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Review article

The use and integration of molecular DNA information in conservation breeding programmes: a review

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Abstract

Conservation breeding programmes often rely on intensive genetic management of the captive population. However, the relatedness between individuals and individual mean kinship are often estimated based on pedigree records, which are frequently incomplete or unreliable. Depending on the quality of a studbook (e.g. expressed as percentage of pedigree known), molecular information can substantially improve knowledge of a population, and therefore contribute to improving the retention of genetic diversity in each generation. As the use of molecular data has been largely under-utilised, this review aims to provide information on the various types of genetic markers that can be used, the estimation of (DNA based) relatedness and pedigrees, their integration in studbooks, the use of molecular information in breeding pair selection, hybridisation issues and population management in general. We discuss recent developments in methodology (e.g. next generation sequencing), theoretical considerations, and software that can aid conservation breeders in each phase of the programme from the founding phase to the (potential) reintroduction, each clarified by various examples from recent literature. Special attention is given to group-managed populations, for which it is difficult to control mating and reconstruct pedigrees as individuals cannot be isolated for management.

Conservation breeding programmes

An increasing number of species are in danger of extinction because of loss of natural habitat, poaching or other, mainly anthropogenic, impacts (Frankham et al. 2010). Largely through conservation breeding programmes, zoos and aquaria have contributed to conservation by reintroduction to the wild, research, fund raising and raising public awareness (Frankham et al. 2010; Lacy 2012). A successful example is the captive European bison (*Bison bonasus*), at one time extinct in the wild, whose captive population grew from seven to 1800 individuals, of which many were successfully reintroduced (Tokarska et al. 2009).

For reintroduction to be successful, the wild population should become self-sustaining in the long term (Frankham et al. 2010). A sustainable population requires sufficient genetic diversity; less genetically diverse populations may suffer from inbreeding depression and reduced ability to adapt (Allendorf et al. 2010; Witzenberger and Hochkirch 2011). Since a decrease in population fitness can lead to reduced genetic diversity via demographic instability, this interaction can force a population into a downward spiral towards extinction (Frankham et al. 2010).

Unfortunately, captive conservation populations typically suffer from two limitations: they are small, and they are descended from few founders (Leberg and Firmin 2008), meaning there is relatively little genetic diversity to start with. Part of this diversity will be lost each generation because of random genetic drift, which is the dominant genetic process in small isolated populations.

Another problem is that the evolutionary force of selection can act on different traits and in different directions in captivity than it does in a species' natural habitat; e.g. individuals carrying alleles that confer more 'docile' behaviour may have relatively high fitness in captivity but low fitness in the wild. Adaption to captivity has already been observed after one generation within a steelhead (*Oncorhynchus mykiss*) hatchery; the most successful individuals in captivity were the least successful in the wild (Christie et al. 2012).

To be sustainable and meet conservation goals, captive populations require genetic management. An important part of this management is the breeding phase. Generally, this consists of selecting breeding pairs and exchanging animals with other institutions. Traditionally, management is based on studbooks. Pedigrees recorded in studbooks are used to calculate the relatedness between individuals and from these values each

Box 1. Genetic markers

Genetic markers are used to measure allelic variation at a given locus (Selkoe and Toonen 2006). Since funds for conservation programmes are very limited, it is desirable to keep analysis costs low (Bömcke et al. 2011; Witzenberger & Hochkirch, 2011). This can be done by selecting the appropriate number of markers and individuals to answer specific research questions; software such as SPOTG can aid in this process (Hoban et al. 2013a). More statistical power to estimate, for example, relatedness, is obtained when markers are used that measure more variable loci, that are evenly divided over the genome and not in linkage disequilibrium (Bomcke and Gengler 2009). Linkage disequilibrium is the process whereby two loci are more likely to be transmitted to offspring as a pair than other loci; for instance, with complete linkage they are just as informative as one locus (Selkoe and Toonen 2006).

