ.* **103**: 49–90.

Simonsen, V. (1982). Electrophoretic variation in large mammals. II. The red fox (*Vulpes vulpes*), the stoat (*Mustela erminea*), the weasel (*Mustela nivalis*), the pole cat (*Mustela putorius*), the pine marten (*Martes martes*), the beech marten (*Martes foina*), and the badger (*Meles meles*). *Hereditas* **96**: 299–305.

Tautz, D. (1989). Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acid Res.* **17**: 6463.

Wahlund, S. (1928). Zusammensetzung von population and korrelation-serscheinungen von standpunkt der vererbungslehre ans betrachtet. *Hereditas* **11**: 65–106.

Weir, B. S. & Cockerham, C. C. (1984). Estimating *F*-statistics for the analysis of population structure. *Evolution* **38**: 1358–1370.

Wright, S. (1965). The interpretation of population structure by *F*-statistics with special regard to systems of mating. *Evolution* **19**: 365–420.

Evolutionary Ecology Research, 2001, **3**: 449–463

Developmental instability in sexually reproducing and parthenogenetic populations of *Bacillus rossius rossius* and *Bacillus rossius redtenbacheri*

C. Pertoldi,[1]* V. Scali[2] and V. Loeschcke[1]

[1] *Department of Ecology and Genetics, University of Aarhus, Ny Munkegade, Building 540, DK-8000 Aarhus, Denmark and* [2] *Dipartimento di Biologia Evoluzionistica Sperimentale, Via Selmi 3, 40126 Bologna, Italy*

ABSTRACT

Developmental instability, estimated by fluctuating asymmetry and morphological variance, was investigated in sexually reproducing and parthenogenetic populations of *Bacillus rossius rossius* and *Bacillus rossius redtenbacheri*. Fluctuating asymmetry was significantly higher in parthenogenetic females than amphigonic females in both sub-species. It was also higher in males and parthenogenetic females of the sub-species *redtenbacheri* than in males and parthenogenetic females of the sub-species *rossius*. When we compared fluctuating asymmetry between males and females in amphigonic populations within each sub-species, we found no significant differences with one exception: the antenna showed higher fluctuating asymmetry in males than in females in both sub-species. There was a higher morphological variance in parthenogenetic females of the sub-species *rossius* than in parthenogenetic females of the sub-species *redtenbacheri*, whereas no significant differences were found between the amphigonic females of the two sub-species. In both sub-species, there was evidence for higher morphological variance in amphigonic than parthenogenetic females. When Lerner's conjecture was tested, fluctuating asymmetry was not significantly higher in individuals with a body size more than one standard deviation from the mean of the size distribution.

Keywords: amphigonic reproduction, developmental instability, fluctuating asymmetry, parthenogenetic reproduction.

INTRODUCTION

Sexually reproducing individuals produce genetically diverse offspring that may be less prone to extinction than genetically uniform offspring in variable environments (see review in Williams, 1975). Hence, sexual reproduction maintains genetic flexibility in a population, especially in variable environments, where different combinations of genes may respond differently to environmental fluctuations. On the other hand, parthenogenesis, which has

* Address all correspondence to C. Pertoldi, Department of Landscape Ecology, National Environmental Research Institute, Kalø Grenåvej 14, DK-8410 Rønde, Denmark. e-mail: cpb@dmu.dk

Consult the copyright statement on the inside front cover for non-commercial copying policies.

© 2001 C. Pertoldi

evolved independently in many animal and plant groups (White, 1973), has the advantage that clonally reproducing individuals maximize their genetic representation in future generations. However, in parthenogens, adaptive gene combinations are maintained at the expense of producing genetically uniform offspring. Under optimal conditions, parthenogenetic individuals have twice the fitness advantage of sexually reproducing individuals, but the cost of clonal reproduction is high in a variable environment (Maynard Smith, 1978; Hurst and Peck, 1996; Barton and Charlesworth, 1998). To quantify the relative cost and consequences of clonal reproduction in a variable environment, we compared developmental instability in sexually reproducing and parthenogenetic populations of two Italian sub-species of *Bacillus rossius*.

Developmental instability, which is influenced by several factors (e.g. pollution, stress during development, genetic stress), appears to be more or less correlated with a number of fitness-related traits (see Møller and Swaddle, 1997, and references therein; but see Clarke, 1997a). Furthermore, it has been suggested that developmental instability is greater in sexually selected traits than in other traits (Møller and Thornhill, 1998; but see Palmer 1999). The genetic basis of developmental instability and canalization is generally unknown, even though many studies have focused on this topic (for canalization, see Wagner *et al.*, 1997; for developmental instability, see Clarke, 1993). No convincing genetic mechanism for developmental instability has been suggested, but hypotheses regarding the genetic and molecular mechanisms of canalization have been proposed. Thoday (1958) proposed that modifier genes could be responsible for the maintenance of canalization of a trait; recent research of heat-shock protein HSP90 mutants in *Drosophila* has provided the first molecular evidence for such a mechanism (Rutherford and Lindquist, 1998).

Bacillus rossius is a very peculiar stick insect, which, over its wide holomediterranean range, builds up both bisexual and facultatively parthenogenetic, all-female populations. The latter obviously derive from nearby amphigonic demes, since their genetic structure reflects more closely that of their nearest bisexuals rather than that of geographically distant parthenogens; two instances of actual shift from bisexual to unisexual reproduction have been witnessed (Tinti, 1993; Scali, 1996). The two sub-species are *B. rossius rossius*, which is spread along the western coast of Italy and throughout Corsica and Sardinia, and *B. rossius redtenbacheri*, which ranges from Sicily and the Adriatic/Ionian coasts of Italy to Croatia, Yugoslavia, Albania and Eastern Greece. About 30 populations, belonging to both sub-species, were analysed for 20 gene–enzyme systems: *Ald*, *aGpdh* (monomorphic); *Adk-1*, *Adk-2*, *Fh*, *Got-1*, *Got-2*, *Gox*, *G6pdh*, *G3pdh*, *Hk-1*, *Hk-2*, *Idh-1*, *Idh-2*, *Mdh-1*, *Mdh-2*, *Mpi*, *6Pgdh*, *Pgi*, *Pgm* (polymorphic) (Nascetti and Bullini, 1983; Scali and Mantovani, 1989; Mantovani and Scali, 1991; Tinti *et al.*, 1992). Heterozygosity estimates from samples that also included populations close to the four under study (see below) gave values of 0.062 for *B. rossius rossius* and 0.033 for *B. rossius redtenbacheri*. Only bisexual samples contribute to heterozygosity, since heterozygous unisexuals are rare due to the parthenogenetic reproduction mechanism that doubles the A + X set of the reduced egg through anaphase restitution to produce a thelytokous offspring homozygous at all loci (Pijnacker, 1969; Scali, 1969).

Two principal methods are commonly used for the estimation of developmental instability: morpholoigcal variance (Zouros *et al.*, 1980; King, 1984; Livshits and Kobyliansky, 1984; Imasheva *et al.*, 1997), even if the estimate can be blurred by additive genetic variation, and fluctuating asymmetry (see Møller and Swaddle, 1997, and references therein).

Some studies have found a negative correlation between heterozygosity and developmental instability using both methods; however, others have failed to find such a relationship (for reviews, see Mitton, 1995; Britten, 1996). Analysis of differences in fluctuating asymmetry between males and females of haplo-diploid taxa has been limited to a few species only (Clarke *et al.*, 1986, 1992; Clarke, 1997b; Crespi and Brett, 1997) and no clear patterns have emerged.

The main aim of the present study was to determine whether there is a genetic relationship between genetic diversity (heterozygosity) and developmental instability (fluctuating asymmetry or morphological variance). We tested this hypothesis in three ways: first, by comparing the amphigonic males and females of two sub-species with different levels of heterozygosity; second, by comparing parthenogenetic and amphigonic females within the same sub-species, with the parthenogenetic individuals being totally homozygous; third, by comparing the fluctuating asymmetry of individuals at the extremes of the size distribution with that of the modal phenotypes. Such a comparison is based on the suggestion that homozygous individuals are more likely to belong to classes with extreme phenotypes and thus should have greater developmental instability (Lerner, 1954). We also wished to establish whether there are differences in developmental instability (fluctuating asymmetry and morphological variance) between the sexes within the same sub-species.

MATERIALS AND METHODS

Collection of specimens

Stick insects are best collected at night, when adults actively feed, mate and lay eggs. During October 1997, we collected 162 *B. rossius rossius* specimens along the Tyrrhenian coast: 68 parthenogenetic females and 94 amphigonic specimens (41 females, 53 males). The parthenogenetic population was collected at Grilli (40 km south of Grosseto, Tuscany) in a degraded zone where the population lives on bramble bushes. The amphigonic sample was collected at Capalbio, about 15 km south of Grilli, where Mediterranean vegetation is much better preserved and the stick insects mainly live and feed on lentisk shrubs, but also on ilex, myrtle and heather. During the same month, we collected 119 *B. rossius redtenbacheri* specimens along the Adriatic coast, at about the same latitude as that of the Tuscan populations: 68 parthenogenetic females, plus 21 females and 30 males of an amphigonic population. The parthenogenetic sample was collected at Villa Rosa (50 km north of Pescara, Abruzzo) in a degraded zone where the population also lives on bramble along a busy road. The amphigonic sample was collected at Torino di Sangro Marina (40 km south of Pescara) in a pine wood area where the insects feed on lentisk, hornbeam, oak and bramble shrubs.

Measurements and statistical analysis

We measured five bilateral traits and three unilateral traits. The bilateral traits were measured using a binocular microscope with a digital filar eyepiece (Los Angeles Scientific Instrument Company, Inc., USA). The following traits were considered: the antenna, the labial palpus, the maxillary palpus, the length of the fore-legs without the segmented tarsus (femur and tibia) and the cercus on females. Asymmetry was estimated as the difference in

length between each bilateral pair of traits (right – left side). Fluctuating asymmetry was calculated as the variance of (right – left), as an absolute value and as a mean value (Palmer and Strobeck, 1986; Palmer, 1994). A two-way analysis of variance was conducted to test for the significance of fluctuating asymmetry relative to measurement error (following Palmer, 1994). Some individuals showed a strong asymmetry exceeding three standard deviations of the signed values of (right – left) on antenna because of a reduction in segments. These individuals were considered phenodeviants and the fluctuating asymmetry values of their antenna were not included in the analysis.

The unilateral traits (mesonotum, metanotum and abdomen) were measured with a digital calliper to the nearest 0.1 mm. In an effort to quantify possible errors when measuring these traits, we chose 20 amphigonic individuals (10 males and 10 females) at random and for each individual we measured the three traits 10 times. The within-individual coefficient of variation for each mean was then taken as an estimate of the measurement error, adding Haldane's (1955) correction for small sample size. The measurement error of the three traits was low with a mean value of 0.13%.

In all analyses, sub-species, populations, sexes and amphigonic and parthenogenetic females were separated and analysed independently. Variances of the unilateral traits and of the difference in length between each bilateral pair as right minus left (right – left) were calculated. An *F*-test (Fowler and Cohen, 1990) was conducted to compare the variances. We did not perform any log-transformation as the traits were normally distributed and we compared variances instead of coefficients of variation (CV), as the range of the traits' size was very narrow.

To check if the data obtained displayed the statistical properties of fluctuating asymmetry (an approximately normal distribution of signed asymmetry scores around a mean of zero), the hypothesis that the mean of right minus left character values equals zero was tested using a one-sample *t*-test. Normality was tested using Lilliefors' test and by inspecting the distributions graphically (following Palmer, 1994).

A Spearman rank correlation (Zar, 1984) was used to test whether the absolute fluctuating asymmetry of the bilateral traits was correlated with the sum of the mesonotum, metanotum and abdomen. The sum was taken as an estimate of the body size of the insect (following Palmer, 1994). A Spearman rank correlation was used to test whether the fluctuating asymmetries of different traits were correlated.

A sequential Bonferroni test (Rice, 1989) was applied to avoid significant results arising as a consequence of a large number of related tests. Following Miller's (1981) suggestions, we made a separate probability statement for each sub-species (when the reproductive systems were tested) or for each reproductive system (when the two sub-species were compared).

Lerner's conjecture

To test the prediction that the most extreme phenotypes of each group should also show the greatest fluctuating asymmetry (Lerner's conjecture), mean values of the insects' body size were calculated in each group. The fluctuating asymmetry of individuals with trait values within one standard deviation of the mean was compared with that of those with trait values outside the range using a *t*-test and a non-parametric Mann-Whitney *U*-test (Zar, 1984; Hoffmann *et al.*, 1999).

RESULTS

Measurement error

The interaction 'mean square' (MS), which contains information about fluctuating asymmetry, was tested against error mean square (reflecting measurement error). Fluctuating asymmetry MS was significantly larger than measurement error MS in all cases ($2.3 <$ interaction $MS < 6.1$; $0.09 <$ error $MS < 0.14$; d.f. = 164, $P < 0.001$) bar one, the cercus (interaction $MS = 0.35$, error $MS = 0.38$; d.f. = 164, $P > 0.05$), which was excluded from further analysis.

Fluctuating asymmetry

No significant deviations from zero of the mean of the trait (right – left) distributions were found for the antenna (one-sample t-test: $0.11 < P < 0.68$; $19 <$ d.f. < 65). The labial palpus showed two significant deviations from zero (one-sample t-test: $0.008 < P < 0.8$; $20 <$ d.f. < 67), the maxillary palpus one significant deviation from zero (one-sample t-test: $0.01 < P < 0.1$; $20 <$ d.f. < 67) and the fore-leg one significant deviation from zero (one-sample t-test: $0.02 < P < 0.7$; $20 <$ d.f. < 67). We decided, however, not to exclude the traits in which fluctuating asymmetry showed a significant deviation from zero, because the deviations could have been due to the small sample sizes and the significance vanished if we removed one or two extreme values.

Deviations from normal distributions were not significant at the 0.01 level for almost all of the traits (Lilliefors' test: $0.02 < P < 0.58$; $20 < n < 68$). Only 6 of 24 Lilliefors' tests gave significant deviations from normality at the 0.05 level. Hence, the distributions of all traits' fluctuating asymmetry were inspected graphically; the deviations from normality were due to a leptokurtic distribution. Only the fore-leg showed a platykurtic distribution with peaks at the extreme values, thus exhibiting anti-symmetry. Therefore, we removed the fore-legs from our analysis.

The fluctuating asymmetries of the different traits did not appear to be correlated ($-0.36 < r_s < 0.32$; $20 < n < 68$; $0.08 < P < 0.95$). No correlations were found between the sum of the mesonotum, metanotum and abdomen (body size of the insect) and the fluctuating asymmetry of the traits ($-0.44 < r_s < 0.35$; $16 < n < 55$; $0.06 < P < 0.97$).

Fluctuating asymmetry between the two sub-species

Some significant differences in variance (F-test) of the signed values of (right – left) of the bilateral traits were noted between the different sub-species (see Table 1). In particular, we found evidence for greater fluctuating asymmetry in the males of the *redtenbacheri* sub-species than the males of the *rossius* sub-species. For amphigonic females only, the fluctuating asymmetry of the maxillary palpus was greater in the *redtenbacheri* than the *rossius* sub-species. Parthenogenetic females had greater fluctuating asymmetry in the sub-species *redtenbacheri* than in *rossius* (Table 1); all significant results shown in Table 1 were still significant after a sequential Bonferroni test ($P < 0.05$, $K = 3$).

Fluctuating asymmetry between the two reproductive systems within sub-species

Significant differences in variance (*F*-test) of the signed values (right – left) of the bilateral traits were found between the two different reproductive systems (see Table 2). In particular, we found strong evidence for a greater fluctuating asymmetry in the parthenogenetic females than in the amphigonic females of the *rossius* sub-species. The same was found for the *redtenbacheri* sub-species, with the exception of the *labial palpus*, for which the fluctuating asymmetry was greater in the amphigonic than in the parthenogenetic females (Table 2). All significant results shown in Table 2 were still significant after a sequential Bonferroni test ($P < 0.05$, $K = 3$).

Fluctuating asymmetry between the sexes within sub-species

When we compared the fluctuating asymmetry between males and females within sub-species, the only significant difference was for the antenna, which had greater fluctuating asymmetry in males than in females in both sub-species (Table 2).

Morphological variance

Morphological variance between the two sub-species

Significant differences in variance (*F*-test) of the unilateral traits were found between sub-species. The morphological variance of parthenogenetic females was higher in the *rossius* sub-species than in the *redtenbacheri* sub-species; no significant differences were

Table 1. *F*-tests of the variances of the signed values (right – left) of the length of the antenna, labial palpus and maxillary palpus within the two sub-species *Bacillus rossius redtenbacheri* and *Bacillus rossius rossius*

	B. r. redtenbacheri	*B. r. rossius*	*F*-test
Parthenogenetic females			
Antenna	1.23E-4 (66)	6.59E-5 (65)	1.87 (+)**
Labial palpus	1.73E-4 (68)	5.32E-5 (65)	3.24 (+)***
Maxillary palpus	2.81E-4 (68)	9.79E-5 (65)	2.87 (+)**
Amphigonic females			
Antenna	1.91E-5 (20)	2.42E-5 (38)	1.26
Labial palpus	7.01E-5 (21)	7.05E-5 (41)	1.00
Maxillary palpus	8.71E-5 (21)	4.64E-6 (41)	18.77 (+)***
Males			
Antenna	1.77E-4 (24)	5.11E-5 (47)	3.46 (+)**
Labial palpus	8.69E-5 (30)	5.02E-5 (53)	1.73 (+)*
Maxillary palpus	7.23E-5 (30)	1.99E-5 (53)	3.66 (+)**

Note: *F*-tests were performed between individuals with the same reproductive system. The values shown are the variances; figures in parentheses are sample sizes. In the right-hand column, '+' indicates a higher variance of the trait being considered for the sub-species *redtenbacheri* than for the sub-species *rossius*.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 2. *F*-test of the variances of the signed values (right – left) of the length of the antenna, labial palpus and maxillary palpus within the two sub-species *Bacillus rossius redtenbacheri* and *Bacillus rossius rossius*

	Parthenogenetic *vs* amphigonic females	Amphigonic females *vs* males
B. r. redtenbacheri		
Antenna	6.43 (+)***	9.26 (–)***
Labial palpus	8.25 (–)***	1.23
Maxillary palpus	21.09 (+)***	1.20
B. r. rossius		
Antenna	2.72 (+)***	2.11 (–)**
Labial palpus	5.53 (+)***	1.40
Maxillary palpus	68.75 (+)***	1.22

Note: The *F*-test was performed between individuals of different sex or with different reproductive systems. The values shown are the *F*-values. The '+' indicates a higher variance of the denominator of the comparison, the '–' indicates a higher variance of the numerator of the comparison.
** $P < 0.01$, *** $P < 0.001$.

found between the amphigonic females of the two sub-species (Table 3). The morphological variance of one trait, the metanotum, was significantly greater in the males of the *redtenbacheri* sub-species than in those of the *rossius* sub-species (Table 3). All significant results shown in Table 3 were, with one exception, still significant after a sequential Bonferroni test ($P < 0.05$, $K = 3$).

Morphological variance between reproductive systems within sub-species

Significant differences in variance (*F*-test) of the unilateral traits between the two reproductive systems were found in both sub-species. There was strong evidence for greater morphological variance among amphigonic females than parthenogenetic females in both sub-species (Table 4). All significant results shown in Table 4 were still significant after a sequential Bonferroni test ($P < 0.05$, $K = 3$).

Morphological variance between the sexes within sub-species

The amphigonic females of both sub-species had greater morphological variance than the males (Table 4), except for the metanotum in the *redtenbacheri* sub-species.

Statistical analysis of Lerner's conjecture

There was no significant difference in fluctuating asymmetry between individuals whose body size values were more than one standard deviation around the mean and those whose body size values were within one standard deviation around the mean (*t*-test: $0.025 < t < 0.355$; $5 < n < 27$; $0.075 < P < 0.98$; Mann-Whitney *U*-test: $32.5 < U < 228$; $5 < n < 27$; $0.06 < P < 0.69$).

Table 3. *F*-tests of the variances of the traits mesonotum, metanotum and abdomen between the two sub-species *Bacillus rossius redtenbacheri* and *Bacillus rossius rossius*

	B. r. redtenbacheri	*B. r. rossius*	*F*-test
Parthenogenetic females			
Mesonotum	0.66 (19)	1.29 (58)	1.95
Metanotum	0.68 (19)	1.38 (58)	2.03 (–)*
Abdomen	3.00 (19)	6.77 (58)	2.26 (–)**†
Amphigonic females			
Mesonotum	2.53 (19)	5.10 (22)	2.01
Metanotum	1.36 (19)	2.79 (22)	2.05
Abdomen	17.23 (19)	14.84 (22)	1.16
Males			
Mesonotum	0.89 (19)	1.10 (29)	1.23
Metanotum	11.33 (19)	0.70 (29)	16.18 (+)***
Abdomen	3.97 (19)	4.49 (29)	1.13

Note: *F*-tests were performed between species with the same reproductive system. The values shown are the variances; figures in parentheses are sample sizes. In the right-hand column, '+' indicates a higher variance of the trait being considered for the sub-species *redtenbacheri* than for the sub-species *rossius*; '–' indicates a higher variance of the trait being considered for the sub-species *rossius* than for the sub-species *redtenbacheri*.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, †result no longer significant after Bonferroni correction, which was conducted holding a different probability statement for each reproductive system.

Table 4. *F*-tests of the variances of the traits mesonotum, metanotum and abdomen within the two sub-species *Bacillus rossius redtenbacheri* and *Bacillus rossius rossius*

	Parthenogenetic females *vs* amphigonic females	Amphigonic females *vs* males
B. r. redtenbacheri		
Mesonotum	(–)**†	(+)*†
Metanotum	N.S.	(–)**†
Abdomen	(–)***	(+)**†
B. r. rossius		
Mesonotum	(–)***	(+)***
Metanotum	(–)*†	(+)***
Abdomen	(–)**†	(+)***

Note: *F*-tests were performed between individuals of the same sub-species with different reproductive systems. '+' indicates a higher variance of the denominator and '–' indicates a higher variance of the numerator in the comparison.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, † result no longer significant after Bonferroni correction, which was conducted holding a separate probability statement for each sub-species.

DISCUSSION

Fluctuating asymmetry

There could be two explanations for the deviations from normality of the unsigned values of fluctuating asymmetry due to an excessive leptokurtosis of the distribution. First, when the unit of measurement is large in comparison with the actual asymmetry, there will be a tendency for the population's asymmetry distribution to show leptokurtosis even though the traits display fluctuating asymmetry (Palmer, 1994). Second, there could be intense natural or sexual selection against asymmetric individuals; hence, very asymmetric individuals may be relatively rare in field samples, if selection has already acted against the most asymmetric individuals. The platykurtic distribution of fluctuating asymmetry on the fore-leg is probably due to frequent leg regeneration after an autotomy event in early instars, which can blur the true fluctuating asymmetry of the leg.

The reason for the lack of correlations between fluctuating asymmetry among the different traits could be due to the fact that the traits have different developmental windows of vulnerability and that their development is controlled by different gene complexes (Parsons, 1990). Stresses may be specific to particular metabolic pathways and may not affect fluctuating asymmetry in all traits (Parsons, 1990).

Fluctuating asymmetry between sub-species

The reason why the amphigonic females of the sub-species *redtenbacheri* are more asymmetrical than the amphigonic females of the sub-species *rossius* is unclear, but it may be related to differences in the mean heterozygosity of the two sub-species. This would support the hypothesis that differences in fluctuating asymmetry are related to genetic diversification. Several studies have found a negative relationship between fluctuating asymmetry and protein heterozygosity, especially in poikilotherms. These types of organisms are perhaps more susceptible to temperature fluctuations, which may influence biochemical pathways associated with developmental and metabolic processes (Mitton, 1995; Møller and Swaddle, 1997). However, interpretation of these results must be made with caution, as Chakraborty (1987) has shown that heterozygosity at a few allozyme loci does not provide an accurate estimate of an individual's genomic heterozygosity.

The greater fluctuating asymmetry found in the parthenogenetic females of the sub-species *redtenbacheri* compared with the sub-species *rossius* cannot be due to differences in the mean heterozygosity of the two sub-species, as heterozygosity is zero in both sub-species. It could be the consequence of a different stress-susceptibility of the sub-species *redtenbacheri* as compared to *rossius*, or it could be that the *redtenbacheri* sample lived in a more stressed environment than the *rossius* sample.

Fluctuating asymmetry between reproductive systems within sub-species

It is clear from this study that the parthenogenetic females of both sub-species are much more asymmetric than the amphigonic females. Our results are in accordance with other authors' observations in that homozygous individuals are often less stable than their heterozygous counterparts (for example, Leary *et al.*, 1983, 1984; Clarke and McKenzie, 1987; but see Britten, 1996, for a different view). It is not surprising that parthenogenetic females with an almost complete homozygous genome are developmentally less stable than individuals

with a similar but more heterozygous genome. Furthermore, both parthenogenetic sub-species live in a degraded environment with vegetation made up of bramble shrubs, clearly a 'weedy' habitat (see Bullini and Nascetti, 1990), where the environmental fluctuations are expected to be higher than in the wealthier vegetation on which the amphigonic populations analysed are living. In fact, a degraded environment often displays a less structured ecosystem (Van Valen, 1965; Grant, 1967; Rothstein, 1973).

Fluctuating asymmetry between the sexes within sub-species

The greater fluctuating asymmetry of the males' antenna compared with that of the amphigonic females in both sub-species could be due to the length of the antenna being a sexually selected trait, following the suggestion that fluctuating asymmetry is higher in traits under directional selection (Møller and Thornhill, 1998). Møller and Swaddle (1997) suggested that directional selection will favour modifiers that decanalize the phenotype. However, Houle (1998) described seven distinct evolutionary processes that can affect genetic variance, and concluded that directional selection alone is not sufficient to favour decanalizing modifiers, which are favoured only when fitness rises faster than linearly with trait value (Lande, 1980). Another explanation could be that genes controlling antennal development are located on the sex chromosomes, for which the male sex is wholly hemizygous.

Morphological variance

Morphological variance between the two sub-species

We found evidence that the morphological variance of the parthenogenetic females of the *rossius* sub-species was greater than in the parthenogenetic females of the *redtenbacheri* sub-species. This is in contrast to the fluctuating asymmetry of the *redtenbacheri* parthenogenetic females being greater than that of the *rossius* sub-species. Developmental stability refers to the production of a specific phenotype under a given set of environmental conditions (Møller and Swaddle, 1997). Hence, we should expect that the more asymmetric parthenogenetic females of the *redtenbacheri* sub-species should also have greater morphological variance. These findings support the hypothesis that morphological variance is not a good predictor of developmental stability (see King, 1984) and support also Waddington's (1957) hypothesis that separate mechanisms are responsible for the effect of stress on trait variability and on developmental stability.

The lack of evidence of greater intrasexual morphological variance for males and amphigonic females of the *redtenbacheri* sub-species compared with the males and females of the *rossius* sub-species is contrary to our expectation. In fact, additive genetic variation may be sufficient to explain the phenotypic variation patterns among groups with different degrees of allozyme heterozygosity (Allendorf and Leary, 1986). However, these results must be interpreted with caution, as some allozyme loci do not provide an accurate estimator of an individual's genomic heterozygosity.

Morphological variance between the two reproductive systems within sub-species

The greater morphological variance of the amphigonic females compared with the parthenogenetic females in both sub-species can be explained by the fact that morphological variance

in parthenogenetic females is mainly due to environmental components, whereas that of amphigonic females is of both genetic and environmental origin. Morphological variance in a sexually reproducing population ($V_g > 0$) is given by: $V_p = V_g + V_{env} + (gxe) + cov(ge) + DI$, where (gxe) is the genotype by environment interaction, cov(ge) is the co-variance between the genotypic and environmental source of variance and DI is developmental instability. The interaction term expresses the extent to which genotypic variants differ in their sensitivity to environmental effects. The cov(ge) has long been recognized as a confounding source of experimental error (for instance, when the fastest-growing animals are given the best diet). The term cov(ge) probably contributed to the increased morphological variance and increased developmental instability. The result obtained contrasts with Blum's (1988) hypothesis that stressful conditions may increase morphological variance. Under stressful conditions, minor changes in the environment may have large effects on traits, whereas they may have little impact in a more favourable environment.

Morphological variance between sexes within sub-species

The clearly higher morphological variance of the amphigonic females compared with males in the sub-species *rossius* could be due to the males' body size and its components (mesonotum, metanotum and abdomen) being the target of sexual selection, which reduces the morphological variance of the traits (Bulmer, 1985). The pattern observed in the sub-species *rossius* was the same in *redtenbacheri*, except for one highly significant contrasting result (metanotum) which is difficult to explain. However, Møller and Swaddle (1997) described several works where directional selection has been associated with increased morphological variation, supporting their hypothesis that directional selection will favour modifiers that decanalize the phenotype.

Lerner's conjecture

Lerner's conjecture predicts that individuals that have values for quantitative traits close to the mean of the population should also have the most symmetrical traits. Why we did not find such a pattern could be that body size in the two sub-species *rossius* and *redtenbacheri* is a sexually selected trait and, therefore, is under directional selective forces that could have confounded the expected pattern, or more simply the relationship between functional asymmetry and heterozygosity is too weak or absent. This finding contradicts the suggestion of Soulé and Cuzin-Roudy (1982) that homozygous individuals are less developmentally stable, but agrees with the results of Woods *et al.* (1999).

Future directions

We have provided evidence of greater development instability in the parthenogenetic individuals of two sub-species of *Bacillus rossius* compared with amphigonic individuals, which may be explained by a relatively higher cost of parthenogenetic reproduction compared to sexual reproduction. The higher buffering capacity of the amphigonic populations may be envisaged from a biochemical-genetic point of view: when different allelic products have different optima along a range of environmental conditions, heterozygotes will have the most stable genotype (see Schwartz and Laughner, 1969). When using morphological

variance as a precise estimator of developmental instability, populations should be genetically homogeneous ($V_g = 0$) and should not exhibit variation in environmental conditions across the habitat range of species ($V_{env} = 0$); however, these conditions are rarely fulfilled. Furthermore, if a trait is highly canalized and the peak of the size-fitness function is very narrow, there will be a small range in trait size variance between populations and differences will be difficult to detect. The results of several studies of the relationship between functional asymmetry and loss of genetic diversity have been controversial, but that could be due to a threshold relationship between functional asymmetry and genetic diversity (see Gilligan *et al.*, 2000). A clearer picture is required of the extent to which functional asymmetry is genetically based, as this is still an open debate (Møller and Thornhill, 1997; for criticisms, see Leamy, 1997; Markow and Clarke, 1997; Whitlock and Fowler, 1997). This is a fundamental point, because for functional asymmetry to have an evolutionary significance, it must have a heritable basis. A non-zero heritability is also a prerequisite for some hypotheses linking functional asymmetry to genetic stress (Pomiankowski, 1997). It is possible that non-additive genetical effects (dominance and epistatic interactions of genes) are an important feature in the genetical control of developmental stability (Leamy, 1984; Livshits and Kobyliansky, 1991). However, our study was of parthenogenetic individuals found in degraded environments. In a variable environment, the cost of clonal reproduction is high (Maynard Smith, 1978). Therefore, further studies should address other organisms in which parthenogenetic reproduction takes place under optimal conditions and sexual reproduction takes place under sub-optimal conditions. Similarly, it would be interesting to compare parthenogenetic and amphigonic individuals in species with different types of parthenogenesis where heterozygosity is maintained.

ACKNOWLEDGEMENTS

We are grateful to W. Gabriel and J. Tomiuk for helpful comments on an earlier draft of the manuscript and to the following students for help with collections: A. Galassi, M. Passamonti, M. Pellecchia and C. Zauli. This work was supported in part by grants from the Italian Research Council (#95220905) and by the Ministero Università e Ricerca Scientifica e Tecnologica (#104620901).

REFERENCES

Allendorf, F.W. and Leary, R.F. 1986. Heterozygosity and fitness in natural populations of animals. *Conservation Biology: The Science of Scarcity and Diversity* (M.E. Soulé, ed.), pp. 57–76. Sunderland, MA: Sinauer Associates.

Barton, N.H. and Charlesworth, B. 1998. Why sex and recombination? *Science*, **281**: 1986–1990.

Blum, A. 1988. *Plant Breeding for Stress Environments.* Boca Raton, FL: CRC Press.

Britten, B.H. 1996. Meta-analyses of the association between multilocus heterozygosity and fitness. *Evolution*, **50**: 2158–2164.

Bullini, L. and Nascetti, C. 1990. Speciation by hybridization in phasmids and other insects. *Can. J. Zool.*, **68**: 1747–1770.

Bulmer, M.G. 1985. *The Mathematical Theory of Quantitative Genetics.* Oxford: Clarendon Press.

Chakraborty, R. 1987. Biochemical heterozygosity and phenotypic variability of polygenics traits. *Heredity*, **59**: 19–28.

Clarke, G.M. 1993. Patterns of developmental stability in *Chrysopa perla* L. in response to environmental pollution. *Environ. Entomol.*, **22**: 1362–1366.

Clarke, G.M. 1997a. Developmental stability and fitness: The evidence is not quite clear. *Am. Nat.*, **152**: 762–766.

Clarke, G.M. 1997b. The genetic basis of developmental stability. III. Haplo-diploidy: Are males more unstable than females? *Evolution*, **51**: 2021–2028.

Clarke, G.M. and McKenzie, J.A. 1987. Developmental stability of insecticide resistant phenotypes in blowfly: A result of canalizing natural selection. *Nature*, **325**: 345–346.

Clarke, G.M., Brand, G.W. and Whitten, M.J. 1986. Fluctuating asymmetry: A technique for measuring developmental stress caused by inbreeding. *Aust. J. Biol. Sci.*, **39**: 145–153.

Clarke, G.M., Oldroyd, B.P. and Hunt, P. 1992. The genetic basis of developmental stability in *Apis mellifera*: Heterozygosity *vs* genetic balance. *Evolution*, **46**: 753–762.

Crespi, B.J. and Brett, A.V. 1997. Fluctuating asymmetry in vestigial and functional trait of a haplodiploid insect. *Heredity*, **79**: 624–630.

Fowler, J. and Cohen, L. 1990. *Practical Statistics for Field Biology*. Philadelphia, PA: Open University Press.

Gilligan, D.M., Woodworth, L.M., Montgomery, M.E., Nurthen, R.K., Briscoe, D.A. and Frankham, R. 2000. Can fluctuating asymmetry be used to detect inbreeding and loss of genetic diversity in endangered populations? *Anim. Conserv.*, **3**: 97–104.

Grant, P.R. 1967. Bill length variability in birds of the Tres Marias Islands, Mexico. *Can. J. Zool.*, **45**: 805–815.

Haldane, J.B.S. 1955. The measurement of variation. *Evolution*, **9**: 484.

Hoffmann, A.A., Woods, R.E. and Clarke, G.M. 1999. A comment on ingroup–outgroup comparisons for fluctuating asymmetry based on trait values from the left or right sides of an individual. *Am. Nat.*, **153**: 140–142.

Houle, D. 1998. How should we explain variance in the genetic variance of traits? *Genetica*, **102/103**: 241–253.

Hurst, D.L. and Peck, J.R. 1996. Recent advances in understanding of the evolution and maintenance of sex. *TREE*, **11**: 46–52.

Imasheva, A.G., Loeschcke, V., Zhivotovsky, L.A. and Lazebny, O.E. 1997. Effects of extreme temperatures on phenotypic variation and developmental stability in *Drosophila melanogaster* and *Drosophila buzzatii*. *Biol. J. Linn. Soc.*, **61**: 117–126.

King, D.P.F. 1984. Enzyme heterozygosity associated with anatomical character variance and growth in the herring *Clupea harengus*. *Heredity*, **54**: 289–296.

Lande, R. 1980. Genetic variation and phenotypic evolution during allopatric speciation. *Am. Nat.*, **116**: 463–479.

Leamy, L. 1984. Morphometric studies in inbred and hybrid house mice. V. Directional and fluctuating asymmetry. *Am. Nat.*, **123**: 579–593.

Leamy, L. 1997. Is developmental stability heritable? *J. Evol. Biol.*, **10**: 21–29.

Leary, R.F., Allendorf, F.W. and Knudsen, K.L. 1983. Developmental stability and enzyme heterozygosity in rainbow trout. *Nature*, **301**: 71–72.

Leary, R.F., Allendorf, F.W. and Knudsen, K.L. 1984. Superior developmental stability of heterozygotes at enzyme loci in salmonid fishes. *Am. Nat.*, **124**: 540–551.

Lerner, I.M. 1954. *Genetic Homeostasis*. London: Oliver & Boyd.

Livshits, G. and Kobyliansky, E. 1984. Comparative analysis of morphological traits in biochemically homozygous individuals from a single population. *J. Hum. Evol.*, **13**: 161–171.

Livshits, G. and Kobyliansky, E. 1991. Fluctuating asymmetry as a possible measure of developmental homeostasis in humans: A review. *Human Biol.*, **63**: 441–466.

Mantovani, B. and Scali, V. 1991. Allozymic characterization of Sardinian *Bacillus rossius* (Rossi) and *B. atticus* Brunner (Insecta, Phasmatodea). *Genetica*, **83**: 275–287.

Markow, T.A. and Clarke, G.M. 1997. Meta-analysis of the heritability of developmental instability: A giant step backward. *J. Evol. Biol.*, **10**: 31–37.

Maynard Smith, J. 1978. *The Evolution of Sex*. Cambridge: Cambridge University Press.

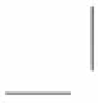
Miller, R.G. 1981. *Simultaneous Statistical Inference.* New York: McGraw-Hill.
Mitton, J.B. 1995. Enzyme heterozygosity and developmental stability. *Acta Theriol.*, suppl. **3**: 33–54.
Møller, A.P. and Swaddle, J.P. 1997. *Asymmetry, Developmental Stability and Evolution.* Oxford: Oxford University Press.
Møller, A.P. and Thornhill, R. 1997. A meta-analysis of the heritability of developmental stability. *J. Evol. Biol.*, **10**: 1–16.
Møller, A.P. and Thornhill, R. 1998. Bilateral symmetry and sexual selection: A meta-analysis. *Am. Nat.*, **151**: 174–192.
Nascetti, G. and Bullini, L. 1983. Differenziamento genetico e speciazione in fasmidi dei generi *Bacillus* e *Clonopsis* (Cheleutoptera, Bacillidae). In *Atti XII Congresso Nazionale Entomologia*, pp. 215–223. Rome: Tipografia Sticca.
Palmer, A.R. 1994. Fluctuating asymmetry analyses: A primer. In *Developmental Instability: Its Origins and Evolutionary Implications* (T.A. Markow, ed.), pp. 335–364. Dordrecht: Kluwer.
Palmer, A.R. 1996. Waltzing with asymmetry. *BioScience*, **46**: 518–532.
Palmer, A.R. 1999. Detecting publication bias in meta-analyses: A case study of fluctuating asymmetry and sexual selection. *Am. Nat.*, **154**: 230–233.
Palmer, A.R. and Strobeck, C. 1986. Fluctuating asymmetry: Measurement, analysis, patterns. *Ann. Rev. Ecol. Syst.*, **17**: 391–421.
Parsons, P.A. 1990. Fluctuating asymmetry: An epigenetic measure of stress. *Biol. Rev.*, **65**: 131–145.
Pijnacker, L. 1969. Automictic parthenogenesis in the stick insect *Bacillus rossius* Rossi (Cheleutoptera, Phasmidae). *Genetica*, **40**: 393–399.
Pomiankowski, A. 1997. Genetic variation in fluctuating asymmetry. *J. Evol. Biol.*, **10**: 51–55.
Rice, W.R. 1989. Analysing tables of statistical tests. *Evolution*, **43**: 223–225.
Rothstein, S.I. 1973. The niche-variation model. Is it valid? *Am. Nat.*, **107**: 598–620.
Rutherford, S.L. and Lindquist, S. 1998. HSP90 as a capacitor for morphological evolution. *Nature*, **396**: 336–342.
Scali, V. 1969. Osservazioni citologiche sullo sviluppo embrionale di *Bacillus rossius*. *Acc. Naz. Lincei Rend. Cl. Sc. Fis. Mat. Nat., Serie VIII*, **46**: 110–116.
Scali, V. 1996. Distribution, genetic diversification and reproductive mechanisms of unisexual *Bacillus* stick insects. In *Abstracts of the Fifth International Congress of Systematic and Evolutionary Ecology*, p. 165. Budapest: Pars Ldt.
Scali, V. and Mantovani, B. 1989. Updating of systematics and speciation mechanisms of *Bacillus* (Insecta, Phasmatodea). *Boll. Zool.*, **56**: 87–98.
Schwartz, D. and Laughner, W.J. 1969. A molecular basis for heterosis. *Science*, **166**: 626–627.
Soulé, M.E. and Cuzin-Roudy, J. 1982. Allometric variation. II. Developmental instability of extreme phenotypes. *Am. Nat.*, **120**: 765–786.
Thoday, J.M. 1958. Homeostasis in selection experiments. *Hereditas*, **12**: 401–415.
Tinti, F. 1993. Morphological, genetic and chromosomal characterization of Corsican and Spanish *Bacillus rossius* (Insecta, Phasmatodea). *Vie Milieu*, **43**: 109–117.
Tinti, F., Mantovani, B. and Scali, V. 1992. Caratterizzazione allozimatica di popolazioni di *Bacillus rossius* dell'Italia centro-meridionale e della Sicilia (Insecta, Phasmatodea). *Boll. Soc. It.* (*Museo Genova*), **123**: 184–194.
Van Valen, L. 1965. Morphological variation and the width of the ecological niche. *Am. Nat.*, **99**: 377–390.
Waddington, C.H. 1957. *The Strategy of the Genes.* New York: Macmillan.
Wagner, G.P., Booth, G. and Bagheri-Chaichian, H. 1997. A population genetic theory of canalization. *Evolution*, **51**: 329–347.
White, M.J.D. 1973. *Animal Cytology and Evolution*, 3rd edn. Cambridge: Cambridge University Press.
Whitlock, M.C. and Fowler, K. 1997. The instability of studies of instability. *J. Evol. Biol.*, **10**: 63–67.

Williams, G.C. 1975. *Sex and Evolution.* Princeton, NJ: Princeton University Press.

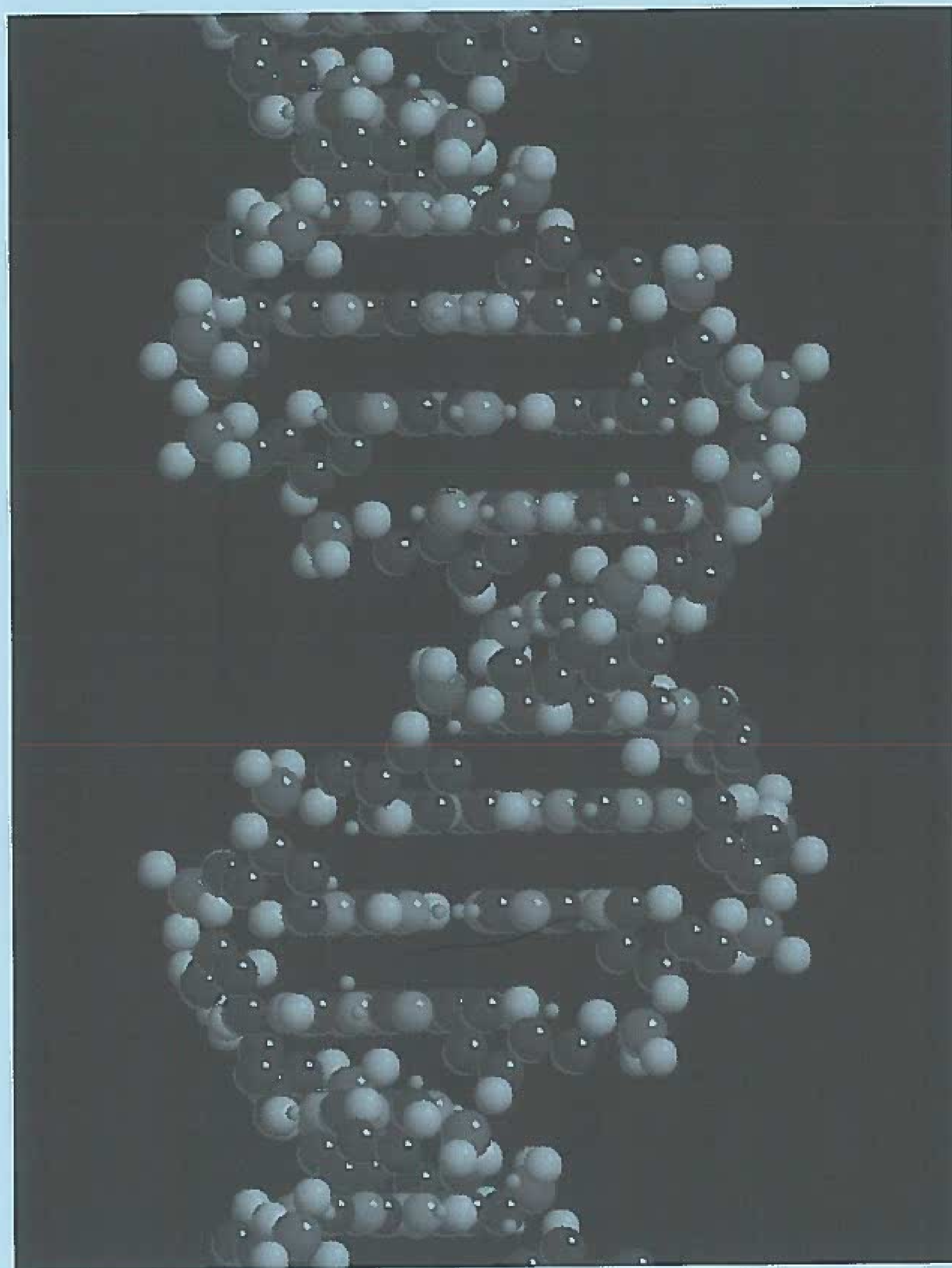
Woods, R.E., Sgrò, C.M., Hercus, M.J. and Hoffmann, A.A. 1999. The association between fluctuating asymmetry, trait variability, trait heritability, and stress: A multiply replicated experiment on combined stresses in *Drosophila melanogaster*. *Evolution*, **53**: 493–505.