The most commonly used markers measure microsatellites: tandem repeats of short DNA sequences found on the non-coding region of the genome. Alleles differ in size; repeat sequences are easily inserted or deleted due to 'slippage' in DNA replication causing microsatellite loci to be highly variable in length. Benefits are that the development of specific primers is relatively easy (Schoebel et al. 2013) and they provide high statistical power per locus (Witzenberger and Hochkirch 2011).

A single nucleotide polymorphism (SNP) is a single base-pair mutation (e.g. C→T) that can be detected with techniques including sequencing, allele-specific PCR or SNP chips (Frankham et al. 2010). The advantages of SNPs over microsatellites are that they can more easily amplify degraded DNA (e.g. museum specimens) due to their shorter target sequence (Allendorf et al. 2010) and they are more useful in parentage analysis when dealing with highly bottlenecked/inbred species, which often have low microsatellite heterozygosity, a common situation in conservation populations (Tokarska et al. 2009). A downside is that many more SNPs are required to acquire the same power as microsatellites.

Analysis of SNPs through chips is relatively inexpensive compared to microsatellites (Tokarska et al. 2009). However, equipment costs are high and development is more costly (Allendorf et al. 2010). Therefore, SNP chips are currently more attractive in species for which genotyping systems already exist but will otherwise take more effort than microsatellites (Frankham et al. 2010).

Next generation sequencing (NGS) allows sequencing of large sections of DNA, up to the entire genome. It may be possible to sequence the entire genome of a population within a reasonable time and budget in the near future (Allendorf et al. 2011). A weakness of NGS is that it is computationally very demanding due to the huge amount of data that is collected (Allendorf et al. 2010). For directly estimating relatedness, the added value of NGS is questionable, since the total sequence of DNA is no more informative than the number of non-linked SNPs it contains; a large number of markers can achieve the same accuracy (Jones and Wang 2010a). For the development of markers, though, it is very useful (Schoebel et al. 2013).

individual's mean kinship (MK) is calculated. MK is defined as the average coefficient of kinship in the population (Ballou & Foose 1995). Individuals are prioritized for breeding based on low MK; in this way founder representation is equalised and the risk of losing unique alleles due to genetic drift is minimised. Simultaneously, this strategy minimises the average increase in inbreeding.

A second type of information is becoming increasingly more accessible: molecular (DNA) information (Abdelkrim et al. 2009). Essentially, molecular markers (Box 1) can measure the specific alleles carried by an entity. This entity can be a chromosome, individual, population, species, etc. This allows for more subtle management through genetic comparisons. For instance, it can assess the relatedness of two individuals compared to the rest of the population. This review will focus on the use of molecular information within the framework of a conservation breeding programme, with emphasis on producing breeding recommendations. Insights gained from modern methods in commercial animal breeding are also discussed.

Estimating relatedness and improving/reconstructing pedigrees

Molecular data can be used to determine the identities of animals with unknown ancestry and correct errors in the studbook (Witzenberger and Hochkirch 2011). First of all, molecular data should be checked for genotype-errors (see supplementary material; www.jzar.org) and then compared with the known pedigree to determine inconsistencies. The program GENOTYPECHECKER assigns probabilities to these inconsistencies, allowing the user to decide whether this is caused by genotyping errors or incorrectly recorded pedigrees (Paterson and Law 2011). For example, analysis of 13 captive Parma wallabies (*Macropus parma*) shows that two dams had been wrongly recorded (Ivy et al. 2009).

Second, molecular data can be used to determine the relationships of individuals with unknown ancestry. Note that there is a difference between the relatedness of a pair and their relationship; a relationship is categorical (e.g. full siblings), while a relatedness value is continuous (e.g. 0.25). Of the multiple definitions of relatedness used in the literature, MKs are normally based on the coefficient of kinship: the probability that an allele chosen randomly in one individual is identical to the allele chosen randomly in another individual, and that these alleles are identical by descent (Malécot 1948). It is important to know which definition is used to prevent comparing the coefficient of kinship with, for instance, Wright's (1922) coefficient, which is twice as high.

Which relatedness estimator will perform best will differ according to the situation because estimator quality depends on many factors. Examples are the quality of genetic and other information, the actual relatedness between individuals and population structure (Blouin 2003; Wang 2011). The best performing estimator is said to be the one with the lowest sampling variance for the greatest number of relationship categories. For instance, all sibling relationships should be assigned similar relatedness values. The program COANCESTRY is able to select the best performing estimator for each data set based on simulations (Wang 2011). When estimating relatedness from molecular data, a challenge is to discriminate between alleles that are identical by descent (IBD) and those that are identical by state (IBS). IBS alleles will be identical because of homologous mutations or because there is no allelic variation on that locus. When IBS alleles are not corrected for, a positive value of relatedness will be produced whenever a pair contains one identical allele (Jones et al. 2002).

Relatedness estimators attempt to adjust for the alleles that are IBS and weigh alleles for the information they provide by using the available information on allele frequencies in the population (Oliehoek et al. 2006). On the extreme side, a locus is not used to estimate relatedness when no variation has been observed on it.

When relatedness is based exclusively on the allelic resemblance of two individuals, corrected for the known allele frequencies in the population, it is called a moment estimator. From this information the most likely relationship category for a pair (e.g. half-siblings) can be determined and with sufficient data the pedigree can be reconstructed. Note that estimates of relatedness/relationship and inbreeding are made through comparisons relative to others. Ideally these are guaranteed unrelated individuals (Witzenberger and Hochkirch 2011). Otherwise, these estimates are too optimistic in bottlenecked populations or inaccurate when there is little variation in the population (Henkel et al. 2012; Santure et al. 2010; see also limitations below under 'Breeding based on molecular information alone').

Often there is more information available than molecular data alone, such as putative relationships. Tests are developed to compare and combine these, and other available data such as social and geographical distance, with molecular data to determine the likelihood of a certain relationship category. These are known as maximum likelihood (ML) tests (Bink et al. 2008). By limiting the possible relations with, for example, incompatible ages, the statistical power of these tests increases (Ford et al. 2011). The statistical power of ML tests may also be increased by fitting the most logical pedigree for multiple individuals at once, preventing conflicting relationships. For multiple parenthoods this may be done using CERVUS (Kalinowski et al. 2007). The likelihood of an entire pedigree can also be determined as implemented in the software COLONY (Jones and Wang 2010b; Wang and Santure 2009). The power of likelihood tests increases in populations that are more related and contain more known or excluded relationships (Wang and Santure 2009).

In the Parma wallaby population discussed previously, the unknown pedigree of seven individuals was resolved using markers. Even for two individuals whose unknown ancestry remained unresolved, relatedness analysis provided useful information; they appeared to be related at the full sibling level, indicating that these lines will produce inbred pairings and are not genetically unique (Ivy et al. 2009).

In most pedigrees founder relationships will be missing. This often results in the assumption that founders are unrelated. Instead, molecular markers can be used to determine founder relatedness in retrospect by molecular analysis of their offspring (Ivy et al. 2009). Of course, better results are obtained if high quality DNA is still available from living founders or from preserved specimens in museums or zoo archives (recommended). The assumption of zero founder relatedness can be replaced by a relatedness estimate of one estimator or by developing a hypothetical pedigree. For the latter, the software MOL COANC is useful (Fernández and Toro 2006).

An interesting, robust method has been proposed for the breeding management of a highly inbred population of Mississippi sandhill cranes (*Grus canadensis pulla*) that uses multiple estimators of founder relatedness (Henkel et al. 2011). In this method, the studbook is first corrected for gaps and errors using DNA analysis. Second, three different studbooks are made in which only the values of founder relatedness differ: (1) founders are assumed unrelated; (2) founder relatedness is based on allelic similarity, which is the uncalibrated proportion of alleles shared; (3) founder relatedness is estimated with G&Q's moment-estimator (Goodnight & Queller 2002). From these studbooks, three different values of MK can also be produced for each individual. Breeding priority is then given to individuals for which all three MK values are lower than the average MK calculated for its respective studbook because all three values provide different information; zero founder relatedness MK gives a qualitative estimate of the part of the genome not measured by molecular markers, while allelic similarity MK indicates how rare the alleles carried by an individual are and breeding pairs are made based on similar Q&G's MK.