Zar, J.H. 1984. *Biostatistical Analysis.* Englewood Cliffs, NJ: Prentice-Hall.

Zouros, E., Singh, M. and Miles, H. 1980. Growth rate in oysters: An overdominant phenotype and its possible explanation. *Evolution*, **34**: 856–867.



Articles in press



J. theor. Biol. (2001) **210**, 000–000
doi:10.1006/jtbi.2001.2317, available online at http://www.idealibrary.com on IDEAL®

A New Method for Estimating Environmental Variability for Clonal Organisms, and the Use of Fluctuating Asymmetry as an Indicator of Developmental Instability

Cino Pertoldi*†, Torsten Nygaard Kristensen‡ and Volker Loeschcke‡

**Department of Landscape Ecology, National Environmental Research Institute, Kalø Grenåvej 14, DK-8410 Rønde, Denmark and ‡Department of Ecology and Genetics, University of Aarhus, Building 540, Ny Munkegade, DK-8000 Aarhus C, Denmark*

(*Received* ■■■)

Environmental science has yet to devise a comprehensive, ecologically relevant, *in situ* biomonitoring system for assessing ecosystem health. Such a system needs to be sufficiently sensitive to provide an early warning of stress. Given the fact that full life table analyses for all threatened species will be extremely time and money consuming and given the urgent need of obtaining as much information as possible about the stress responses of the largest number of species available, we have to consider alternative methods. Here we advocate a biomonitoring technique completely different from traditional physiological or demographical methods. The measure suggested is based upon the concept of developmental instability (*DI*). *DI* refers to the incapacity of producing a specific phenotype under a given set of environmental conditions (Markow, 1994). *DI* is the product of stress, impinging on the individual buffering capacity (Palmer, 1996) and has been suggested as having potential as a stress indicator allowing investigators to identify areas in which ecosystems are stressed (Parsons, 1992). Furthermore, it also allows to estimate the degree of stress, not only in a qualitative, but also in a quantitative way. In this way it is possible to examine populations over time, as investigators will be able to determine temporal changes in the degree of stress experienced.

As for other toxicological tests *DI* as a measure of stress suffers from low reproducibility and large variation in response within populations and between experiments (Woods *et al.*, 1999). Among other things this is caused by the fact that natural populations generally comprise large numbers of genetically diverse individuals inhabiting environments, which are both spatially and temporally heterogeneous (Stearns, 1992). This means that the genetic basis for stress tolerance can only be properly assessed by separating and quantifying the effect of both genes and environment and, most importantly, their interaction on the expression of tolerance. However, when studying stress tolerance, individuals, even from the same population, may have different phenotypic responses. Therefore, investigations should be conducted employing monoclonal strains in order to remove genotypic variability.

In this paper we present ideas based on *DI* that show that the use of monoclonal organisms in toxicological tests (and as a tool as a general stress indicator) might have a huge potential, which, until now, has not been utilized. By using monoclonal strains in stress studies we remove

†Author to whom correspondence should be addressed. E-mail: cpb@dmu.dk

0022-5193/01/000000 + 00 $35.00/0

© 2001 Academic Press

JTBI 20012317 PROD.TYPE: COM
pp. 1–4 (col.fig.: NIL)
ED: Mangala Gowri
PAGN: UMESH TERRENCE— SCAN: NIL

the genetic variance. This has been practised for a long time, but our new method makes the use of monoclonal strains much more efficiently, because it allows one to estimate the confounding effects of environmental variance (V_E). By doing this we believe that the usefulness of DI and other tools for measuring stress will be strongly increased.

Two principal methods are commonly employed for the estimation of DI. Some studies use V_P (David *et al.*, 1994), which is the variance of a trait in a population, others use fluctuating asymmetry (FA), which are random deviations from perfect symmetry in bilaterally paired traits (Møller & Swaddle, 1997 and references therein). However, when working with sexual populations, the estimation of DI is biased because both V_P and FA might be blurred by genotypic variance (V_G). Two other main problems are afflicting the FA concept. If FA should be used as an estimator of environmental conditions the heritable component should be low, because if FA is heritable, it would not provide a reliable bioassay. The heritability (h^2) of FA is currently the subject of some debate (for conflicting views see Leamy, 1997). However, most researchers agree that FA often has a heritable component, but that this is typically small (Whitlock & Fowler, 1997). The problems connected to $h^2 > 0$ can, however, be avoided using a monoclonal strain with $V_G = 0$. The second problem afflicting FA estimates at the population level (especially when measured directly in nature) is that the population under investigation may consist of a mixture of individuals exhibiting different levels of DI (Van Dongen, 1999). This heterogeneity of DI values is due to the V_E within the population, which invalidates the FA estimate (see below). Therefore, the major part of the FA investigations have been limited to laboratory experiments in the attempt to reduce the V_E bias.

In a sexually reproducing population, where $V_G \neq 0$, V_P corresponds to: $V_P = V_G + V_E + (G \times E) + \mathrm{cov}(GE) + DI$, where $(G \times E)$ is the genotype environment interaction and $\mathrm{cov}(GE)$ is the genotype-environment covariance. The $(G \times E)$ expresses the extent to which genotypic variants differ in their sensitivity to environmental effects. The $\mathrm{cov}(GE)$ has long been recognized as a confounding source of experimental error (for instance, when the fastest-growing animals are given the best diet).

V_P of a quantitative character in individuals of a single genotype ($V_G = 0$), reared in a given set or range of environmental conditions, is often taken as a measure of DI of the genotype ($V_P = DI + V_E$) (Møller & Swaddle, 1997 and references therein). However, having removed the genetic component we still have the confounding effect produced by V_E. This explains why some studies even when they are conducted with a monoclonal strain display V_P at a level comparable to that found in sexually reproducing populations (Forbes *et al.*, 1995).

FA is estimated following Palmer (1996), as

$$FA = \sigma^2(r - l), \tag{1}$$

where r and l are, respectively, the trait values on the right- and left-hand side and σ^2 is the variance of the difference.

V_P is estimated as:

$$V_P = \sigma^2(r + l). \tag{2}$$

Statistically, the value of the variance of the sum of right and left [eqn (2)], is equal to the value of the variance of the difference [eqn (1)], if the correlation between right and left is zero (Sokal & Rohlf, 1995). Correspondingly, if the correlation increases, the variance for the sum becomes higher than the variance for the difference. If r and l are negatively correlated, then the variance for the difference will be higher than the variance for the sum. Hence, if there is only one source of variation in a group of individuals (chance disturbances during individual development), then the total V_P (measured by the variance of the sum), will be equal to FA (variance of the differences):

$$\sigma^2(r + l) = \sigma^2(r - l). \tag{3}$$

If there is no genetic variance as in monoclones ($V_G = 0$), an eventual correlation between right and left can only be due to environmental factors. Hence, if there is a non-zero environmental variance (V_E), eqn (3) becomes

$$\sigma^2(r + l) = \sigma^2(r - l) + V_E. \tag{4}$$

As the variance of $(r + l)$:

$$\sigma^2(r + l) = \sigma_r^2 + \sigma_l^2 + 2\,\mathrm{cov}(r, l) \qquad (5)$$

(σ_r^2 and σ_l^2 are the variances of the right- and left-hand side, respectively) and FA which is the variance of $(r - l)$ is equal to

$$\sigma^2\,(r - l) = \sigma_r^2 + \sigma_l^2 - 2\,\mathrm{cov}(r, l). \qquad (6)$$

From eqn (6) it is also clear that the FA estimate is confounded by $\mathrm{cov}(r, l)$ even if the genetic component ($V_G = 0$) is removed. Substituting eqns (5) and (6) into eqn (4) we get

$$\sigma_r^2 + \sigma_l^2 + 2\mathrm{cov}(r, l) = \sigma_r^2 + \sigma_l^2 - 2\,\mathrm{cov}(r, l) + V_E. \qquad (7)$$

Simplifying eqn (7) by deleting the same terms on the right- and left-hand side, we obtain

$$V_E = 4\,\mathrm{cov}(r, l). \qquad (8)$$

Therefore, for each stress group replicate V_E can be calculated as four times the covariance between right- and left-side, $\mathrm{cov}(r, l)$, with

$$\mathrm{cov}(r, l) = R(r, l)\sigma_r\sigma_l, \qquad (9)$$

where R is the Pearson product moment coefficient of the correlation between r and l values, whereas σ_r and σ_l are the standard deviations of right- and left-hand side. Hence, if the regression coefficient is not significantly different from zero, the covariance term (and consequently also $4\,\mathrm{cov} = V_E$) will not be significantly different from zero.

For traits with high functionality (traits for which the level of FA is minimized by strong canalizing selection) our method may have some limitations because a canalizing effect on FA (e.g. when an increased biomechanical cost is associated with increased FA) may increase the $\mathrm{cov}(r, l)$. Investigation of traits with low functionality (traits which have low correlation with fitness) is therefore suggested.

The method proposed in this paper for partitioning out the different components of V_P can easily be applied in the field, because this does not require collections over several generations, nor controlled laboratory conditions. It gives an instantaneous picture of the environmental conditions where the investigated population is collected. The method can therefore be applied to all apomictic or vegetatively reproducing populations in plants, e.g. the aquatic *Helodea canadensis*, as well as in animals, e.g. the terrestrial collembolan *Folsomia candida* and the cladoceran *Daphnia magna*. By using monoclones occupying large areas like *Folsomia candida* it is possible to compare the stress level in specific habitats within this area. It can also be applied to sympatric polyphyletic clonal strains, which then have to be separated using morphometrical or molecular techniques (as $V_G \neq 0$).

We believe that the method presented here will increase the value of FA as an indicator of environmental stress, and also that the method could be of great value in connection with other biomarkers and ecotoxicological tests. Estimating V_E and removing samples where a significant V_E is observed, can increase efficiency and reproducibility of the standard ecotoxicological tests performed today.

We thank Ary Hoffmann, Jürgen Tomiuk and Pamela Hall for critical comments on the manuscript.

REFERENCES

DAVID, J. R., MORETEAU, B., GAUTHIER, J. P., PÉTAVY, G., STOCKEL, A. & IMASHEVA, A. G. (1994). Reaction norms of size characters in relation to growth temperature in *Drosophila melanogaster*: an isofemale lines analysis. *Genet. Sel. Evol.* **26**, 229–251.

FORBES, V. E., MØLLER, V. & DEPLEDGE, M. H. (1995). Intrapopulation variability in sublethal response to heavy metal stress in sexual and asexual gastropod populations. *Funct. Ecol.* **9**, 477–484.

LEAMY, L. (1997). Genetic analysis of fluctuating asymmetry for skeletal charaters in mice. *J. Hered.* **88**, 85–92.

MARKOW, T. A. (1994). *Developmental Instability: Its Origins and Evolutionary Implications.* Dordrecht: Kluwer.

MØLLER, A. P. & SWADDLE, J. P. (1997). *Asymmetry, Developmental Stability and Evolution.* Oxford: Oxford University Press.

PALMER, A. R. (1996). Waltzing with asymmetry. *BioScience* **46**, 518–532.

PARSONS, P. A. (1992). Fluctuating asymmetry: a biological monitor of environmental and genomic stress. *Heredity* **68**, 361–364.

SOKAL, R. R. & ROHLF, F. J. (1995). *Biometry*. New York: W. H. Freeman.

STEARNS, S. C. (1992). *The Evolution of Life Histories*. Oxford: Oxford University Press.

VAN DONGEN, S. (1999). Accurancy and power in the statistical analysis of fluctuating asymmetry: effects of between-individual heterogeneity in developmental instability. *Ann. Zool. Fenn.* **36,** 45–52.

WHITLOCK, M. C. & FOWLER, K. (1997). The instability of studies of instability. *J. Evol. Biol.* **10,** 33–67.

WOODS, R. E., SGRO, C. M., HERCUS, M. & HOFFMANN, A. A. (1999). The association between fluctuating asymmetry, trait variability, trait heritability, and stress: a multiple replicated experiment on combined stresses in *Drosophila melanogaster*. *Evolution* **53,** 493–505.

Effects of habitat fragmentation on the Eurasian badger (*Meles meles*) subpopulations in Denmark

Cino Pertoldi[1], Volker Loeschcke[1], Aksel Bo Madsen[2], Ettore Randi[3] and Nadia Mucci[3].

1: Department of Ecology and Genetics, University of Aarhus, Building 540, Ny Munkegade, DK-8000 Aarhus C, Denmark.

2: Department of Landscape Ecology, National Environmental Research Institute, Kalø Grenåvej 14, DK-8410 Rønde, Denmark.

3: Istituto Nazionale per la Fauna Selvatica, via Ca´ Fornacetta 9, I-40064 Ozzano Emilia (Bo), Italy.

Corresponding author: Cino Pertoldi, Department of Ecology and Genetics, University of Aarhus, Building 540, Ny Munkegade, DK-8000 Aarhus C, Denmark. Fax: ++4586127191, e-mail: Cino.Pertoldi@biology.aau.dk

Abstract

Genetic variation in five populations of the Eurasian badger from Denmark was screened, using the hyper-variable minisatellite DNA probe 33.15. Very low genetic variability was found within populations. This lack of variability could be related to the fragmentation of the Danish landscape which reduces the effective population size of local populations and the gene flow between the different subpopulations. The possibility of managing the Danish badger subpopulations as a metapopulation is discussed.

Key words: *Meles meles*, minisatellites, landscape fragmentation, Denmark.

Riassunto

La variabilità genetica in cinque popolazioni danesi di tasso è stata esaminata utilizzando la sonda minisatellitare 33.15. Una variabilità genetica estremamente bassa è stata riscontrata entro le diverse popolazioni. Questa mancanza di variabilità botrebbe essere dovuta all frammentazione del territorio danese che riduce la popolazione effettiva e riduce il flusso genico fra le varie subpopolazioni. La possibilità di considerare le subpopolazioni danesi di tasso come parte di un sistema a metapopolazione è discusso.

Parole chiave: *Meles meles*, minisatelliti, frammentazione del territorio, Danimarca

Introduction

The Eurasian badger, *Meles meles* (Mustelidae) has a restricted limited dispersal (Kruuk & Parish, 1982, Cheeseman *et al.* 1987), and forms highly stable social groups (Kruuk 1978). Badger populations in Denmark, estimated to consist altogether of about 25,000 individuals, are thought to be declining (Sørensen 1995). Denmark, with few local exceptions, shows a relatively low density of badgers (Sørensen 1995, Taastrøm 1993). Further, Cheeseman *et al.* (1988) found that dispersal rates are low in rural areas like the Danish landscape.

Badgers suffer supposedly for habitat fragmentation as they need different habitats that include water, wood and pasture land. Moreover, human disturbance and outdoor activities have increased strongly in the last decades together with an increased number of straying dogs that may cause olfactive stress. Also road traffic has become a major threat. From 1983 to 1991 road traffic, measured by the number of kilometres driven on the roads, increased by 38% in Denmark (Sørensen 1995). Killings by traffic have sharply increased in the last 20 years (Sørensen 1995).

Whether the genetic structure of natural populations of badgers is disrupted by isolation, is a question of concern for their conservation. Small and isolated populations have a high risk of extinction and may suffer from the fixation of deleterious genes (Soulé 1987 and references therein). The consequences of isolation and small population size include inbreeding depression and loss of genetic variation. Inbreeding is usually deleterious in species that normally outbreed, whereas when inbreeding is part of the natural social system of a species, inbreeding depression is far less severe, and the genetic load is usually low (Soulé 1987 and references therein).

The aim of this paper is to screen the genetic structure of the Danish badger populations examined in populations from five different zones (with a maximum distance of 40 km between individuals, within the same zone) on Denmark (see Fig. 1). The nuclear genomes were screened using the DNA fingerprint (Jeffreys *et al.* 1985a, Jeffreys *et al.* 1985b, Jeffreys *et al.* 1988) which has proven a powerful technique in the study of the genetic structure of populations (Smith & Wayne 1996 and references therein).

Materials and methods

Fifteen male badger bodies were collected with reports of the exact place of death, and could be mapped within 10x10 Km squares. Samples of muscle and liver were stored in freezers at -30 °C and thereafter preserved in 100% ethanol. Genomic DNA was extracted from tissue following standard procedures including proteinase K, phenol-chloroform, chloroform-isoamyl alcohol extraction (Sambrook *et al.* 1989). DNA samples were run on a test gel to check for degradation and to adjust concentrations. Ten micrograms genomic DNA were restricted with an excess of enzyme Alu I overnight at 37 °C. A second gel was run to test for completeness of digestion and for even concentration of samples. Electrophoresis was performed in 1% agarose gels and TBE buffer. Gels were run at 25 V for 27-28 hours.

The banding pattern obtained with Jeffreys' probe 33.6 (Jeffreys *et al.* 1988) were faint and confused and therefore could not be used for our investigation Only the probe 33.15 gave interpretable results. Deviations of 1 mm were allowed for bands from two

individuals to be considered shared. Comparisons between individuals on different gels were made by photocopying one autoradiograph to the exact size of the other.

The similarity coefficient (S_{xy}) between each pair of individuals (x and y) was calculated as the number of common bands in their fingerprint profiles (n_{xy}) divided by the average number of bands in their fingerprint profiles: $S_{xy} = 2n_{xy}/(n_x + n_y)$. The average number of bands can be used for estimating the degree of inbreeding (Lynch 1991). Therefore the mean number of bands in the five populations was compared by an analysis of variance.

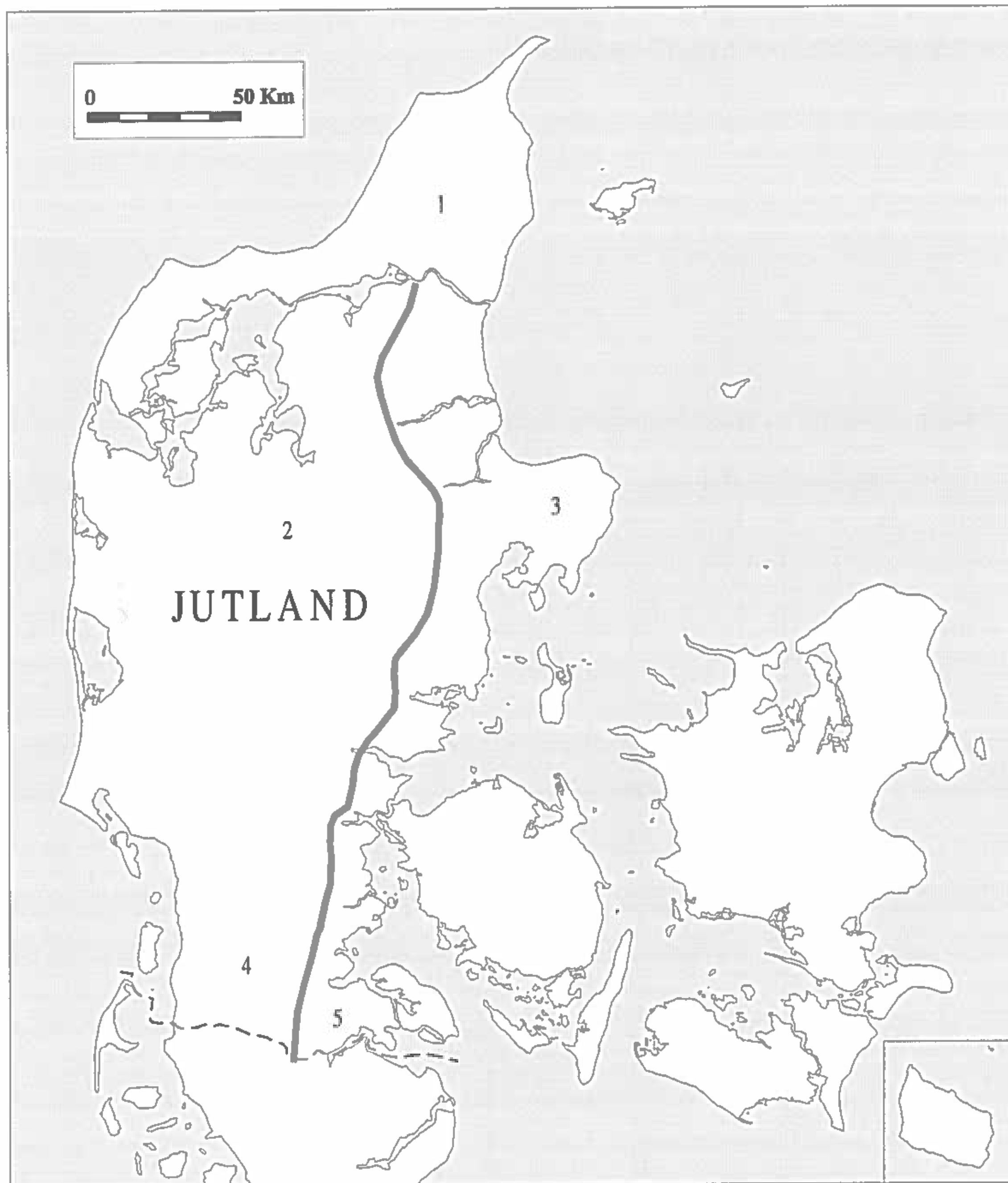


Figure 1: Map of Denmark with the five zone of collection of badgers. Full line = motorway

Results

We found low genetic differences within populations, indicating that populations were genetically rather homogeneous (see Table 1). The same banding pattern was, however, never found in two different populations (see Table 1).