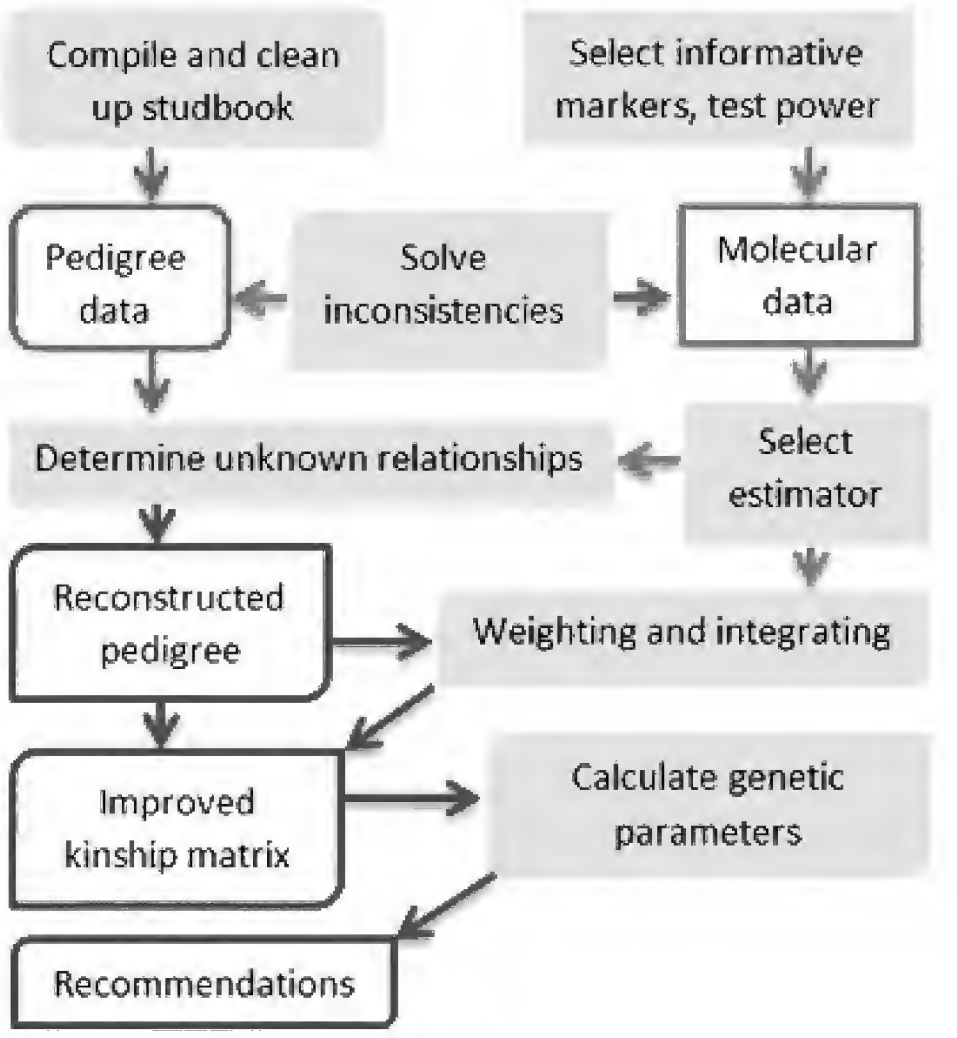

Figure 1. Pedigree reconstruction and integration with molecular data. Flowchart summarising each step from preparatory phase (top) to breeding recommendations.

Because the default assumption in breeding programmes is zero founder relatedness, the effect of knowing this value increases with increasing founder relatedness. When relatedness is very low, it will not affect management at all (Rudnick and Lacy 2008). A simulation of the captive Parma wallaby population shows that implementing the (low) founder relatedness in management hardly influences the genetic diversity maintained over 100 years (Ivy et al. 2009).