The genetic similarity coefficient (S_{xy}) was different in the different populations, suggesting a higher level of inbreeding and genetic isolation (lower gene flow between the other populations) in zone 1 and 3 (Table 2). The mean number of bands was 17.3 ± 1.2, the maximum number of bands was 19 and the minimum 16, and only six bands were shared by all individuals. The differences between the number of bands in the different populations were significant (Table 3).

Table 1: Banding pattern of the badgers collected in the five different populations (Pop.).

	Pop. 1			Pop. 2			Pop. 3					Pop. 4		Pop. 5	
Individual	1	2	3	1	2	3	1	2	3	4	5	1	2	1	2
	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1
	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1
	1	1	1	1	1	1	0	0	0	0	0	1	1	1	1
	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1
	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
	1	1	1	1	1	1	0	0	0	0	1	1	0	1	0
	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0
	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1
	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
	1	1	1	1	1	1	0	0	0	0	0	1	1	1	0
	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1
	1	1	1	1	1	0	0	0	0	0	0	1	1	1	1

Table 2. Average genetic similarity D ± S.D. of the badger between individuals within the five different populations.

Zone	*n*	D ± *S.D.*
1	3	1 ± 0
2	3	0.884 ± 0.004
3	5	0.975 ± 0.033
4	2	0.947
5	2	0.875

Table 3. Comparison of average number of bands between subpopulations.

Zone	Number of individuals	Average number of bands ± *S.D.*	Source of variation	*df*	MS	*p*
1	3	18 ± 0	Between	4	4.04	0.0006
2	3	17.667 ± 1.155	Within	10	0.32	
3	5	16 ± 0				
4	2	19				
5	2	17.5				

Discussion

The low genetic variability found in the Danish badgers makes DNA fingerprinting unsuitable for paternity testing, especially in those zones (populations 1 and 3) where the banding patterns were all monomorphic or very near to being monomorphic. In order to avoid close inbreeding, the adult badger spontaneously transfers, sometimes permanently between adjacent social groups and mating occurs between males of one group and females of another, but habitat fragmentation nevertheless reduces the dispersal and increases the genetic heterogeneity between the different subpopulations. Therefore, we can expect close inbreeding in the isolated patches and the obtained results (the extremely low genetic variability within the single subpopulation) confirmed this hypothesis.

The low genetic variability found in population 1 was an expected result, because the region is totally isolated by the Limfjord. The low genetic variability found in population 3 (population 3 is confined by the sea to the north, east and south) indicates a high degree of isolation from the other zones. The reason could be the primary road and now the highway (with rather high traffic intensity), west of the population which separates the population living in zone 3 from the nearest population living in zone 2. The genetic difference found between the adjacent populations 4 and 5 could also indicate a degree of isolation due to the highway that divides the two populations. However, the higher genetic similarity found in zone 4 compared to that found in zone 5 could suggest a higher degree of isolation in zone 4. That could be the consequence of a prevalent agricultural landscape that is thought to reduce the migration rate and density of badgers. The extremely high genetic similarity that was found within the single populations, is consistent with evidence of inbreeding and limited dispersal obtained from field studies of ecology and behaviour of badgers. The fact that within populations genetic variability was low, and that the same banding pattern was never found in two populations together, could indicate a geographic partitioning, which can reveal some fragmentation effect, like the increased genetic difference between subpopulations. The different average number of bands found in the different populations could also indicate that the populations differ in effective population size (N_e). The road killing should also play an important role in reducing the genetic variability, because it reduces the effective population size (N_e) by about 10% (Sørensen 1995). Dutch findings indicate that the loss of badgers to road traffic is particularly significant, and these sources of mortality equal the annual level of cub production (Griffiths *et al.* 1993). Therefore, fauna passages should be built in zones where the gene flow is interrupted or reduced. However, the fauna-tunnels built to prevent animals from getting killed on the road have not worked on badgers (see Madsen 1996, for review).

The preliminary results suggest (also if strong conclusions cannot be drawn because of the small sample size) that the Danish badger can be managed as a metapopulation with a gene flow of different intensities between the subpopulations living in the patches. In the metapopulation, we have local extinction and local recolonization, therefore population parameters of the Danish badger could be very useful in predicting the extinction risk in the different zones and for calculating the impact that road killings have on its population structure.

Acknowledgements

We thank Dr. Vibeke Simonsen for her constructive critique of the manuscript. Further, we wish to thank Bo Gaardmand and Kirsten Zaluski, of the National Environmental Research Institute, for invaluable suggestions, help with figures and improving our text.

References

Cheeseman, C. L., Cresswell, W. J., Harris, S., Mallinson, P. J. 1988: Comparisons of dispersal and other movements in two badger (Meles meles) populations. -- Mammal Rev. 18: 51-59.

Cheeseman, C. L., Wilesmith, J. W., Ryan, I. & Mallinson, P. J. 1987: Badger population dynamics in a high-density area. -- Symp. Zool. Soc. Lond. 58: 279-294.

Griffiths, H. I., Griffiths, C. A. & Thomas, D. H. 1993: The Badger (Meles meles) An Assessment of the Population Status, Conservation Needs and Management Requirements of the Species in the Western Palaearctic. -- A report to the Standing Committee of the Convention on the Conservation of European Wildlife and Natural Habitats. (Convention on the Conservation of European Wildlife and Natural Habitats).

Jeffreys, A. J. Royle, N. J. Wilson, V. & Wong, Z. 1988: Spontaneous mutation rates to new length alleles at tandem-repetitive hypervariable loci in human DNA.-- Nature 332: 278-281.

Jeffreys, A. J., Wilson, V. & Thein, S. L. 1985a: Hypervariable "minisatellite" regions in human DNA. -- Nature 314: 67-73.

Jeffreys, A. J., Wilson, V. & Thein, S. L. 1985b: Individual-specific "fingerprints" of human DNA. -- Nature 316: 76-79.

Kruuk, H. 1978: Spatial organisation and territorial behaviour of the European badger (Meles meles). -- J. of Zool., Lond. 184: 1-19.

Kruuk, H. & Parish, T., 1982: Factors affecting population density, group size and territory size of the European badger, (Meles meles).-- J. of Zool., Lond. 196: 31-39.

Lynch, M. 1991: Analysis of population genetics structure by DNA fingerprinting. -- In Burke, T., Dolf, G., Jeffreys, A. J., Wolff, R. (eds.), DNA Fingerprinting Approaches and Applications, Birkhäuser Verlag, Basel, p. 113-126.

Madsen, A. B. 1996: Odderens (Lutra lutra) økologi og forvaltning i Danmark. The Ecology and Conservation of the Otter (Lutra lutra) in Denmark. PhD Thesis. Danish Nat. Env. Res. Inst. 84 p.

Sambrook, J., Fritsch, E. F. & Maniatis, T. 1989. Molecular cloning: a laboratory manual. -- 2nd edn. New York: Cold Spring Harbor Laboratory Press.

Smith, T. B. & Wayne, R. K. 1996: Molecular Genetic Approaches in Conservation. -- (Smith B. T. & Wayne R. K. eds.) Oxford University Press, Oxford.

Soulé, M. E. 1987: Viable populations for conservation. -- Cambridge University Press, Cambridge.

Sørensen, J. A. 1995: Road-kills of badgers (Meles meles) in Denmark. -- Ann. Zool. Fennici 32: 31-36.

Taastrøm, H. 1993: Bestandsvurdering, home-range og gruppestørrelse hos grævlinger (Meles meles) i et udvalgt område, samt diskussion af problemer med grævlinger i kunstgrave. -- M. Sci. thesis, University of Århus.

GENETIC CONSEQUENCES OF POPULATION DECLINE IN EUROPEAN OTTER (*Lutra lutra*): AN ASSESSMENT OF MICROSATELLITE DNA VARIATION IN DANISH OTTERS FROM 1883 TO 1993.

CINO PERTOLDI[1,3#], MICHAEL MØLLER HANSEN[2], VOLKER LOESCHCKE[1], AKSEL BO MADSEN[3], LENE JACOBSEN[2] AND HANS BAAGOE[4]

1: Department of Ecology and Genetics, University of Aarhus, Building 540, Ny Munkegade, DK-8000 Aarhus C, Denmark.

2: Danish Institute for Fisheries Research, Department of Inland Fisheries, Vejlsøvej 39, DK-8600 Silkeborg, Denmark.

3: Department of Landscape Ecology, National Environmental Research Institute, Kalø Grenåvej 14, DK-8410 Rønde, Denmark.

4: Mammal Section, Zoological Museum, Universitetsparken 15, DK-2100 Copenhagen, Denmark

#Corresponding author. Fax: +45 86 127191, e-mail: CPB@DMU.DK

Running head: Temporal genetic variation in otter

Key words: Ancient DNA, conservation, *Lutra lutra*, microsatellites, population bottleneck, temporal variation

SUMMARY

The European otter (*Lutra lutra*) was common in Denmark until the 1960s, but its present distribution encompasses only a minor part of the country. The aim of the study was to assess if the recent population decline had resulted in loss of genetic variability and to gain further insight into the dynamics of the population decline. This was done by analysing microsatellite DNA variation in contemporary and historical samples, the latter encompassing DNA samples extracted from museum specimens covering a time span from the 1880s to the 1960s. Tests for differences in expected heterozygosity and numbers of alleles in contemporary versus historical samples and a test for detecting population bottlenecks provided few indications of a recent bottleneck and loss of variability. However, a procedure for detecting population expansions and declines, based on the genealogical history of microsatellite alleles, suggested that a drastic long-term population decline had taken place, which could have started more than two thousand years ago, possibly due to ancient anthropogenic pressure. Finally, assignment tests and pairwise F_{ST} values suggested weak but statistically significant genetic differentiation between the extant population and historical samples of otters from other regions in Denmark, more likely reflecting differentiation among original populations rather than recent drift.

INTRODUCTION

Many species and populations are in danger of going extinct or losing significant proportions of their genetic variability (Avise & Hamrick 1996). Typically, species have been studied, which were previously abundant and distributed over large geographical areas but are now found in only a few small isolated populations (e.g. Vrijenhoek 1994; Bouzat *et al.* 1998). Fragmentation and reduced population sizes will eventually lead to loss of genetic diversity and fitness (Lande & Barrowclough 1987). However, the assessment of the loss of variability that has actually taken place is often hampered by lack of information on the genetic composition of the same

populations prior to the fragmentation and bottleneck. Moreover, there exists generally little knowledge about the variation that has been lost when populations have gone entirely extinct. Some recent studies have solved this problem by extracting "ancient" DNA and analysing molecular markers from museum or other types of historical samples and comparing the results to present populations (Taylor *et al.* 1994; Nielsen *et al.* 1997; Bouzat *et al.* 1998).

The European otter (*Lutra lutra*) is endangered in several regions of Europe (Macdonald & Mason 1994). It was previously distributed all over Denmark, but declined in number severely during the last four decades. Thus, the Danish Game Bag Record which covers the period from 1941 to 1968 (when the species became totally protected) shows a drastic decline from about 500 individuals bagged in 1941 to 100 individuals bagged in 1967 (Strandgaard and Asferg 1980). National surveys in 1984-1991 indicated that the species had almost or totally disappeared from Sealand and Funen and is now restricted to northern and western Jutland around the Limfiord catchment (Madsen 1996; see Fig. 1).

In the present study we focused on the genetic consequences of this population decline. The census population size of otters in the Limfiord catchment was estimated to be 200 in the 1980's (Madsen 1996) but the effective population size (N_e) was perhaps considerably lower (cf. Frankham 1995). Therefore, we wanted to assess if the present Limfiord population has suffered a significant loss of genetic variability and if it experienced a population bottleneck. In addition, we wanted to assess the genetic relationship between the surviving otter population in the Limfiord catchment and (extinct) otters from elsewhere in Denmark. In question is whether the remaining population was part of one big population that had declined, or if genetically distinct population segments had previously been present, one of which was the Limfiord population. These issues were addressed using analysis of microsatellite DNA from museum samples of otters from Jutland, Funen and Sealand, covering a time-span from the 1880s to the 1960s, and from fresh tissue samples collected from the present otter population.

MATERIAL AND METHODS

a) *Samples*

The historical samples consisted of 67 otter skulls from the collections of the Zoological Museum, Copenhagen (period 1: 1883-1949, N = 32, with eight individuals dating back before 1910) and the Natural History Museum, Aarhus (period 2: 1960-1963, N = 35). These samples were from the islands of Funen and Sealand and the Jutland peninsula. Contemporary samples (N = 58), all from the Limfiord catchment, consisted of frozen muscle, kidney or liver tissue samples obtained mainly from road kills during the years 1989 to 1993 (period 3) (Fig. 1). We further subdivided the samples according to geography (see Fig. 1) and defined the following groups as Sealand, time period 1 (abbreviated SEA-1), Sealand, time period 2 (SEA-2), various unknown sites in Jutland, time period 1 (JUT-1), southern Jutland and Funen, time period 2 (JUTS-2), eastern Jutland (Djursland), time period 2 (JUTD-2), Jutland, Limfiord catchment, time period 2 (JUTL-2) and Jutland, Limfiord catchment, time period 3 (JUTL-3).

b) *Molecular analyses*

DNA was extracted from fresh tissue using standard phenol/chloroform extraction (Sambrook *et al.* 1989). The DNA from museum specimens was extracted by removing a canine tooth from the skull and drill out the tooth root using a 2 mm drill. Approximately 0.1 gram of tooth and tooth root were collected and DNA extraction followed the procedure of Nielsen *et al.* (1997). In order to avoid cross-contamination, the drill was heated until glowing following each collection of tooth root. Also, no extraction of DNA from recent tissue samples was conducted in the laboratory during the time period we worked with historical samples, and PCR reagents were exposed to UV-radiation in an UV-crosslinker to degrade possible contaminating DNA. Finally, PCR amplification of fresh and historical samples were for the most part done in separate laboratories (Danish Institute for Fisheries Research and University of Aarhus, respectively).

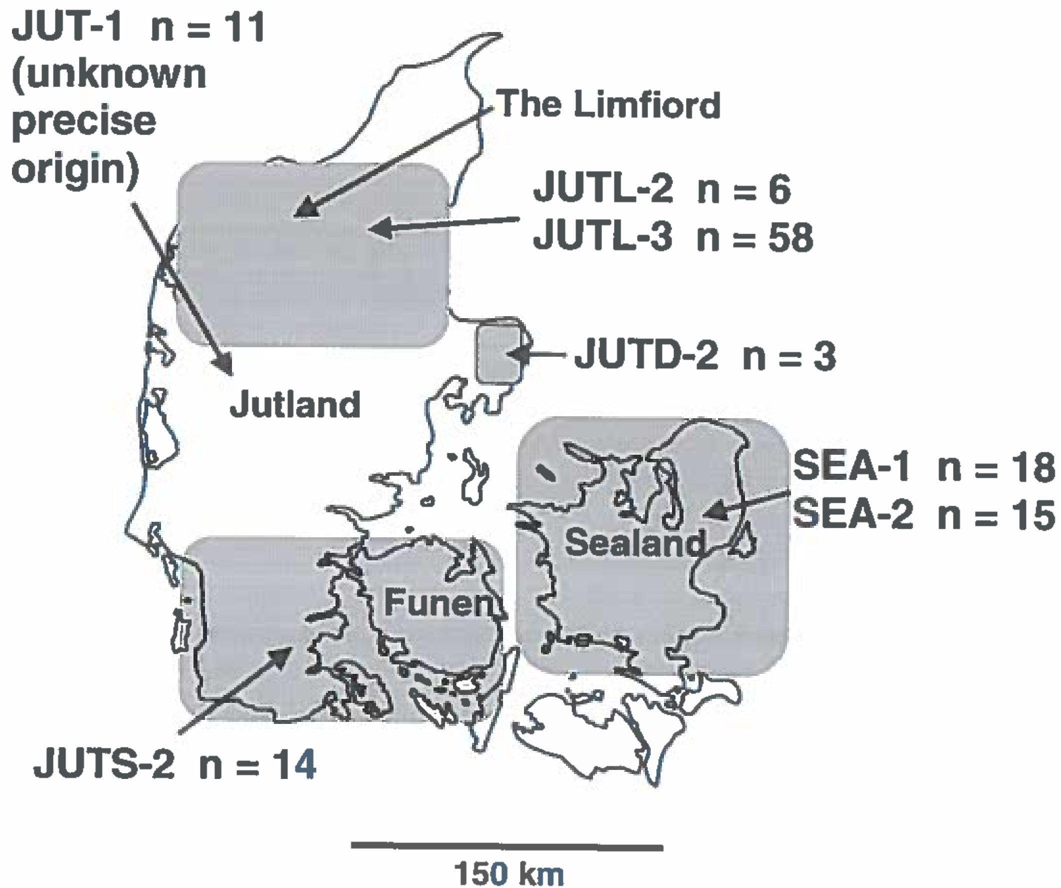


Figure 1: Map showing the approximate location of sampling sites for contemporary and historical samples of otters in Denmark. See Materials and Methods for sample abbreviations.

The following microsatellite loci were assayed: Lut435, Lut457, Lut701, Lut717, Lut733, Lut782, Lut818, Lut832, Lut453, Lut833, Lut715 (Dallas & Piertney 1998) and Lut902 (Dallas *et al.* 1999). Lut453 did not yield reliable amplification and therefore was excluded from further investigations. All loci were amplified at an annealing temperature of 58 °C, using 30 (contemporary samples) or 40 (historical samples) PCR cycles. The amplified loci were analysed on a Pharmacia ALFexpress automated sequencer.

c) *Statistical treatment*

Deviations from Hardy-Weinberg equilibrium (HWE) were tested by exact tests (Guo & Thompson 1992), using the program GENEPOP 3.1 (Raymond & Rousset 1995a). For testing

equality of heterozygosity (H_e) in the contemporary sample relative to the historical samples, all historical samples were pooled and a one-tailed t-test was performed based on arcsine-square root transformed H_e values. Loss of allelic variation in the contemporary sample was tested by a randomisation test. For each locus, 1,000 samples equal in size to the contemporary sample of otters were drawn at random from the pooled historical samples. We constructed a frequency distribution of the number of alleles in the generated samples, and assessed the probability of observing a number of alleles equal to or less than that observed in the contemporary sample (for further details see Nielsen *et al.* 1999). To test for recent population bottlenecks, we applied the test by Cornuet & Luikart (1996), using the software Bottleneck 1.2. We performed the tests assuming both an infinite allele model (IAM), a step-wise mutation model (SMM), and a two-phase model of mutation (TPM, with 95% SMMs). We tested both the contemporary and historical samples, as we could not *a priori* exclude the possibility that Danish otters had experienced a bottleneck prior to the time span covered by the samples. Finally, we used the procedure by Beaumont (1999) for detecting population declines and expansions. It assumes a SMM and estimates the posterior probability distribution of several genealogical and demographic parameters, using Markov Chain Monte Carlo simulations, based on the observed distribution of microsatellite alleles and their repeat numbers. The most important output parameters are r, defined as N_o/N_1, where N_o is the current effective number of chromosomes and N_1 is the number of chromosomes at some point back in time, t_f. t_f is defined as t_a/N_o, where t_a denotes the number of generation that have elapsed since the decline or expansion began. Finally, the procedure estimates theta, defined as 2 $N_o\mu$, where μ denotes the mutation rate. We performed the analyses for both the contemporary sample (JUTL-3) and the pooled samples from time period 1, assuming both linear and exponential modes of decline.

Genetic differentiation between samples was assessed using exact tests (Raymond & Rousset 1995b), using GENEPOP 3.1, and by calculating pairwise F_{ST} values and testing their significance by permuting individuals between samples with the program ARLEQUIN 1.1 (Schneider *et al.* 1997). The very small samples JUTL-2 and JUTD-2 were excluded from the analyses. The population of origin of individuals was assessed using assignment tests (Paetkau *et al.* 1995), where individuals were assigned to the sample in which they had the highest "probability of belonging", based on their multilocus genotypes. We used the program GENECLASS 1.0 (Cornuet *et al.* 1999) and chose the "Bayesian approach", as recommended by the authors.

RESULTS

a) *Genetic variability and population declines*

Two of the loci were monomorphic, and the nine remaining loci amplified well and were polymorphic. Even among the historical samples the success of amplification ranged between 96% and 100% among loci. Previous studies have shown that allelic drop-outs (amplification of just one of two alleles) may occur in analysis of degraded DNA obtained from old teeth and bones (Zierdt *et al.* 1996), presumably due to scarcity of intact DNA templates (Hummel and Herrmann 1995). However, when the quality and quantity of extracted DNA from fresh and historical samples was compared on a 2% agarose gel, the quantity of DNA from historical samples did not differ much from that obtained from fresh samples. The historical DNA samples were more degraded than fresh samples, but there was clearly a proportion of larger DNA fragments present (> 500 bp). In six specimens where some of the loci were found to be homozygotic, we extracted DNA from another canine tooth belonging to the same skull and observed the same genotypes (i.e. same homozygotic alleles). Hence, the quality and quantity of the DNA extracted from teeth and the reproducibility of results leads us to assume that problems with allelic drop-outs have not significantly affected our results. Four significant deviations from HWE were observed and all were found in the contemporary sample, all due to heterozygote deficiency (JUTL-3; see Table 1). Levels of polymorphism were low with total numbers of alleles per locus ranging from two to five, both in the contemporary and historical

samples (Table 1). The mean number of alleles per locus was 3.9 in the pooled historical samples and 3.1 in JUTL-3. The t-test for equality of expected heterozygosity in JUTL-3 compared to the historical samples yielded a non-significant result ($p = 0.19$). The randomisation tests for reduced allelic variability in JUTL-3 yielded a significant outcome for the loci Lut435, Lut457 and Lut782 (all $p < 0.001$). Tests for population bottlenecks did not result in significant outcomes, neither in the case of JUTL-3 nor in the historical samples.

Table 1. Summary of observed number of alleles per microsatellite locus, outcome of tests for deviations from expected Hardy-Weinberg proportions (H.-W. test), expected (He) and observed heterozygosity (Ho) and sample sizes (n) of the studied populations. Table-wide significance levels were applied, using the sequential Bonferroni technique (Rice, 1989) (initial k = 44). See Materials and Methods: or explanation of sample abbreviations.

Locus		SEA-1	SEA-2	JUT-1	JUTL-2	JUTS-2	JUTD-2	JUTL-3
Lut818	No. Alleles	4	3	3	3	3	3	3
Total no.	H.-W. test	n.s.	n.s.	n.s.	#	n.s.	#	n.s.
alleles: 4	Ho	0.688	0.533	0.364	0.500	0.643	1	0.527
	He	0.615	0.508	0.329	0.439	0.505	0.733	0.462
	n	16	15	11	6	14	3	55
Lut701	No. Alleles	3	3	3	3	3	2	3
Total no.	H.-W. test	n.s.	n.s.	n.s.	#	n.s.	#	n.s.
alleles: 3	Ho	0.500	0.600	0.727	0.500	0.538	0.333	0.158
	He	0.417	0.476	0.515	0.439	0.526	0.333	0.210
	n	18	15	11	6	13	3	57
Lut733	No. Alleles	4	4	2	2	3	2	5
Total no.	H.-W. test	n.s.	n.s.	n.s.	#	n.s.	#	***
alleles: 5	Ho	0.389	0.400	0.091	0.167	0.214	0.333	0.421
	He	0.456	0.559	0.455	0.167	0.204	0.333	0.636
	n	18	15	11	6	14	3	57
Lut435	No. Alleles	5	4	1	2	3	2	3
Total no.	H.-W. test	n.s.	n.s.	#	#	n.s.	#	n.s.
alleles: 5	Ho	0.333	0.400	0	0	0.231	0.667	0.121
	He	0.598	0.453	0	0.303	0.335	0.533	0.203
	n	18	15	10	6	13	3	58
Lut832	No. Alleles	4	4	3	2	4	2	4
Total no.	H.-W. test	n.s.	n.s.	n.s.	#	n.s.	#	*
alleles: 4	Ho	0.556	0.467	0.500	0.800	0.462	0.333	0.397
	He	0.554	0.605	0.484	0.533	0.554	0.600	0.577
	n	18	15	10	5	13	3	58
Lut717	No. Alleles	2	2	2	2	2	2	2
Total no.	H.-W. test	n.s.	n.s.	n.s.	#	n.s.	#	*
alleles: 2	Ho	0.556	0.667	0.545	0.667	0.571	0.667	0.610
	He	0.489	0.46	0.485	0.485	0.423	0.533	0.424
	n	18	15	11	6	14	3	55
Lut782	No. Alleles	3	4	5	2	4	1	2
Total no.	H.-W. test	n.s.	n.s.	n.s.	#	n.s.	#	n.s.
alleles: 5	Ho	0.333	0.333	0.545	0.667	0.286	0	0.351
	He	0.375	0.407	0.528	0.485	0.429	0	0.335
	n	18	15	11	6	14	3	57
Lut902	No. Alleles	4	3	3	4	3	3	4
Total no.	H.-W. test	n.s.	n.s.	n.s.	#	n.s.	#	n.s.
alleles: 4	Ho	0.600	0.267	0.545	0.667	0.429	1	0.464
	He	0.572	0.543	0.589	0.561	0.521	0.733	0.563
	n	15	15	11	6	14	3	56
Lut457	No. Alleles	3	3	3	3	3	3	2
Total no.	H.-W. test	n.s.	n.s.	n.s.	#	n.s.	#	***
alleles: 3	Ho	0.118	0.333	0.182	0.2	0.143	0.333	0.187
	He	0.348	0.517	0.385	0.511	0.265	0.600	0.416
	n	17	15	11	5	14	3	55

*** $p < 0.001$, * $p < 0.05$, # test which was not performed due to small sample sizes (< 10).

The procedure used for assessing population declines and expansions (Beaumont 1999) suggested that the population sizes both in time period 1 and 3 had declined drastically to approximately 1 - 3% of the original population size [modes of log(r) = -1.91 (time period 1) and log(r) = -1.555 (time period 3); Table 2]. Furthermore, the analysis suggested that this decline had taken place over a long time period [modes of $\log(t_f)$ = 0.989 (time period 1) and $\log(t_f)$ = 0.98 (time period 3); Table 2]. If we roughly assume a current effective number of chromosomes, N_0, = 100, and a generation time of 3 years, this suggests that the decline started approximately 2 - 3,000 years ago. These results were based on a linear model of population decline. Simulations assuming an exponential model also pointed to a drastic decline over a long time-span (Table 2), but as this model is primarily valid for short-term strong declines (Beaumont 1999), we emphasize the results based on a linear model.

b) ***Genetic population structure***

The exact tests for differences in allele frequencies between samples showed significant differentiation between JUTL-3 and all other samples, whereas no significant differentiation was observed between historical samples (Table 3).

Similarly, pairwise F_{ST} values were low (0 – 0.043) and non-significant between historical samples, but slightly higher (0.023 – 0.064) and in all but one case significant between JUTL-3 and the other samples. It is of course possible that some differentiation existed between otters from Sealand and southern Jutland, but that sample sizes were too small to obtain sufficient statistical power. We assessed the magnitude of this problem by randomly reducing the sample size of JUTL-3 to 18 and performing again the exact tests for differences in allele frequencies. Two of four tests involving this sample were still significant at the 5% level.

Table 2. Summary statistics for otter data collected in period 1 and period 3.The lower 0.9 HDP limit, mode, and upper 0.9 HDP limit for three parameters, analyzed using the linear and exponential model declining trends. The parameter values (respectively lower and upper bounds) used for running the simulations are shown in the first column.

parameter	values		period 1			period 3	
linear model							
log (r)	(-2.3, 1)	(-1.848)	(-1.91)	(-1.836)	(-1.655)	(-1.555)	(-1.651)
log (tf)	(-5, 4)	(0.851)	(0.989)	(0.86)	(0.866)	(0.98)	(0.869)
log theta	(-2, 0)	(-0.823)	(-0.635)	(-0.799)	(-0.949)	(-0.837)	(-0.937)
exponential model							
log (r)	(-2.3, 1)	(-2.162)	(-2.291)	(-2.138)	(-2.212)	(-2.14)	(-2.194)
log (tf)	(-5, 4)	(0.323)	(0.353)	(0.332)	(0.56)	(0.585)	(0.567)
log theta	(-2, 0)	(-0.769)	(-0.845)	(-0.741)	(-0.78)	(-0.791)	(-0.922)

Table 3. Pairwise F_{ST} values (above diagonal) and exact tests for homogeneity of allele frequencies with probabilities combined over – loci using Fisher's method (below diagonal).

	SEA-1	SEA-2	JUT-1	JUTS-2	JUTL-3
SEA-1		0	0.029	0.004	0.034*
SEA-2	n.s.		0.022	0	0.023
JUT-1	n.s.	n.s.		0.043	0.064**
JUTS-2	n.s.	n.s.	n.s.		0.056**
JUTL-3	***	***	***	***	

For the assignment tests we used two baseline samples, i.e. JUTL-3 and the pooled historical samples SEA-1, SEA-2 and JUTS-2. This pooling was justified by the observation of no significant differences between the samples and by the fact that they all represented a coherent geographical region separated from the Limfiord catchment (Fig. 1). Assignment tests showed that 74% and 83% of the individuals from the two baseline samples respectively, were assigned correctly (data not shown). The individuals from the historical sample from Jutland, but of unknown precise geographical origin (JUT-1), and the three individuals from eastern Jutland (JUTD-2) were all assigned to the pooled historical sample. Conversely, four of the six individuals sampled from the Limfiord catchment in the 1960s (JUTL-2) were assigned to the present population (JUTL-3).

DISCUSSION

a) *Genetic variability and population decline*

The number of alleles detected per locus (between 1 and 5) must be considered low compared to the levels of variability that are normally reported for microsatellite loci (Goldstein & Pollock 1997). Dallas & Piertney (1998) observed higher variability at the same loci with numbers of alleles ranging from 5 to 10. However, they screened individuals from a much larger area (UK, Ireland and Germany). In a more intensive study of microsatellite diversity in Scottish otters, Dallas *et al.* (1999) observed mean numbers of alleles per locus ranging from 2.1 to 5.3. The mean numbers of alleles observed in the present study (3.9 for the historical samples and 3.1 for JUTL-3) fall within this range. Other studies of otter populations from Denmark and Germany employing analyses of mitochondrial DNA have also revealed low variability (Effenberger & Suchentrunk 1999; Mucci *et al.* 1999; Cassens *et al.* 2000). Thus, it must be concluded that otter populations, at least from northern Europe, exhibit low genetic variability. The present study suggests that genetic variability was low even before the recent major decline of otter populations in Denmark took place. This could be a result of the drastic population decline started approximately 2 - 3,000 years ago, as suggested by the analyses using Beaumont's (1999) method. Alternatively, the low variability could be due to founder events during postglacial recolonisation (approximately 10,000 years ago). This explanation is supported by data by Cassens *et al.* (2000). These authors sequenced the mitochondrial DNA d-loop in otters from eastern Germany and Central Europe and in most cases found the same single dominant haplotype and a few rare, local haplotypes derived from the most common one by single point mutations.

There were a few indications of recent loss of variability in the extant otter population compared to the historical samples. Tests for bottlenecks and differences in expected heterozygosity did not provide evidence of reduced variability. Only the tests for reduced numbers of alleles yielded three significant outcomes, but this may simply reflect that the historical samples represent a much larger geographical range, and consequently more genetically divergent population segments, compared to the contemporary sample. This does not imply, however, that loss of variability in Danish otters has not taken place. First, the bottleneck test by Cornuet & Luikart (1996) can detect only a severe and relatively recent reduction, which has taken place within $0.2N_e$ to $0.4N_e$ generations. In addition, the analysis suggested by Beaumont (1999) for detecting population declines and expansions suggested that a severe decline had in fact taken place and that the current population size had been reduced to only a few percent of the original population size. Both the contemporary samples and the historical samples from the 1880's to the 1940's yielded qualitatively similar results, showing that the decline had taken place over a much longer time span than that covered by the analysed samples. In fact, the analysis suggested that the decline may have started more than 2,000 years ago. This is not an unreasonable estimate given that Denmark has been relatively densely populated by men approximately for the past 2,000 years. Another riverine mammal, the European beaver (*Castor fiber*), went extinct in Denmark approximately 2,500 years ago,

presumably due to man-induced habitat destruction or hunting (Aaris-Sørensen 1988). However, as pointed out by Beaumont (1999) results obtained by his method should also be interpreted with some caution as the analysis assumes a strict SMM, which is probably invalid for microsatellite loci. In conclusion, the low variability in Danish otters could be explained either by postglacial founder events or a more recent population decline, which started approximately 2 – 3,000 years ago. The two explanations are not mutually exclusive, but given the strong signal of a drastic, long-term population decline obtained by the analysis of Beaumont (1999), we assume that historical population declines have played an important role for the observed low variability in extant Danish otters.

b) *Genetic population structure*

The tests for genetic differences between samples indicated that the present JUTL-3 population is genetically divergent from the historical samples from southern Jutland and Sealand (Table 3). The genetic divergence of JUTL-3 relative to the other samples could be due to either geographical variation or shifts in allelic frequencies in JUTL-3 caused by genetic drift during the recent population decline. However, as discussed previously, there were few, if any, indications of a recent population bottleneck in JUTL-3. Furthermore, the assignment tests showed that individuals from the historical samples of unknown precise origin in Jutland (JUT-1) and from eastern Jutland (JUTD-2) were all assigned to the pooled historical baseline sample. In contrast, four of six individuals from the historical sample from the Limfiord (JUTL-2) were assigned to the present Limfiord population, suggesting that genetic differentiation was also present in the past. The deviations from HWE observed in JUTL-3 suggest that even within this region the otter population may not be homogenous (Table 1), and there may be some sort of population structure even at a finer geographical scale. This could also be the case in some of the other regions, where sample sizes may have been too small to detect deviations from HWE.

c) *Conclusions*

In conclusion, our study shows that canine teeth are reliable sources of historical DNA samples. As skull collections are often maintained in high numbers at museums, analysis of DNA from canine teeth may be a way to obtain sufficient sample sizes, a problem otherwise hampering studies based on analysis of DNA from historical samples (e.g. Nielsen *et al.* 1999). Even though historical sample sizes in the present study were higher than in previous studies (n = 5; Taylor *et al.* (1994), n = 15; Bouzat *et al.* (1998)) the design of the study was limited by the availability of samples, which have reduced the power of several statistical tests (e.g. HWE tests). A direct comparison of contemporary and historical genetic variation in the extant Limfiord population would have been preferable, but too few samples were available for that purpose. Nevertheless, despite these limitations the data presented here indicate that the extant otter population has not suffered a recent severe loss of genetic variability and that some geographical variation was present in the past. There were indications that a drastic population decline had taken place, but this had happened on a time-scale covering hundreds or thousands of years. This suggests that the recent population decline of otters only represents an acceleration of a negative development that may date back even into prehistoric times.

ACKNOWLEDGEMENTS

We thank two anonymous reviewers for helpful suggestions on a previous version of the manuscript, Mark Beaumont for kindly providing the software for conducting his analysis of population declines and expansions and for discussing the results of the analyses, Einar Eg Nielsen for advice on extracting DNA from historical samples, Karen-Lise Mensberg and Dorte Meldrup for technical assistance and Bo Gaardmand, Kirsten Zaluski, Miriam Hercus and Robert A. Krebs, for help with figures and linguistic suggestions.

REFERENCES

Aaris-Sørensen, K. 1988. *Danmarks forhistoriske dyreverden. Fra istid til vikingetid.* Copenhagen: Gyldendals Forlag.

Avise, J. C. & Hamrick, J. L. 1996 *Conservation Genetics. Case Stories from Nature.* New York: Chapman & Hall.

Beaumont, M. A. 1999 Detecting Population Expansion and Decline using Microsatellites. *Genetics* 153, 2013-2029.

Bouzat, J. L. Lewin, H.A. & Paige, K.N. 1998 The ghost of genetic diversity past: Historical DNA analysis of the greater prairie chicken. *Am. Nat.* 152, 1-6.

Cassens, I. Tiedemann, R. Suchentrunk, F. & Hartl, G. B. 2000 Mitochondrial DNA variation in the European otter (*Lutra lutra*) and the use of spatial autocorrelation analysis in conservation. *J. Heredity* 91, 31-35.

Cornuet, J. M. Piry, S. Luikart, G. Estroup, A. Solignac, M. 1999 Comparisons of methods emplying multilocus genotypes to select or exclude populations as origins of individuals. *Genetics* 153, 1989-2000.

Cornuet, J. M. & Luikart, G. 1996 Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics* 144, 2001-2014.

Dallas, J. F. & Piertney, S. B. 1998 Microsatellite primers for the Eurasian otter. *Mol. Ecol.* 7, 1248-1251.

Dallas, J. F. Bacon, P. J. Carss, D. N. Conroy, J. W. H. Green, R. Jefferies, D. J. Kruuk, H. Marshall, F. Piertney, S. B. & Racey, P.A. 1999 Genetic diversity in the Eurasian otter, (*Lutra lutra*), in Scotland. Evidence from microsatellite polymorphism. *Biol. J. Linn. Soc.* 68, 73-86.

Effenberger, S. & Suchentrunk, F. 1999 RFLP analysis of the mitochondrial DNA of otters (*Lutra lutra*) from Europe, implications for conservation of a flagship species. *Biol. Cons.* 90, 229-234.

Frankham, R. 1995 Effective population size/adult population size ratios in wildlife: A review. *Genet. Res.* 66, 95-107.

Goldstein, D. B. & Pollock, D. D. 1997 Launching microsatellites: A review of mutation processes and methods of phylogenetic inference. *J. Heredity* 88, 335-342.

Guo, S. W. & Thompson, E. A. 1992 Performing the exact test for Hardy-Weinberg proportion for multiple alleles. *Biometrics* 48, 361-372.

Hummel, S. & Herrmann, B. 1995 aDNA Analysis in Palaeopathology: Mini-Review and Prospects. *Palaeopathology Newsletter* 91, 6-9.

Lande, R. & Barrowclough, G. F. 1987 Effective population size, genetic variation and their use in population management. In *Viable Populations for Conservation* (ed. M. Soulé), pp. 87-123. New York: Cambridge University Press.

Macdonald, S.M. & Mason, C. F. 1994 Status and conservation needs of the otter (*Lutra lutra*) in the western Palaearctic. *Nature and Environment* 67, 1-54.

Madsen, A. B. 1996 The ecology and conservation of the otter (*Lutra lutra*) in Denmark. *PhD Thesis. Danish National Environmental Research Institute.*

Mucci, N. Pertoldi, C. Madsen, A. B. Loeschcke, V. & Randi, E. 1999 Extremely low mitochondrial DNA control-region sequence variation in the otter (*Lutra lutra*) population of Denmark. *Hereditas* 130, 331-336.

Nielsen, E. E. Hansen, M. M. & Loeschcke, V. 1997 Analysis of microsatellite DNA from old scale samples of Atlantic salmon: A comparison of genetic composition over sixty years. *Mol. Ecol.* 6, 487-492.

Nielsen, E. E. Hansen, M. M. & Loeschcke, V. 1999 Analysis of DNA from old scale samples: Technical aspects, applications and perspectives for conservation. *Hereditas* 130, 265-276.

Paetkau, D. Calvert, W. I. Stirling, I. & Strobeck, C. 1995 Microsatellite analysis of population structure in Canadian polar bears. *Mol. Ecol.* 4, 347-354.

Raymond, M. & Rousset, F. 1995a Genepop (version 3.1): population genetics software for exact tests and ecumenicism. *Journal of Heredity* 86, 248-249.

Raymond, M. & Rousset, F. 1995b An exact test for population differentiation. *Evolution* 49, 1280-1283.

Rice, W. R. 1989 Analyzing tables of statistical tests. *Evolution* 43, 223-225.

Sambrook, J. Fritsch, E. F. & Maniatis, T. 1989 *Molecular cloning.* 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Lab Press.

Schneider, S. J. Kueffer, M. Roessli, D. & Excoffier, L. 1997 *Arlequin. Version 1.1.* Switzerland: Genetics and Biometry Lab, Dept. of Anthropology, University of Geneva.

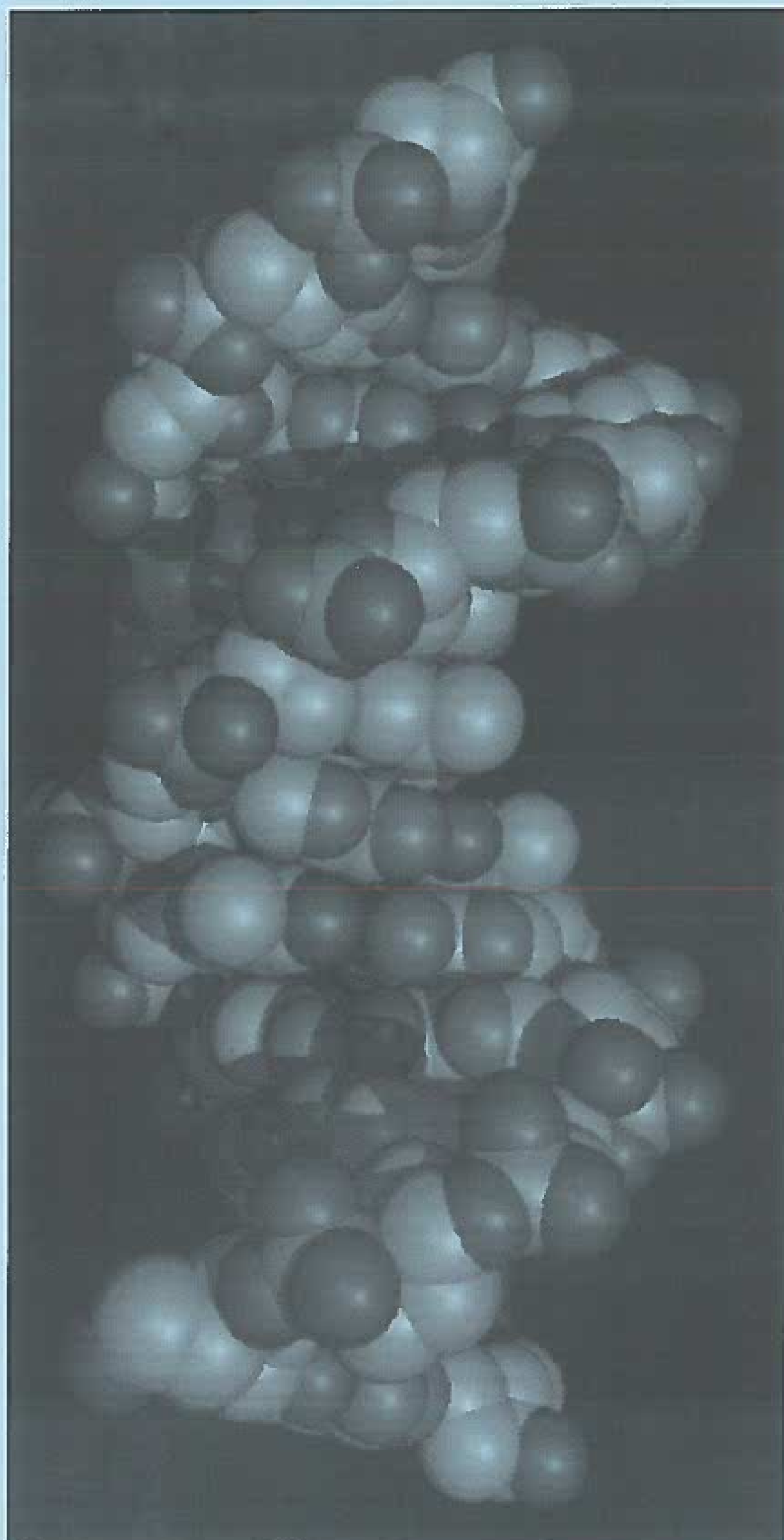
Strangaard, H. & Asferg, T. 1980 The Danish Bag Rwcord II. Fluctuations and Trends in the Game Bag Record in the Years 1941-1976 and the Geographical Distribution of the Bag in 1976. *Danish Review of Game Biology* 11, 1-112.