Breeding based on molecular information alone

A relatively complete pedigree may give better estimates of relatedness than molecular analysis using up to 100 microsatellite markers, even without taking into account biases caused by genotyping errors, mutations and sampling errors for allele frequencies (Baumung and Solkner 2003; Fernández et al. 2005). However, with the development of SNP-chips and NGS (see Box 1), the accuracy of molecular data has greatly improved (Allendorf et al. 2010). Furthermore, while pedigree-based relatedness assumes no variation in inheritance, in reality there is such variation due to random inheritance and linkage (Engelsma et al. 2011). For example, the relatedness between full siblings in a zebra-finch (*Taeniopygia guttata*) population, analysed with a genome-wide array of markers, had a standard error of 20% (Santure et al. 2010). In other words, for determining the relationship (e.g. full or half sibling) pairwise molecular data will often be inaccurate, but for determining relatedness (e.g. 27% of alleles identical by descent) molecular data can give more accuracy than pedigrees can achieve, on condition that the molecular data is sufficiently comprehensive (de Cara et al. 2011). Based on more accurate relatedness values, a greater amount of genetic diversity can theoretically be maintained (de Cara et al. 2011; Henkel et al. 2011). Whether marker-based management can also achieve lower

levels of inbreeding (depression), though, is still debated (de Cara et al. 2011; Santure et al. 2010; Townsend and Jamieson 2013). Molecular analysis allows for tracking specific alleles in the population and so can be used to prevent unique alleles from being lost from the population (de Cara et al. 2011; Jones et al. 2002). There is a risk associated with blindly equalising allele frequencies, however. Some alleles will be rare because they are deleterious and for some alleles the population's fitness will be optimal at a non-equal frequency (Charlesworth and Charlesworth 2012; Ivy and Lacy 2010). The chances of success of a reintroduced population will be highest with certain allele frequencies best adapted to that environment, such as that of the historical population (Miller et al. 2010). Molecular analysis and the method of Saura et al. (2008) allows the determination of the offspring each individual should leave to the next generation in order to maintain allele frequency distribution at each locus as close as possible to a certain distribution, while simultaneously maintaining acceptable levels of genetic diversity. However, even if historical frequencies can be retrieved, this method is hazardous: genetics in endangered populations are usually more subjected to genetic drift than selection and the habitat of a species is likely to have changed since their capture (Frankham et al. 2010). The safest strategy may therefore simply be to equalise allele-frequencies and so minimise the chance of losing alleles (Miller et al. 2010).

Integrating pedigree and molecular information

Pedigree and molecular relatedness are different types of information. Pedigrees give theoretical information on relatedness over the entire genome while molecular data give empirical evidence on specific parts of the genome. In other words, they give values of relatedness for different parts of the genome and information is lost when both sources are available but only one is used. When combining these two values it is important that the same definition of relatedness is used (preferably the coefficient of kinship; Malécot 1948). Also, if a combined value of pedigree and molecular relatedness is calculated as the average of the two, information will be lost due to unequal accuracy of the two coefficients. Instead, the two values can be weighted for the information that they provide (Bömcke and Gengler 2009; Bömcke et al. 2011; Fernández et al. 2012).

A relatively simple method is described by Fernández et al. (2012); pedigrees and molecular information are given relative weights of 10 and 1 respectively. The added power of molecular markers in this study is used to discriminate between two equally related mates, based on the pedigree (e.g. full siblings), of which one has a lower degree of molecular relatedness. The weight attributed to markers and pedigrees, however, can be made more fit to the situation; Bömcke and Gengler's (2009) method weights pedigree data by the depth of the pedigree (number of known generations and missing parentage in these generations), and weights molecular data by the number of unlinked markers and their polymorphism information content (PIC; useful software: CERVUS, Kalinowski et al. 2007). The PIC value is based on the number of polymorphisms of a certain marker and their distribution in the population. After weighting, the average of the molecular and pedigree coefficients is then the relatedness between two individuals and breeding pairs can be selected based on these values.