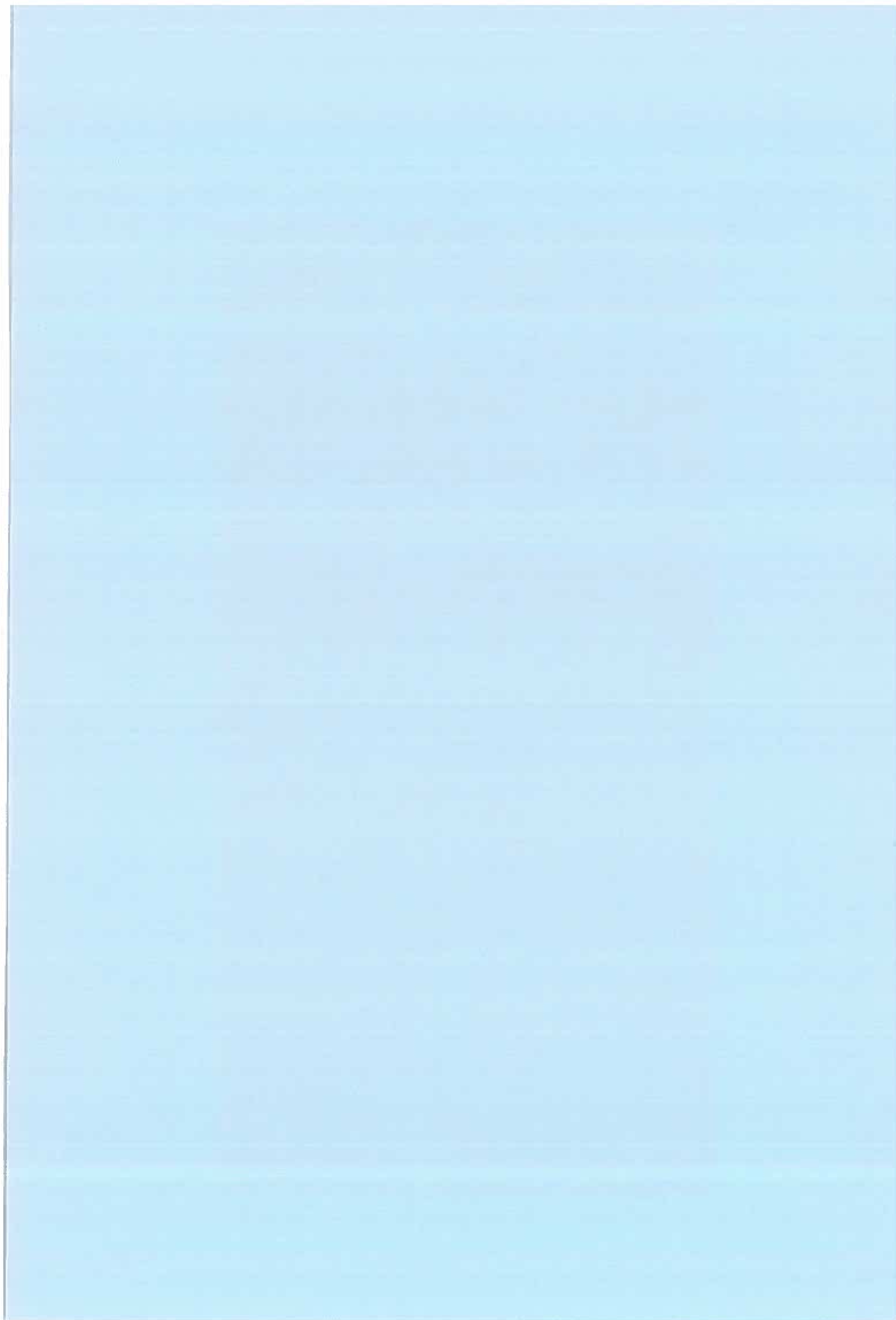
Taylor, A. C. Sherwin, W. B. & Wayne, R. K. 1994 Genetic variation of microsatellite loci in a bottlenecked species, the hairy-nosed wombat,(*Lasiorhinus krefftei*). *Mol. Ecol.* 3, 277-290.

Vrijenhoek, R. C. 1994 Genetic diversity and fitness in small populations. In *Conservation Genetics* (eds. V. Loeschcke, J. Tomiuk, & S. K. Jain), pp.37-53. Basel: Birkhäuser.

Zierdt, H. Hummel, S. & Herrmann, B. 1996 Amplification of human short tandem repeats from medieval teeth and bone samples. *Hum. Biol.* 68, 185-99.

Articles in prep





Analysis of the effects of different fertilisers on developmental stability in *Folsomia candida*

by Cino Pertoldi, Torsten Nygaard Kristensen and Volker Loeschcke

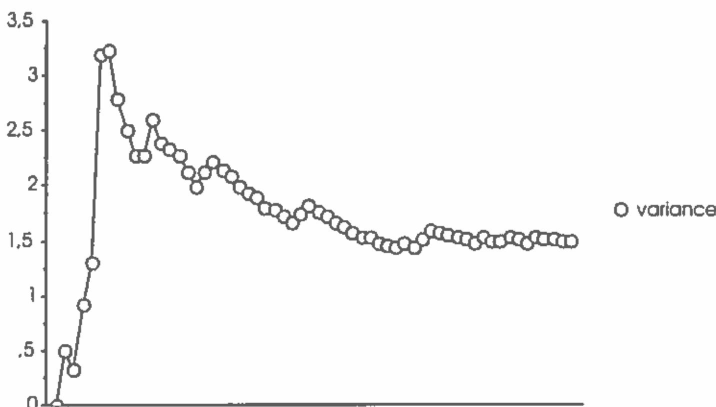
Introduction

To predict the distribution and effects of organic pollutants in the environment, long-term monitoring is required. Pollutants with properties such as persistency and lipophilicity tend to end up in organisms and, therefore, the accumulation of residues may indicate the order of magnitude of exposure. However, before the pollutants are taken up by organisms, the abiotic part of the ecosystem (water, sediment, soil, air, etc.) may be sampled in order to predict exposure and to estimate risks. In order to concentrate certain persistent pollutants already during sampling in the field, their lipophilic properties may be utilised. The principles behind the use of laboratory studies to monitor pollutants in the air, in air-borne fallout, in aquatic surface microlayers and in the water will be discussed.

Given the fact that full life table experiments for all the species of a given ecosystem under study will be extremely time and money consuming and given the urgent need of obtaining as much information as possible about the stress responses of the largest number of species possible, we have to consider alternative methodologies. One of these methodologies is to measure developmental stability, DS, of the species in presence of stressors. DS, the ability of a genotype to produce a targeted phenotype despite different disruptive influences, is often used as an indicator of individual fitness or population well being. A common estimator of DS is fluctuating asymmetry, FA, the random deviation from perfect symmetry of bilateral traits. FA can be used as an indicator of stress level when dose-response relationships have been validated. Thus in order to credibly extrapolate from individuals in the laboratory to field populations, it is necessary to understand how genetic and environmental factors in the field potentially modify responses measured in the laboratory. Indeed this is a crucial aspect of the measurement of biological effects in the context of ecological risk assessment. This means that the genetic basis for stress tolerance can only be properly assessed by separating and quantifying the effect of both genes and environment and, most importantly, their interaction on the expression of tolerance. One of the biggest problems when studying stress tolerance is the fact that different genotypes, even from the same population, may have different phenotypic responses. Natural populations generally comprise large numbers of genetically diverse individuals inhabiting environments, which are both spatially and temporally heterogeneous. Progress on standardising indicator organisms for the risk assessment of possible toxic chemicals, however, has been made in the last few years by making use of asexually reproducing organisms.

Eliminating the genetic variability does not solve the problem of the within population environmental variability (Venv), which can alter the estimates of the population's DS. We have developed an approach whereby it is possible to partition out Venv. By doing this, estimates of DS can become highly useful as a bioindicator if dealing with asexually reproducing organisms.

Here we present two experiments done with collembolans. We show the confounding effect that Venv can have in toxicological experiments and the usefulness of FA as an indicator of potentially stressful conditions. In order to estimate how many collembolans should be used for each test we have done, we made a simulation on how the variance of the signed value of FA behaves with increasing number of individuals counted. From Fig. 1 it is clear that stabilisation of the variance begins to occur around 50 observations. To be above this critical value we chose to measure FA on 60 collembolans for each experimental set-up:



Project 1

The aim of project 1 was to detect variation in the degree of DS and the environmental variability (Venv) of a mono-clonal strain of a parthenogenetic collembolan (*Folsomia candida*). The parent strain has been synchronised and was allowed to stay in petri dishes with a diameter of 12 cm (20 *Folsomia candida* for each dish) from the age of three days to the age of 22 days. During this period the individuals were fed with yeast (50 mg), which was renewed every three days in order to avoid bacterial and fungal contamination of the cultures. When 22 days old, the adult individuals were removed and the offspring were allowed to stay in the petri dishes for 20 additional days and then analysed for DS with 60 individuals analysed from each petri dish.

For every soil sample we prepared three petri dishes (three replicates). The soil samples in which *Folsomia candida* were allowed to stay, came from three sampling sites in a field consisting of sandy soil. This field had not been fertilised this year before the first samples were collected. Three samples containing soil from the same sandy field but collected the day after the field had been fertilised with sludge from a waste water treatment plant and cultivated by a harrow, were also collected. DS for each replicate was estimated by calculating FA in the number of the ventral setae of the manubrium. As *Folsomia candida* were fed with yeast during all the experiments, we consider any eventual change in the degree of FA to be due to the absorption of the contaminant via *cuticula*. For counting purposes the specimen were depigmented in hot lactic acid and covered with a cover glass, and studied under a microscope with an ordinary light contrast, with a magnification of 200 to 400 times. A Panasonic video camera mounted on the microscope and connected to a computer, registered all the pictures. These were later processed with an object image analyser (IBAS), which allowed the setae counting. The same procedure was also used for project 2.

Results

Of the 18 petri dishes (three sites, three replicates per site, two treatments per replicate) we have used for our investigation, five were removed from further analysis, because we detected bacterial and fungal contamination, and of the 13 remaining dishes, only eight were considered for our statistical analysis, as in five petri dishes we detected with our methodology the significant presence of Venv.

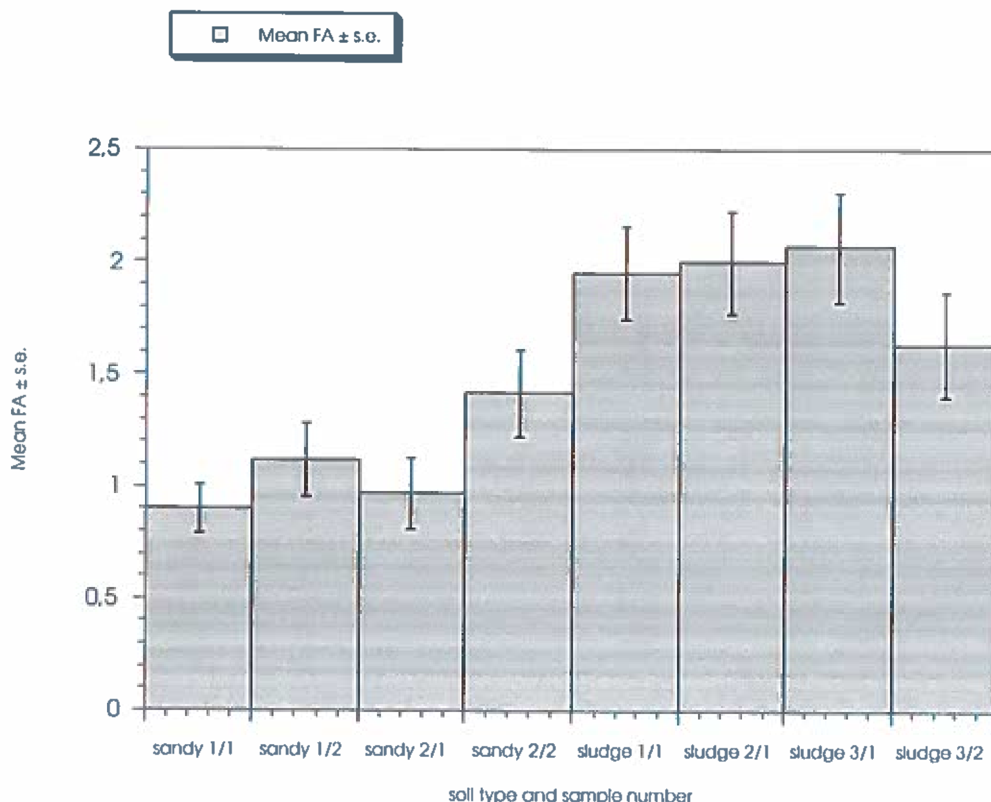


Figure 2 shows the mean and the standard error, s.e., of the mean absolute value of FA in the eight petri dishes. We found evidence for a lower FA in the offspring from parents exposed to sandy soil compared to those offspring from parents exposed to sandy soil fertilised with sludge. The two ways ANOVA revealed that there was no significant variation and interaction within replicates (results not shown). Furthermore, we tested if there was a significant difference in mean FA among samples for offspring where parents were exposed to sandy soil and if there were significant differences in mean FA among samples of offspring whose parents were exposed to sandy soil with sludge. No significant differences were found (results not shown). Therefore we pooled the replicates and the samples from the same treatment together and made a two tailed t-test in order to test if there were significant differences between mean FA for the treatments sandy soil and sandy soil with sludge (mean FA ± s.e. sandy soil = 1.1 ± 0.079, n = 240 and mean FA ± s.e. sandy soil with sludge = 1.9 ± 0.114, n = 239, two tailed t-test: t = 5.85, p = ***). The mean FA was nearly twice as high in offspring from parents exposed to sandy soil with sludge as compared to the mean FA of offspring from parents exposed to sandy soil only.

Project 2

The aim of this project was to detect variation in the degree of DS and the Venv of a mono-clonal strain of a parthenogenetic collembolan (*Folsomia candida*). The strain has been synchronised and was maintained in petri dishes of 12 cm diameter (60 *Folsomia candida* for each dish, two replicates for each experiment) from the age of three days to the age of 22 days. During this period the individuals were allowed to live on a coal substrate and were fed with either yeast only (*ad libitum*), with pure sludge mixed with yeast (50 mg of a homogeneous mixed substrate), with pure cattle manure (50 mg of a homogeneous mixed substrate) or with pure cattle manure mixed with yeast (50 mg of a homogeneous mixed substrate). The substrate was renewed every three days in order to avoid bacterial and fungal contamination of the cultures. When 22 days old, the adult individuals were removed and the offspring were allowed to stay in the petri dishes for 20 additional days and then analysed for DS (with 60 individuals analysed for each petri dish). DS was estimated calculating FA in the number of the ventral setae of the manubrium. We consider any eventual change in the degree of DS among the different groups as being due to effects of contaminants via direct ingestion.

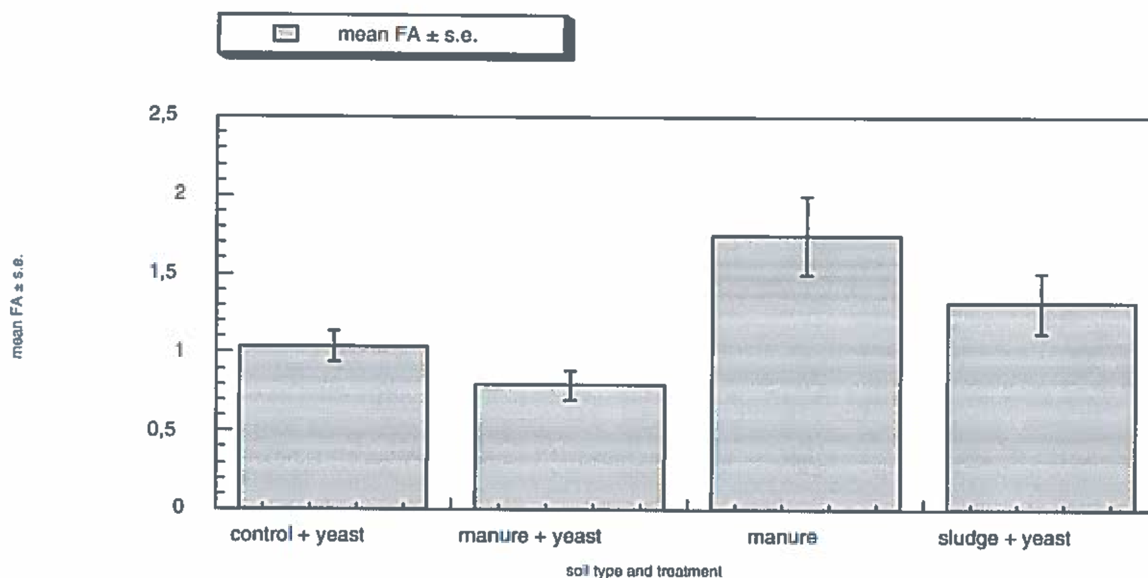
Results

In two of the eight petri dishes we used in our experiment, we detected the presence of a significant Venv (sludge with yeast and cattle manure). Therefore, we analysed only one petri dish with sludge with yeast and one petri dish with cattle manure, whereas we had two control dishes with yeast and two cattle manure dishes with yeast. Because no significant differences between replicates within treatments were found, we pooled the results of the two replicates. Out of the 120 pooled collembolans used in the two treatments, some were damaged during the preparation, which reduced the number of individuals studied in both pooled treatments to 106.

We found strong evidence for differences of mean FA among the four treatments (one way ANOVA, $F_{3,328}$ = 7.316, p = ***). Fig. 3 shows the mean and the standard error (s.e.) of the mean absolute value of FA in the four treatments.

1) control with yeast FA = 1.038 ± 0.1, n = 106
2) cattle manure with yeast FA = 0.792 ± 0.094, n = 106
3) cattle manure FA = 1.750 ± 0.252, n = 60
4) sludge with yeast FA = 1.317 ± 0.195, n = 60

The pairwise comparisons (Fisher's test) revealed that individuals exposed to cattle manure treatment had a significantly higher mean FA than individuals exposed to the control treatment (***) and to cattle manure with yeast (***). Furthermore, the mean FA of the treatment sludge with yeast was significantly higher than that of the treatment cattle manure with yeast (*).



Conclusions of the two investigations

The general conclusions that can be drawn from the above experiments are promising. With our new method of detecting Venv we are able to detect and remove those replicates where the significant presence of Venv has confounding effects on the results. The investigations revealed that there is a detectable effect of the fertilisers employed for agricultural practice, and that these fertilisers have an effect both via absorption and by direct ingestion. Apparently, the effect of sludge is stronger in experiment 1, where the contaminants have been absorbed through the surface, as compared to the effects revealed in experiment 2, where the sludge has been directly ingested. In order to detect which of the toxicants in the fertilisers had these effects on FA of the collembolans, one has to conduct separate experiments with single toxicants.

Future directions

Future work could be done in the terrestrial, aquatic, and aerial environment. In the terrestrial and aerial environment, two asexually reproducing organisms, *Drosophila mercatorum* (one clonal line) and *Folsomia candida* (several clonal lines), could be used. In the aquatic environment *Daphnia magna* (several clonal lines) will be well-suited.

Terrestrial environment

Several experiments could be conducted using different stress factors. Model organisms can be exposed to single toxicants or a combination of different toxicants and with different concentrations, in order to detect synergistic effects. Strains from the two parthenogenetic species, *Drosophila mercatorum* and *Folsomia candida,* could be used for that purpose.

Aquatic environment

Daphnia is well suited for experiments of this kind for a number of reasons. Due to asexual reproduction, separate clones may easily be isolated and kept for a number of generations. For the present experiments, the species *Daphnia magna* would be suitable, due to its rapid growth and good performance in cultures. Moreover, an image analysis design has been constructed for rapid enumeration and surface imaging of live individuals, allowing continuous determination of reproductive success (size distribution, number of individuals) as well as rapid determination of major phenotypic characteristics such as length to width ratios. For a more detailed analysis of morphological characteristics the left and right side appendages may be useful. The *antennae* of *Daphnia* have several morphological characteristics and *setae* that may be easily assessed and measured on a large number of individuals for screening of FA.

There is a set of primers and DNA-sequences available for *Daphnia* that may allow for more detailed genetic studies. A set of enzymes is also available for allozyme screening of clonal affinities and levels of heterozygosity. These genetic studies could allow us to collect *Daphnia* directly in the field and then to use the genetic markers to separate the different clonal lines for measuring DS within each line.

Aerial environment

The incomplete combustion of petrol in e.g. car engines gives rise to the emission of organic pollutants, polycyclic aromatic hydrocarbons (PAH). *Drosophila* possess genes which react to the presence of these pollutants. These genes, which are responsible for the synthesis of proteins in a clearly determined family, cytochrome P450, are little active under normal conditions. However, in the presence of pollutants, the genes are activated and cytochrome P450 is synthesised in large quantities. The problem is that no univocal relationship exists between the presence of a particular type of PAH in the atmosphere and the synthesis of a specific type of cytochrome P450.

Quite apart from detecting the presence of pollutants (which could be achieved using standard chemical detectors), the principal value of cytochrome P450 is to reveal their toxic nature. In fact, once synthesised, cytochrome P450 oxidises the PAHs, which then becomes chemically reactive. This has the effect that PAHs interact with proteins and DNA, thus compromising the correct functioning of the cell. It is well known that incorrect functioning of the cell reduces individual DS. Therefore, we presume that *Drosophila mercatorum* and possibly also *Folsomia candida* could be investigated for DS in order to detect the effect of these air pollutants in a more specific way.

Acknowledgements: We are grateful to Susanne Boutrup, Århus Amt, for providing us with the soil samples studied, to Henning Krogh, DMU, for providing us with colembolans (*Folsomia candida*) used as test organisms, to Martin Holmstrup for advice on handling cultures of *Folsomia candida* and to Ditte Holm Andersen and to Christina Weideick Kærsgaard for help with counting bristles. We also thank Kirsten Zaluski for linguistic improvements to the text. The project was financially supported by Østjysk Innovation.

Developmental stability of wing size in *Drosophila melanogaster* under food stress

Jørgen Bundgaard#, Cino Pertoldi# and Agnès Lefranc*

Department of Ecology and Genetics, Institute of Biological Sciences, Aarhus University, Ny Munke gade, Building 540, DK-8000 Aarhus C, Denmark

**Present address:*
Laboratoire d'Ecologie
Université Pierre et Marie Curie
Bâtiment A, 7ème étage
7, quai Saint Bernard
Case 237
75232 Paris cedex 05
France

#Corresponding author: biojb@biology.aau.dk

ABSTRACT

Fluctuating asymmetry (FA) and phenotypic variance of wing length in *Drosophila melanogaster* which were collected at three different hatching stage (early, middle and late stages) was investigated at two larval food stress levels. The stress levels ranged from severe food stress with only 16.1 % larva-to-adult survival to a moderate stress with 29.5 % larva-to-adult survival.