Bömcke et al. (2011) use a different approach: molecular markers only give relatedness information on the parts of the genome in linkage disequilibrium with the markers used, therefore molecular data is given a weight that is relative to the proportion of the genome that the markers measure. This proportion is determined through simulation as the power of a set of molecular markers to predict the pedigree. For the unmeasured part of the genome, (incomplete) pedigree data can be used to determine its theoretical relatedness. Again, simulations can be used to determine the power of an incomplete pedigree to predict the actual pedigree. These measures can then be combined into one value using these two weights. A method to fit in individuals for whom only pedigree data is available is also provided. When using these methods it is important to be aware that estimates will still in practice be based on one parameter if the quality of either pedigree or molecular data is relatively low (Bömcke et al. 2011; Bömcke and Gengler 2009). For new integration methods, it may be useful to weigh the relative value of molecular information using software KININFOR (Wang 2006), which uses four measurements of information value for each molecular marker. In addition, this software enables the statistical power of a relatedness analysis to be tested for. The program PMx may be used to determine population genetic parameters, and so produce breeding recommendations (Lacy et al. 2012). It provides the option to combine the pedigree-based relatedness matrix with a molecular (empirical) based matrix, using a chosen weight. A new relatedness matrix can also be inserted directly. For a summary of the steps described above for the use of molecular data for breeding recommendations, see Figure 1.

The methods proposed by Bömcke and Gengler (2009), Bömcke et al. (2011) and Fernández et al. (2012) for integrating molecular data with studbook data are superior over methods that simply reconstruct the pedigree because they use more of the available information. Nevertheless, further improvement may be possible in the future; management methods minimising the population's MK do not discriminate between adaptive variation and neutral variation (Marsden et al. 2013). Traditional molecular markers (e.g. microsatellites) measure neutral DNA directly, but can also measure coding DNA indirectly due to linkage disequilibrium. Novel methods such as NGS (see Box 1) make it possible to measure coding DNA specifically, which opens doors for research and possibly also for maintaining diversity at specific adaptive parts of the genome (Allendorf et al. 2010; Engelsma et al. 2011).

It is questionable, though, whether it is useful to develop methods focused solely on maintaining adaptive diversity. Assessing which adaptive variation is important for each species is likely to require an enormous amount of general and specific research (Witzenberger and Hochkirch 2011). Furthermore, if adaptive variation is evenly spread over the genome, it is much simpler to manage on MK. Nonetheless, there is evidence that genetic diversity is unevenly divided over the genome, so that it may be useful to use a strategy that conserves diversity at specific important regions where it is disappearing (Engelsma et al. 2012). This can be important for loci where variation has major adaptive effects. For example, individuals heterozygous for the major histocompatibility complex (MHC) genes are less susceptible to diseases than homozygous individuals (Hughes 1991). In addition, pairs with dissimilar MHC are thought to be more attracted to each other (Havlicek and Roberts 2009), which could potentially decrease the number of failed pairings in breeding programmes. Marsden et al. (2013) used molecular information on the MHC in combination with information on neutral diversity and the pedigree to evaluate and improve the breeding programme of the African wild dog (*Lycaon pictus*). Results show no significant difference between the neutral and MHC diversity maintained, indicating that the MHC is not under selection in captivity. The authors conclude that MK-based management performs well in the maintenance of MHC diversity.

Comparable to the identification of adaptive variation is the identification of deleterious alleles causing diseases (Maher et al. 2012). This information can be used for purging deleterious alleles from the population: deliberately selecting related breeding pairs to create inbred offspring. Since offspring with two (recessive)

deleterious alleles are less likely to survive, this will decrease its frequency. However, the effects of purging are unpredictable and will often cause the loss of valuable genetic diversity (de Cara et al. 2013; Leberg and Firmin 2008; Boakes et al. 2007). Even when specific carriers are known, excluding these from a small population is not thought to be worth the loss of genetic diversity (Allendorf et al. 2010). In addition, much is still unclear on how multiple deleterious alleles interact (Charlesworth and Willis 2009). Hence, purging is not recommended, even if individual molecular marker data are available (Witzenberger and Hochkirch 2011).

Hybridisation

Because founders for a population are often scarce it often occurs that individuals of different populations are used as founders (Frankham et al. 2010). This introduces the risk of crossing different species and so creating hybrids. Even though hybrids are undesirable, managers should not be overly cautious about including individuals in their breeding programme. This is discussed further in the section 'Conserving the species' below. In case of doubt, molecular analysis can assist in determining if founders are of the same species. In this way, for example, a Nile crocodile (*Crocodylus niloticus*) was discovered within the Philippine crocodile (*C. mindorensis*) breeding programme (Hauswaldt et al. 2013).

When a number of individuals in a population carries unwanted hybrid (exogenous) alleles, simply removing them from the breeding programme is not recommended because this will usually cause an undesirable amount of native diversity to be lost as well (Grobler et al. 2011). Instead, breeding with hybrids can be continued while artificially selecting against hybrid (exogenous) alleles. Native individuals can be given relatively higher breeding priority, based on either studbook or molecular information (Amador et al. 2011, 2012). In this method, markers outperform pedigrees if ten or more informative alleles are used that are private/diagnostic for the conserved species, or if more than 20 alleles are used with a much higher frequency in the conserved species (Amador et al. 2012).

To identify hybrids, genetic data must be available on guaranteed pure individuals. In populations with widespread hybridisation this can cause problems. Complementing genetic data with morphological data can be a solution, as currently done for the black wildebeest (*Connochaetes gnou*). Its wild population seems to mainly consist of hybrids with the blue wildebeest (*C. taurinus*) due to mismanagement in the past (Grobler et al. 2011). Breeding to obtain certain morphology can also be a conservation tool in some cases e.g. breeding-back of the auroch (*Bos primigenius*, van Vuure 2005). Techniques developed for livestock breeding can then be of use. Based on a combination of molecular and pedigree information, Fernández et al. (2012) achieved a 43% increase in frequency of a specific trait of interest while only losing 4% genetic diversity on the rest of the genome in an Iberian pig (*Sus scrofa domesticus*) Dorado strain. Any selection procedure in a conservation programme, however, needs careful consideration since levels of genetic diversity are usually already very low.

Group management

Optimal genetic management requires the availability of individual pedigrees, the ability to breed all individuals and control over breeding pairs. In reality, however, this is often not the case. A common restriction is that a captive population is held on several continents and as a result is managed as multiple sub-populations with limited migration. The programs PMx and METAPOP are able to manage these sub-populations separately, while aiming to maintain genetic diversity for the entire population (Lacy et al. 2012; Pérez-Figueroa et al. 2008). In the absence of shared pedigrees, molecular data can be used to determine the kinship between sub-populations.

For species held as multi-male, multi-female groups, breeding pairs cannot be controlled and deducing pedigrees is not feasible (e.g. shoals of fish). As a result, these populations are managed at the group level, instead of the individual level. Genetic management for these populations consists of artificially exchanging individuals between groups. The genetics of a group change each generation and this change is not visible to the human eye. Fortunately, molecular analysis makes it possible to determine the actual loss of diversity by genetic drift (expressed as the effective population size), identify selective forces and estimate the reproductive success of immigrants (Leus et al. 2011; Hasler et al. 2011; Wang 2004). In this way, relatedness between groups can be determined and individuals can be exchanged at random or, through the use of detailed tests, can even be selected on their genetic suitability (McGreevy et al. 2010; Miller et al. 2010).

When groups are large and fecundity is high, founder representation can be equalised by selecting a relatively small part of the population based on molecular kinship to produce the next generation. Software that can be used includes GENCONT and EVA (Berg & Nielsen 2006; Meeuwissen 2002).

When continuous molecular analysis in each generation is too costly, genetic research can facilitate the use of low-intensity management through models such as Wang's (2004) migration model, by providing knowledge of breeding behaviour and subpopulation structure (Smith 2010).