A significant reduction in wing size and a concomitant increase of FA was found with increasing larval food stress, whereas no significant increase of phenotypic variance was found. Significant reduction of the wing size with a concomitant increase of FA and phenotypic variance was found for *Drosophila* which hatched in the late stage as compared to those which hatched in the earlier stages.

We found evidence for the fact that females are less stress tolerant than males. A negative association between FA and the wing size in both sexes was found for *Drosophila* which was grown in severe food stress conditions. The selective forces that may have produced these patterns are discussed.

1. INTRODUCTION

1.1. Developmental stability

Two principal methods are commonly employed for the estimation of developmental stability; some studies use variances of morphological characters (Zouros *et al.* 1980, King 1984, Livshits and Kobliansky 1984, Imasheva *et al.*, 1997), where the estimate can be blurred by additive genetic variation, while other studies use fluctuating asymmetry (FA) (Møller and Swaddle 1997 and references therein).

Measures of developmental instability, such as FA, are often much more sensitive indicators of current environmental conditions than measures traditionally used for monitoring, such as various life history components like growth, fecundity and survival. Several authors have suggested that FA tends to be higher in sexually selected than in non-sexually selected traits because of their recent history of intense directional selection (Møller 1992, Manning and Chamberlain 1993, see Møller and Swaddle 1997, for review). In addition, several fitness components may be negatively correlated with FA (Møller and Swaddle 1997).

1.2. Fluctuating asymmetry

FA occurs when an individual for some reason is unable to undergo identical development on both sides of a bilaterally symmetrical trait (Van Valen 1962, Palmer & Strobeck 1986). FA tends to become elevated under stress (Leary & Allendorf 1989). Stress factors known to raise FA include various chemicals, e.g. pesticides (Valentine & Soulé 1973; Clarke and McKenzie 1987), polluted habitats (Weiner & Rago 1987), extreme temperatures (Parsons 1962, Siegel & Doyle 1975, Sciulli et al. 1979; Imasheva *et al.*, 1997), audiogenic stress (Sciulli et al. 1979) and food deficiency, either in terms of quality or quantity (Parsons 1990). Increased levels of nutritional stress have been shown to increase FA in *Drosophila* (Parsons 1964), and phenotypic variance has been found to increase in *D. melanogaster* in a varying environment (Gibson and Bradley 1974, Mackay 1980), and under extreme conditions (Hoffmann and Parsons 1991; Imasheva et al. 1997, 1998). Rasmusson (1960) observed that the frequency of the phenodeviants increased with larval density in *Drosophila melanogaster*, and that the frequency of these phenodeviants was positively related to both asymmetry and the coefficient of variation. In his study of the common housefly, *Musca domestica,* Møller (1996) detected stabilising sexual selection for wing symmetry. In addition, the relatively common finding that asymmetry increases as trait size decreases, has lead to the view that FA plays a significant role in the evolution of sexual signals (Møller and Pomiankowski, 1993).

1.3. Phenotypic variance

In *Drosophila,* body size (as measured by thorax size, wing size or weight) can be influenced by genes (Robertson and Reeve, 1952; Robertson, 1962; Coyne and Beecham, 1987; Prout and Barker, 1989), developmental temperature (Parsons, 1961; Tantawy, 1964; David et al., 1994; Barker and Krebs, 1995; Imasheva et al., 1997), nutrition (Parsons, 1961; Robertson, 1963; David et al., 1983; Hillesheim and Stearns, 1991), or a combination of these factors. In the present study, we measured wing length of both wings in *Drosophila melanogaster* reared at two different food stress levels, keeping the temperature and the genetic variation as constant as possible.

Variance of wing length at different larval food stress levels, were investigated because of the more recent advanced hypothesis that under stressful condition, both phenotypic and genetic variation of fitness related traits would increase (Parsons 1987, Hoffmann and Parsons 1991). This evolutionary strategy would increase the populations potential adaptiveness.

2. MATERIAL AND METHODS

2.1.1. Experimental design

One to two thousand of three to five day old *Drosophila melanogaster* males and females from uncrowded bottle cultures (of the stock Groningen 83) were introduced into a 35x35x20 cm plexiglass box where the females layed eggs for 10-12 hours in petri dishes with yest-agar-sugar medium in the bottom. When the first eggs started to hatch some 18 hours later, all larvae were removed from the petri dishes and all new first instar larvae were collected from the petri dishes at 3 hour intervals and placed in food vials with varying amounts of food (see Table 1). In vials with less than 7 ml of food, the medium was layered on top of a complementary volume of 1.5% agar-water solution in order to have a total volume of 7 ml in each vial. The agar solution prevented excessive desiccation in those tubes containing low quantities of medium.

All larvae in a vial were collected at three different hatching stage (early, middle and late stages). The procedure resulted in unequal food stress among larvae, from a light food stress of the larva which hatched at the early stage to middle food stress of the larvae which hatched at the middle stage, up to the relatively high food stress of larvae which emerged at the late stage. In this way we have obtained two increasing levels of food stress, from level 1 with relatively high food amount (4 ml per vial) and moderate stress to level 2 with a low amount of food (2.5 ml per vial) and high stress (Table 1).

For both stress level, the wings of approximately 150 females and 150 males were measured, of these individuals, 50 hatched at the early stage, 50 at the middle stage and 50 at the late stage. Before the wing size measurements, the flies were killed by etherization, both of their wings pulled off and each wing placed in a drop of water on a microscope slide covered with a cover slip.

2.1.2. Wing measurement

The length of the wing was measured from the intersection between the anterior cross vein and the third longitudinal vein (L3) to the intersection between L3 and the distal wing margin using a binocular microscope with a digital filar eyepiece (Los Angeles Scientific Instrument Company, Inc., USA). All the wings were measured three times and the median of the three measurements was considered.

2.2. Fluctuating asymmetry

2.2.1. Measurement error

The measurement error was obtained by measuring every wing twice at two independent trials. The magnitude of asymmetry was estimated from the difference in length between the two pairs of wings as right minus left (r-l). We then calculated the contribution of measurement errors (the difference between two independent estimates of FA) in relation to FA.

2.2.2. Statistical properties of fluctuating asymmetry

In all the analyses which will be conducted below, the two sexes and the two stressing levels were analysed separately. The three hatching stages were pooled. To see if the data display the statistical properties of FA (display an approximate normal distribution of signed asymmetry scores around a mean of zero). The hypothesis that the mean of right minus left character values equals zero was tested in a one sample *t*-test. Normality was tested by a Lilliefors' test and by inspecting the distributions graphically.

2.2.3. Dependency of fluctuating asymmetry by the size of the wing

A Spearman rank correlation test and a linear regression analysis (Zar 1984) were used to test if the absolute value of FA of the wings was correlated with wing length. For these tests we pooled the three hatching stages.

2.2.4. Comparisons of FA among the three hatching stages

FA of flies who hatched at the early middle and late stage were calculated using index FA1 and FA4 of Palmer and Strobeck (1986). FA1 is the absolute value of (r-l), and FA4 is the variance of (r-l). A Levene's test (Zar 1984) was conducted to test if the absolute FA (FA1) were significantly different among the three hatching time. Because the absolute values of FA are expected to be half-normally distributed, we also performed a Kruskal-Wallis one-way non-parametric analysis of variance (K-W) (Zar 1984). Furthermore, multiple comparison tests were made with a Scheffé *F*-test (Zar 1984), for comparing the differences in FA in pairwise comparisons. Comparisons of FA between sexes of individuals which hatched in the same hatching stages were made with a *F*-test, comparing the signed value of (r-l) (FA4).

2.2.5. Comparisons of FA between the two larval food stress levels

FA of flies grown at the two larval food stress levels was calculated using index FA4. A *F*-test was conducted to test if the variance of (r-l) (FA4), was significantly different between the two stress levels. The *F*-test were conducted comparing individuals which hatched in the same stage.

2.3. Phenotypic variance

2.3.1. Comparisons of phenotypic variance among the three hatching stages

A *F*-test was conducted to test whether the variance of the log-transformed length of the left wing was significantly different among the three hatching stages. A log-transformation of the wing length was performed, since the variance is in general proportionate to its mean.

The coefficients of variation (CVs) of the left wing length were calculated for the three hatching time and plotted graphically. Comparisons of phenotypic variance between sexes of individuals which hatched in the same hatching stages were made with a *F*-test.

2.3.2. Comparisons of phenotypic variance between the two larval food stress level

Differences of phenotypic variance between the two larval food stress level were compared with a *F*-test. The *F*-test were conducted comparing individuals which hatched in the same stage.

2.3.3. Comparisons of means of wing length among the three hatching stages

A Levene's test (Zar 1984) was conducted to test if the mean of the left wing length was significantly different among individuals which hatched in the three different hatching stages. Multiple comparison tests were made with a Scheffé *F*-test.

Comparisons of the mean of the wing length between sexes of individuals which hatched in the same stage, were made with a *t*-test.

2.3.4. Comparisons of the mean wing length between the two larval food stress level

Differences of the mean wing length between the two larval food stress level were compared with a *t*-test.

3. RESULTS

3..2. Fluctuating asymmetry

3.2.1. Measurement error

The overall mean ± sd of the absolute value of FA for the wings were 0.01 ± 0.09 for males, and 0.011 ± 0.011 for females when pooled over all stress levels. The contribution of measurement errors (the difference between two independent estimates of FA) had a mean of 0.0004 for the males, and 0.0009 for females. The mean measurement error in relation to the mean FA estimate was for males 4% and for females 8%. The measurement error in relation to mean FA was therefore within the range of 25%, suggested as a reasonable range by Palmer (1994, 1996).

3.2.2. Statistical properties of fluctuating asymmetry

No significant deviations from zero of the mean of the trait (r -l) distributions were found for both sexes (one sample *t*-test: -1.837 • *t* • 1.24, 0.07 • *p* • 0.80, 152 • *df* • 163).

Deviations from normal distributions of the signed values of (r-l) were not significant in both sexes for *Drosophila* which was grown in 4 ml vial, whereas it were highly significant for both sexes of *Drosophila* which was grown in 2.5 ml vial (Lilliefors' test, males: $p < 0.001$ $n = 155$, females: $p < 0.0001$ $n = 155$). The two distributions of the traits FA were inspected graphically and we could see that the deviations from normality was due to a leptokurtic distribution (males' kurtosis = 3.142, females' kurtosis = 6.667) and we did not find evidence of antisymmetry.

3.2.3. Dependency of fluctuating asymmetry by the size of the wing

(2.5 ml) males: A highly significant negative correlations (linear regression analysis) were found between wing length and the absolute value of FA (FA1) of the wing of *Drosophila* males grown in vial with low food amount (2.5 ml), ($r = -0.253$, $n = 154$, $p = 0.0015$), (see Fig. 1). Also the non parametric (Spearman test) correlation analysis shows a highly significant negative correlation ($r_S = -0.226$, $n = 154$, $p = 0.051$), in complete concordance with the result obtained from the linear regression analysis.

(2.5 ml), females: A highly significant negative correlations (linear regression analysis) were found between wing length and the absolute value of FA (FA1) of the wing in *Drosophila* females grown in vial with low food amount (2.5 ml), ($r = -0.217$, $n = 165$, $p = 0.005$), (see Fig. 1). Whereas the non parametric (Spearman test) correlation analysis was not significant ($r_S = -0.136$, $n = 165$, $p = 0.0815$).

(4 ml), males: No correlations were found between wing length and the absolute value of FA (FA1) of the wing of *Drosophila* males grown in vial with relatively high food amount (4 ml), ($r = 0.093$, $n = 153$, $p = 0.25$). Also the non parametric (Spearman test) correlation analysis was not significant ($r_S = 0.081$, $n = 153$, $p = 0.317$),

(4 ml), females: No correlations were found between wing length and the absolute value of FA (FA1) of the wing of *Drosophila* females grown in vial with relatively high food amount (4 ml), ($r = -0.109$, $n = 155$, $p = 0.175$). Also the non parametric (Spearman test) correlation analysis was not significant ($r_S = -0.115$, $n = 155$, $p = 0.155$),

3.2.4. Comparisons of FA among the three hatching stages

(2.5 ml), males: The Levene's tests, which was conducted for the absolute value of FA (FA1) of the wing of the *Drosophila* grown in vial with low food amount (2.5 ml), were highly significant for males (Table 2). The one-way non-parametric analysis of variance (Kruskal-Wallis test) was also highly significant for males (Table 2), in complete concordance with the result obtained from the Levene's test. The multiple comparisons (see Scheffé's *F*-test: Table 2) revealed evidence of a significantly higher FA for males which hatched in the late stage compared to those which hatched in the early and middle stage.

(2.5 ml), females: The Levene's tests, which was conducted for the absolute value of FA (FA1) of the wing of the *Drosophila* grown in vial with low food amount (2.5 ml), were significant for females (see Table 2). Whereas, the one-way non-parametric analysis of variance (Kruskal-Wallis test) was not significant for females (Table 2).

(4 ml), males: The Levene's tests, which was conducted for the absolute value of FA (FA1) of the wing of the *Drosophila* grown in vial with relatively high food amount (4 ml), were not significant for males (see Table 2). The one-way non-parametric analysis of variance (Kruskal-Wallis test) was also not significant for males (Table 2).

(4 ml), females: The Levene's tests, which was conducted for the absolute value of FA (FA1) of the wing of the *Drosophila* grown in vial with relatively high food amount (4 ml), were not significant for females (see Table 2). The one-way non-parametric analysis of variance (Kruskal-Wallis test) was also not significant for males (Table 2).

3.2.5. Comparisons of FA between the two larval food stress level

There was some evidence for an increase FA between the two larval who hatced in late stage food stress levels (Table 3) and there was some evidence for higher FA (FA4) of those females who hatced in the early stage and was grown in 2.5ml and 4 ml vial as compared to males (Table 4).

3.3. Phenotypic variance

3.3.1. Comparisons of phenotypic variance among the three hatching stages

There was evidence for differences in variance of the log-transformed length of the left wing among the three hatching stages in both males and females, with a clear increase in variance of *Drosophila* which hatched in the later stages as compared to those which hatched earlier (see Table 5).

3.3.2. Comparisons of phenotypic variance between the two larval food stress level

There was no evidence for differences in variance of the log-transformed length of the left wing between the two larval food stress levels (2.5 ml and 4 ml) of *Drosophila* which hatched at the same stage (1.14 • F • 1.5, 44 • n • 58, $p > 0.05$) with only one exception(late females: $F_{58,49} = 1.9$, $p < 0.05$).

Significant differences in variance (*F*-test) of the log-transformed length of the left wing between the two sexes grown at the same stress level, were found. There was evidence for a significantly higher phenotypic variance in females as compared to males (see Table 6).

3.3.3. Comparisons of means of wing length among the three hatching stages

There were large evidence for a reduction of the wing size of Drosophila which hatched in the late stage as compared to those which hatched in the earlier stage in both sexes and in both the two larval food stress levels (2.5 ml and 4 ml) (see Table 7).

3.3.4. Comparisons of the mean wing length between the two larval food stress levels

There were large evidence in both sexes and in all the hatching stages for a reduction of the wing size of *Drosophila* which was grown in 2.5 ml vial as compared to those which was grown in 4 ml vial (Table 8).

4. DISCUSSION

4.2. Fluctuating asymmetry

4.2.1. Statistical properties of fluctuating asymmetry

Deviations from normality of the unsigned value of FA due to an excessive leptokurtosis of the distribution could have two explanations. First, when the unit of measurement is large in comparison with the actual asymmetry, there will be a tendency for the population asymmetry distribution to show leptokurtosis even though the traits display FA. Second, it could be that there is an intense natural or sexual selection against asymmetric individuals, and hence very asymmetric individuals may be relatively rare in field samples if selection has already acted against the most asymmetric individuals. We would also expect the biomechanical cost of asymmetry on wing size to be extremely high. The high leptokurtosis of the distributions of the signed values of FA found in our investigation in both sexes could confirm this strong selection event.

If larger sexual traits are more costly to produce and maintain under stressful conditions compared to more favourable conditions, and asymmetry itself is a selected trait, then fewer individual should be able to achieve both larger and symmetrical traits under stressful conditions. Differences in food abundance are likely to result in greater variation in individual quality under stressful conditions than under optimal conditions. Stressed (and therefore relatively smaller as compared to unstressed *Drosophila*)*Drosophila* individuals cannot allocate their resource to the wing grown as unstressed *Drosophila*.

4.2.3. Dependency of fluctuating asymmetry by the size of the wing

The negative relationship between FA and wing size which was found in both male and females of *Drosophila* grown in vial with low food amount (2.5 ml), suggest that the wings are under strong directional selection (see Møller & Swaddle, 1997, for review). The lack of relationship of *Drosophila* grown in vial with relatively high food amount (4 ml), could be due to the fact that the intensity of directional selection is reduced under optimal condition, confounding therefore the expected relationship. We have not used scaled index also if we found a dependency of FA with the size because we was aware of obscure the relationship between FA and fitness. However, there is a large body of evidence indicating a negative association between fitness and individual asymmetry. Since poor environmental conditions increase asymmetry, a negative relationship between FA and growth performance found in females may arise simply because the insects grow poorly and become very asymmetric.

4.2.4. Comparisons of FA among the three hatching stages

The higher FA of the wing of the late hatched (as compared to the early and middle hatched) *Drosophila* which growned in vial with low food amount (2.5 ml), indicate clearly that individual which hatched first found better condition as compared to the individuals which hatched in the later stages. However, we cannot exclude that there are differences of developmental stability which are independent of the food amount that the larvae found when they hatch. But the fact that no differences of FA was found among *Drosophila* 's wing which growned in vial with relatively high food amount (4 ml), seems to confirm the hypothesis that the differences in developmental stability are only due to food stress, in fact the food stress of the late hatched *Drosophila* which hatched in the 4 ml vial will suffer a relatively lower food stress as compared to the late hatched *Drosophila* which hatched in the 2,5 ml vial.

4.2.5. Comparisons of FA between the two larval food stress level

The onlyest differences which was found between individuals which was grown in the 2.5 ml vial and 4 ml vial was found in both sexes of *Drosophila* which hatched in the late stage. This fact indicate that

Drosophila which hatch in the late stage are the most sensitive to stress as compared to *Drosophila* which hatced before.

4.3. Phenotypic variance

4.3.1. Comparisons of phenotypic variance among the three hatching stages

The clear increase of the variance of the wing of the *Drosophila* which hatched in the later stage as compared to those which hatched earlier stage are concordant with the FA investigation.

4.3.2. Comparisons of phenotypic variance between the two larval food stress level

The non significant differences found between *Drosophila* which was grown in the two larval food stress levels, could be due to the fact that the stress difference produce was to low. One of the reasons could be as said in the introduction that the estimate can be blurred by additive genetic variation.

The clear higher variance of the wing of female *Drosophila* as compared to females, is concordant with the FA investigation and support the hypothesis that females are less stress tolerant than males (Loeschcke et al. 1994).

4.3.3. Comparisons of means of wing length among the three hatching stages

The clear reduction of the wing of the *Drosophila* which hatched in the later stage as compared to those which hatched earlier stage are concordant with the increased FA and phenotypic variance.

4.3.4. Comparisons of the mean wing length between the two larval food stress level

The significant reduction of the wing found between *Drosophila* which was grown in the two larval food stress levels, indicate that also the size reduction is a sensitive indicator of stress. A clear reduction of the wing size with increasing stress level indicate also that food deficiency acts strongly on *Drosophila*'s wing size which shows high phenotypic plasticity in both sexes.

REFERENCES

Barker. J.S.F. and R. Krebs 1995. Genetic variation and plasticity of thorax length and wing length in *Drosophila aldrichi* and *D. buzzatii.* J. Evol. Biol. 8:689-709.

Britten, B. H. 1996. Meta-analyses of the association between multilocus heterozygosity and fitness. Evolution 50: 2158--2164.

Clarke, G. M., Brand, G. W. and Whitten, M. J. 1986. Fluctuating asymmetry: a technique for measuring developmental stress caused by inbreeding. Aust. J. Biol. Sci., 39, 145-153.

Clarke, G. M. and McKenzie, J. A. 1987. Developmental stability of insecticide resistant phenotypes in blowfly; a result of canalizing natural selection. Nature, Lond. 325: 345-346.

Clarke, G. M., Oldroyd, B.P. and Hunt, P. 1992. The genetic basis of developmental stability in *Apis mellifera*: Heterozygosity vs. genetic balance. Evolution, 46, 753-762.

Coyne, J. A. and E. Beecham, 1987. Heritability af two morphological characters within and among natural populations of *Drosophila melanogaster*. Genetics 117: 727-737

Crespi B. J. and Brett, A. V. 1997. Fluctuating asymmetry in vestigial and functional trait of a haplodiploid insect. Heredity, 79, 624-630.

David, J., R. Allemand, J. van Herrewege and Y. Cohet 1983. Ecophysiology: abiotic factors. In: *The Genetics and Biology of* Drosophila. (M. Ashburner, H.L. Carson and J.N.Thompson (eds). Vol. 3d. pp.106-109. Academic Press, London.

David, J.R., B. Moreteau, J.R. Gautier, A. Stockel and A.G. Imasheva 1994. Reaction norms of size characters in relation to growth temperature in *Drosophila melanogaster*: an isofemale line analysis. Génét. Sél. Evol. 26:229-251.

Fowler, J. & Cohen L. 1990. *Practical statistics for field biology*. Philadelphia: Open University Press.

Gibson, J. B. and Bradley, B. P. 1974: Stabilizing selection in constant and fluctuating environments. Heredity 33, 293-302.

Haldane, J. B. S. 1955. The measurement of variation. *Evolution* 9, 484.

Hillesheim, E. and S.C. Stearns 1991. The responses of *Drosophila melanogaster* to artificial selection on body weight and its phenotypic plasticity in two larval food environments. Evolution 45:1909-1923.
Hoffmann, A. A. and Parsons, P.A. 1991: Evolutionary Genetics and Environmental Stress. Oxford University Press, Oxford.
Imasheva, A.G., V. Loeschske, O. Lazebny and L.A. Zhivotovsky 1997. Effects of extreme temperatures on quantitative variation and developmental stability in *Drosophila melanogaster* and *Drosophila buzzatii.* Biol. J. Linn. Soc. 61:117-126.
Imasheva, A. G. Loeschcke, V. Lazebny, O. and Zhivotovsky, L. A. 1998: Stress temperatures and quantitative variation in Drosophila melanogaster. Heredity, in press.
King, D. P. F. 1985: Enzyme heterozygosity associated with anatomical part character variance and growth in the herring (Clupea harengus L.) Heredity 1954, 289-296.
Leary, R. F., Allendorf, F. W. and Knudsen, K. L. 1983. Developmental stability and enzyme heterozygosity in rainbow trout. Nature 301, 71-72.
Leary, R. F. & Allendorf, F. W. 1989: Fluctuating asymmetry as an indicator of stress: Implications for conservation biology. -- Trends Ecol. Evol. 4: 214-217.
Leary, R. F., Allendorf, F. W. and Knudsen, K. L. 1984. Superior developmental stability of heterozygotes at enzyme loci in salmonid fishes. Am. Nat. 124: 540-551.
Lerner, I. M. 1954. Genetic Homeostasis. Oliver and Boyd, London.
Livshits, G. & Kobyliansky, E. 1984: Biochemical heterozygosity as a predictor of developmental homeostasis in man. Ann. Human Genet. 48, 173-184.
Loeschcke, V. Krebs, R. A. and Barker, J. S. F. 1994: Genetic variation and acclimation to high temperature stress in Drosophila buzzatii. Biol. J. Linn. Soc. 52: 83-92.
Mackay, T.F.C. 1980: Genetic variance, fitness, and homeostasis in varying environments: an experimental check of the theory. Evolution 34, 167-185.
Manning, J. T. and Chamberlain, A. T. 1993. Fluctuating asymmetry in gorilla canines: a sensitive indicator of environmental stress. Proc. R. Soc. Lond. B 255, 189-193.
Maynard Smith, J. 1978. The Evolution of Sex. Cambridge University Press, Cambridge.
Mitton, J. B. 1995. Enzyme heterozygosity and developmental stability. Acta Theor., Suppl. 3: 33-54.
Møller, A.P. 1992: Female swallow preference for symmetrica male sexual ornaments. Nature 357, 238-240.
Møller, A. P. 1993: Morphology and sexual selection in the barn swallow Hirundo rustica in Chernobyl, Ukraine. Proc. R. Soc. Lond. B 252: 51-57.
Møller, a. P. 1996: Sexual selection, viability selection, and developmental stability in the domestic fly Musca domestica. Evolution 50: 746-752.
Møller, A. P. and Pomiankowksi, A. 1993: Fluctuating asymmetry and sexual selection. Genetica, 89, 267-279.
Møller, A. P. and Swaddle, J. P. 1997 Asymmetry, developmental stability and evolution. Oxford: Oxford University Press.
Norry, F. M. Vilardi, J. C. and Hasson, E. 1997: Genetic and phenotypic correlations among size-related traits, and heritability variation between body parts in Drosophila buzzatii. Genetica 101: 131-139.
Norry, F. M. Vilardi, J. C. and Hasson, E. 1998: Sexual selection related to developmental stability in Drosophila buzzatii. Hereditas 128: 115-119.
Palmer, A. R. 1994. Fluctuating asymmetry analyses: a primer. In: Developmental Instability: Its Origins and Evolutionary Implications.
T. A. Markow (Ed.), pp. 335 - 364. Kluwer, Dordrecht.
Palmer, A. R. 1996. Waltzing with asymmetry. BioScience 46: 518 - 532.
Palmer, A. R. & Strobeck, C. 1986 Fluctuating asymmetry: measurement, analysis, patterns. *Annu. Rev. Ecol. Syst.* 17, 391-421.
Parsons, P.A. 1961. Fly sice, emergence time and sternopleural chaeta number in *Drosophila.* Heredity 16:455-473.
Parsons, P. A. 1962: Maternal age and developmental variability. -- J. Exp. Biol. 39: 251-260.
Parsons, P. A. 1964 Parental age and the offspring. Q. Rev. Biol. 39, 258-275.
Parsons, P. A. 1987: Evolutionary rates under environmental stress. Evolutionary Biology 21: 311-347.
Parsons, P. A. 1990: Fluctuating asymmetry: An epigenetic measure of stress. -- Biol. Rev. 65: 131- 145.
Prout, T. and J. S. F. Barker, 1989. Ecological aspects of heritability of body size in *Drosophila buzzatii.* Genetics 123: 803-813
Rasmuson, M. 1960: Frequency of morphological deviations as a criterion of developmental stability. Hereditas 46: 511-536.

Robertson, F.W. and E.C.R. Reeve 1952. Studies in quantitative inheritance. I. The effects of selection of wing and thorax length in *Drosophila melanogaster*. J. Genet. 50:414-448.

Robertson, F. W., 1962. Changing the relative size of the body parts of *Drosophila* by selection. Genet. Res. Camb. 3: 169-180

Robertson, F. W., 1963. The ecological genetics of growth in *Drosophila*. 6. The genetic correlation between the duration of the larval period and body size in relation to larval diet. Genet, Res. 4: 74-92

Sciulli, P. W., Doyle, W. J., Kelley, C., Siegel, P. & Siegel, M. I. 1979: The interaction of stressors in the induction of increased levels of fluctuating asymmetry in the laboratory rat. -- Am. J. Phys. Anthropol. 50: 279-284.

Siegel, M. I. & Doyle, W. J. 1975: The differential effect of prenatal and postnatal audiogenic stress on fluctuating dental asymmetry. -- J. Exp. Zool. 191: 211-214.

Sokal, R. R. & Rohlf, F. J. 1981. Biometry. W. H. Freeman & Co., San Francisco.

Soulé, M. E. 1982: Allomeric variation. I. The theory and some consequences. Amer. Nat. 120: 751-764.

Spieth, H.T. and J.M. Ringo. 1983. Mating Behavior and Sexual Isolation in *Drosophila* in Ashburner, Carson and Thompson (eds.) volume 3c "The Genetics and Biology of Drosophila" pp. 223-284. Academic Press

Tantawy A.O. 1964. Studies on natural populations of *Drosophila*. III. Morphological and genetical differences of wing length in *Drosophila melanogaster* and *D. simulans* in relation to season. Evolution 18:560-570.

Valentine, D. W. & Soulé, M. E. 1973: Effect of p,p' DDT on developmental stability of pectoral fin rays in the grunion (Leuresthes tenius). -- Fishery Bull. 71: 920-921.

Van Valen, L. 1962: A study of fluctuating asymmetry. -- Evolution 16: 125-142.

Watson, P. J. and Thornhill, R. 1994: Fluctuating asymmetry and sexual selection. Trends. Ecol. Evol. 9: 21-25.

Weiner, J. G. & Rago, P. J. 1987: A test of fluctuating asymmetry in Environ. Pollut. 44: 27-36.

White, M. J. D. 1973. Animal Cytology and Evolution. 3rd edition. Cambridge University Press, Cambridge.

Williams, G. C. 1975. Sex and Evolution. Princeton UniversityPress, Princeton.

Zar, J. H. 1984: Biostatistical Analysis. Prentice Hall, New Jersey.

Zouros, E., Singh, S. M. & Miles, H. E. 1980: Growth rate in oystres: An overdominant phenotype and its possible explanation. Evolution 34, 856-867.

Table 1. Crowding regime and larval survival.

number of vials	amount of food per vial (ml)	number of larvae per vial	total number of larvae	total number of adult emerging	egg-to adult survival
8	2.5	400	3,2	516	16.1
4	4	400	1,6	472	29.5

Table 2. Levene's test for comparing the absolute value of FA (FA1) of the wing of males and females hatced in the early midle and late stage with a food amount of 2.5 ml or 4 ml, Kruskal-Wallis one non parametric analysis of variance and Scheffe´test for multiple comparisons.

sex	food amount	early stage (n), mean ± sd	middle stage (n), mean ± sd	late stage (n), mean ± sd	source of variation	df	MS	Levene's test (F value)	p	K-W test (H value)	p	Scheffe´test
males	2.5	(48), 0.007 ± 0.005	(53), 0.009 ± 0.007	(53), 0.016 ± 0.014	between	2	0.001	12.114	***	12.933	**	(late > early)*** (late > middle)***
					within	151						
females	2.5	(56), 0.01 ± 0.01	(51), 0.011 ± 0.009	(58), 0.016 ± 0.017	between	2	4.96E-01	3.04	*	4.587	ns	
					within	162						
males	4	(58), 0.008 ± 0.006	(51), 0.008 ± 0.006	(44), 0.008 ± 0.007	between	2	1.20E-03	0.03	ns	0.409	ns	
					within	150						
females	4	(54), 0.009 ± 0.009	(52), 0.01 ± 0.007	(49), 0.012 ± 0.009	between	2	1,10E-01	1.599	ns	3.795	ns	
					within	152						

$p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***

Table 3. F-test of the variance of (r-l) FA4 index of males and females hatced at different stage and with different food amount.

hatcing stage	sex	food amount (2.5 (n), FA4	food amount (4 (n), FA4	F-test	p
early	males	(48), 9.15E-5	(58), 1.06E-4	1.16	ns
middle	males	(53), 1.25E-4	(51), 1.06E-4	1.18	ns
late	males	(53), 4.77E-4	(44), 1.35E-4	3.53	**
early	females	(56), 2.06E-4	(54), 1.73E-4	1.19	ns
middle	females	(51), 2.14E-4	(52), 1.53E-4	1.4	ns
late	females	(58), 4.7E-4	(49), 1.98E-4	2.37	**

$p < 0.01$ = **

Table 4. F-test of the variance of (r-l) FA4 index of males and females hatced at different stage and with different food amount.

hatcing stage	food amount	males (n), FA4	females (n), FA4	F-test	p
early	2.5	(48), 9.15E-5	(56), 2.06E-4	2.25	**
middle	2.5	(53), 1.25E-4	(51), 2.14E-4	1.712	*
late	2.5	(53), 4.77E-4	(58), 4.7E-4	1.01	ns
early	4	(58), 1.06E-4	(54), 1.73E-4	1.63	*
middle	4	(51), 1.06E-4	(52), 1.53E-4	1.44	ns
late	4	(44), 1.35E-4	(49), 1.98E-4	1.46	ns

$p < 0.05$ = *, $p < 0.01$ = **

Table 5. F-test for compare the variance of log-transformed size of the wing of males and females hatced in the early middle and late stage with a food amount of 2.5 ml or 4 ml.

sex	food amount	early stage (n), variance	middle stage (n), variance	late stage (n), variance	F-test
males	2.5	(48), 6.67E-5	(53), 6.83E-5	(53), 1.22E-4	(early < late: F = 1.832)* (middle < late: F = 1.79)*
females	2.5	(56), 1.23E-4	(51), 7.01E-5	(58), 2.73E-4	(early > middle: F = 1.75)* (early < late: F = 2.22)** (middle < late: F = 3.89)**
males	4	(58), 5.43E-5	(51), 4.78E-5	(44), 8.9E-5	(early < late: F = 1.639)* (middle < late: F = 1.862)*
females	4	(54), 8.18E-5	(52), 6.13E-5	(49), 1.44E-4	(early < late: F = 1.76)* (middle < late: F = 2.35)**

$p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***

Table 6. F-test of the variance of the log-transformed length of the left wing of males and females emerging at different eclosion stages and raised on different amounts of food.

eclosion stage	food amoun ml	males (n)	males variance	females (n)	females variance	F-test	P
early	2.5	(48)	6.67E-5	(56)	1.23E-4	1.84	*
middle	2.5	(53)	6.83E-5	(51)	7.01E-5	1,03	ns
late	2.5	(53)	1.22E-4	(58)	2.73E-4	2.24	**
early	4	(58)	5.43E-5	(54)	8.18E-5	1.51	*
middle	4	(51)	4.78E-5	(52)	6.13E-5	1.28	ns
late	4	(44)	8.90E-5	(49)	1.44E-4	1.62	ns

*: P < 0.05; **: P < 0.01

Table 7. Levene's test for comparing the mean size of the wing of males and females hatced in the early midle and late stage with a food amount of 2.5 ml or 4 ml, and Scheffe´test for mul

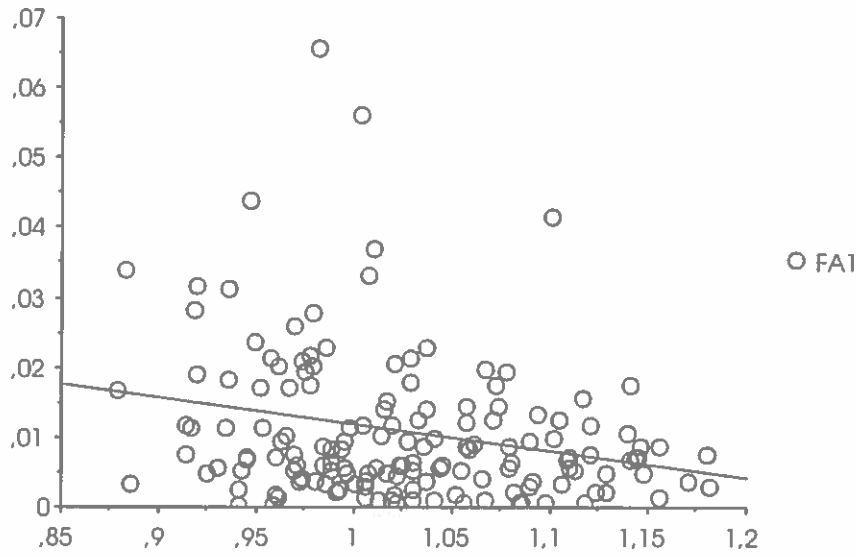
sex	food amount	early stage (n), mean ± sd	middle stage (n), mean ± sd	late stage (n), mean ± sd	source of variation	df	MS	Levene's test (F value)	p	Scheffe´test
males	2.5	(48), 1.103 ± 0.04	(53), 1.001 ± 0.038	(53), 0.978 ± 0.05	between	2	0.219	118.034	***	(early > middle)***, (early > late)** (middle > late)**
					within	151				
females	2.5	(56), 1.217 ± 0.057	(51), 1.104 ± 0.04	(58), 1.062 ± 0.079	between	2	0.362	95.38	***	(early > middle)***, (early > late)** (middle > late)**
					within	162				
males	4	(58), 1.191± 0.037	(51), 1.104 ±0.033	(44), 1.096 ± 0.046	between	2	0.148	98.718	***	(early > middle)***, (early > late)**
					within	150				
females	4	(54), 1.299 ± 0.048	(52), 1.232 ± 0.04	(49), 1.205 ± 0.061	between	2	0.122	48.479	***	(early > middle)***, (early > late)** (middle > late)*
					within	152				

p < 0.05 = *, p < 0.01 = **, p < 0.001 = ***

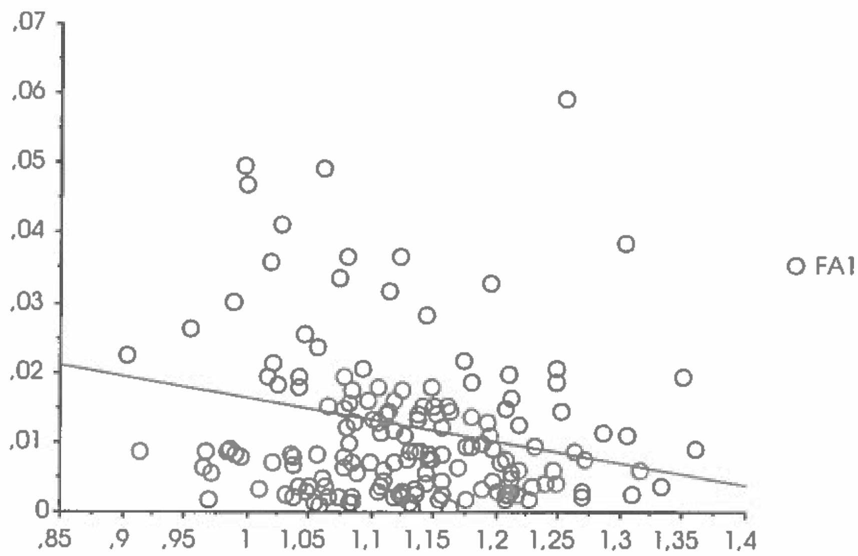
Table 8. t-test for compare the wing length of males and females hatced at different stage and with different food amount.

hatcing stage	sex	food amount (2.5 ml) (n), mean ± sd	food amount (4 ml) (n), mean ± sd	t-test	p
early	males	(48), 1.103 ± 0.04	(58), 1.191± 0.037	11.808	***
middle	males	(53), 1.001 ± 0.038	(51), 1.104 ±0.033	14.688	***
late	males	(53), 0.978 ± 0.05	(44), 1.096 ± 0.046	12.012	***
early	females	(56), 1.217 ± 0.057	(54), 1.299 ± 0.048	8.242	***
middle	females	(51), 1.104 ± 0.04	(52), 1.232 ± 0.04	16.141	***
late	females	(58), 1.062 ± 0.079	(49), 1.205 ± 0.061	10.364	***

p < 0.001 = ***

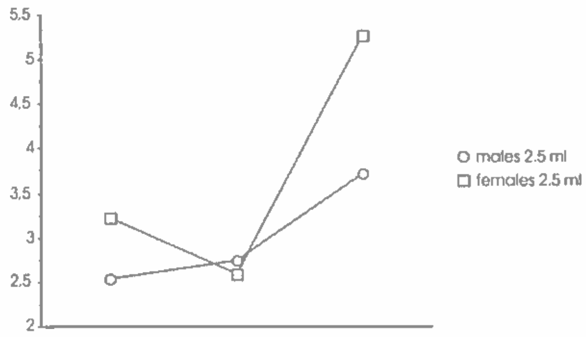


(1a)

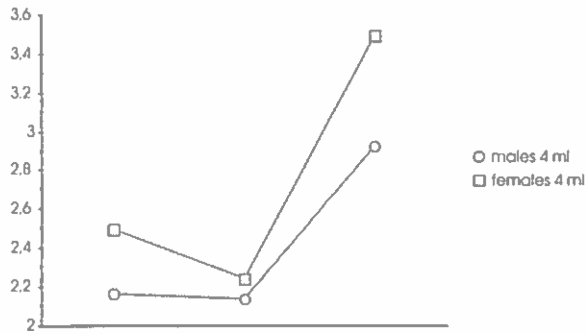


(1b)

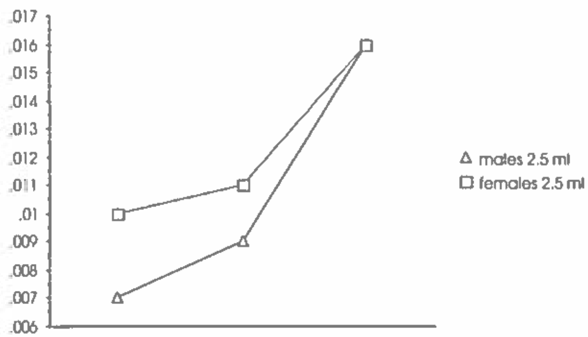
Figure 1: Linear rgression analysis between wing length and the absolute value of FA (FA1) of the wing of *Drosophila* males (1a) and females (1b) grown in vial with low food amount (2.5 ml).



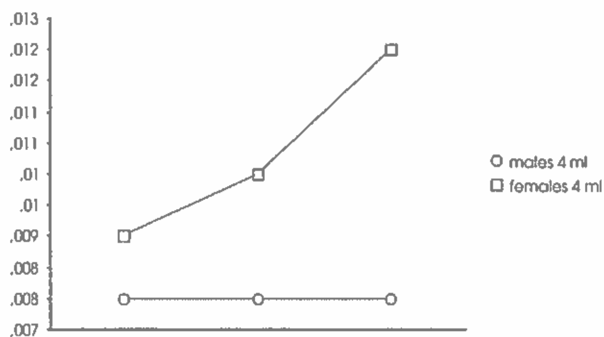
(2a)



(2b)



(2c)



(2d)

Figure 2: CV (2a, 2b) and FA1 (2c, 2d) of wing size of males and females grown at two larval food stress levels, which were collected at three different hatching stages (early, middle and late stages).

National Environmental Research Institute

The National Environmental Research Institute, NERI, is a research institute of the Ministry of Environment and Energy. In Danish, NERI is called *Danmarks Miljøundersøgelser (DMU).*
NERI's tasks are primarily to conduct research, collect data, and offer advise on problems related to the environment and nature.

Addresses:

URL: http://www.dmu.dk

National Environmental Research Institute
Frederiksborgvej 399
Postboks 358
4000 Roskilde
Tlf.: 46 30 12 00
Fax: 46 30 11 14

Management
Personnel and Economy Secretariat
Research and Development Section
Department of Atmospheric Environment
Department of Environmental Chemistry
Department of Policy Analysis
Department of Marine Ecology
Department of Microbial Ecology and Biotechnology
Department of Arctic Environment

National Environmental Research Institute
Vejlsøvej 25
Postboks 314
8600 Silkeborg
Tlf.: 89 20 14 00
Fax: 89 20 14 14

Environmental Monitoring Co-ordination Section
Department of Lake and Estuarine Ecology
Department of Terrestrial Ecology
Department of Streams and Riparian areas

National Environmental Research Institute
Grenåvej 14, Kalø
8410 Rønde
Tlf.: 89 20 17 00
Fax: 89 20 15 15

Department of Landscape Ecology
Department of Coastal Zone Ecology

Publications:
NERI publishes technical reports, technical instructions, and the annual report. An R&D project catalogue is available in an electronic version on the World Wide Web.
Included in the annual report is a list of the publications from the current year.

The topics covered in this PhD dissertation comprise a comprehensive re-examination of the interaction between genetics, demography and different types of stochasticity. This PhD illustrates that caution is essential in interpreting data based on different kinds of genetic markers, as such markers may be associated with genotypes that are completely different from those involved in some future responses to selection, and at the same time underline the importance of quantitative genetics (QG) investigations as an indispensable complementary tool in conservation biology projects.

I should like to stress, however, that the arguments discussed and the results presented are only the tip of an iceberg, the bottom of which represents a large amount of frustrating accumulated nill-results which have yet to be published. What dolphins and banana flies have in common has opened my eyes to the world of possibilities available when applying modelling principles to the present knowledge of DNA and population genetics.

Cino Pertoldi

Ministry of Environment and Energy
National Environmental Research Institute

ISBN: 87-7772-621-9

PhD thesis Population genetics: comparisons of different techniques in conservation projects