Conserving the species

At the time of reintroduction, a conservation breeding population ideally represents 95% of the natural gene-pool and is self-sustaining (Frankham 2009; Miller et al. 2010).

A breeding programme will not reach these goals if the founder population was inadequate in the first place. Miller et al.'s (2010) method uses molecular data to select a group of founders that are representative for a (sub)-species, including all its sub-populations. However, analyses of a large number of wild individuals are then required, which will often be unrealistic. It will also often be unrealistic and unnecessary to set up breeding programmes for a large number of subspecies (Frankham 2009; Hedrick and Fredrickson 2009). Instead, conservation actions may best be aimed at preserving a management unit that is both feasible and has the greatest conservation impact (Frankham 2009). If the choice is made to conserve only one sub-species, a combination of genetic and demographic data can be used to determine the extinction risk of subspecies and prioritise the need for a captive breeding programme (FAO 2010; note: developed for breeds of livestock).

If a choice is made to cross multiple populations, the risk of a depression of fitness (outbreeding depression) can be estimated from molecular and ecological information (see the decision tree of Frankham et al. 2011). It is important to note that significant genetic differentiation does not necessarily cause outbreeding depression and that the risk of outbreeding depression will often be low compared to the risk of inbreeding depression in endangered species (Frankham et al. 2011; FAO 2010).

Programme coordinators may be convinced that their programme has started with a large enough proportion of a species' genetic diversity and that their programme is adequately maintaining it. However, without qualitative evaluation of both the wild and captive population, this conclusion cannot be made (Witzenberger and Hochkirch 2011). While pedigrees are often missing for wild populations, samples for DNA analysis can easily

be extracted from hair and faeces (Oliveira and Gaiotto 2011). This has already led to a large number of captive breeding programme evaluations (Gonçalves da Silva et al. 2010; McGreevy et al. 2010; Shen et al. 2009; Tzika et al. 2008). In some studies this did not lead to any concerns (Gonçalves da Silva et al. 2010), but in others it became apparent that diversity levels were dangerously low and additional founders from the wild were required; three out of five groups of yellow-breasted capuchin monkeys (*Cebus xanthosternos*) had dangerously low diversity levels (Oliveira and Gaiotto 2011).

On the other hand, molecular analysis can also reveal a need for reintroduction due to low genetic diversity of the wild population. A way to improve the genetic health of both the wild and captive population is an 'open' system with continuous exchange between the two (Lacy 2012). Such an open system is used for the giant panda (*Ailuropoda melanoleuca*), where molecular data showed that both the captive and wild population were at risk without genetic exchange (Shen et al. 2009). In this case, molecular analysis may be used to construct a pedigree covering both the wild and captive population and individuals can be selected for exchange that optimises genetic diversity of the species as a whole (Allendorf et al. 2010).

DNA analysis in zoos and aquaria has so far been restricted by financial requirements and lack of expertise. Fortunately, these techniques are becoming increasingly simple and exponentially more affordable (Allendorf et al. 2010). Zoos and aquaria currently underestimate the interest of research institutes in collaboration. Geneticists can benefit from zoo studies through overlapping research questions and publications. Their research is often restricted by the number of DNA samples, while zoos and aquaria can provide these with relative ease, possibly combined with historical quantitative and medical data. Communication between breeding programme managers and geneticists is not only important in discovering opportunities on both sides, but also in preventing unforeseen restrictions after analysis (Hoban et al. 2013b). General guidance and case studies for the use of molecular markers in conservation breeding programs and related topics can be found on www.ConGRESSgenetics.eu and a summary of the methods and software described in this review is available as supplementary material (www.jzar.org).

Molecular information has huge conservation potential, but instead of blindly replacing all other information, it should be cleverly integrated into population management. This will facilitate intelligent and informed decisions, which can save money, resources and, most importantly, species.

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The use and integration of molecular DNA information in conservation breeding programmes: a review

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Supplementary information

Useful software (Table 1) and methods (Table 2) for the integration of molecular information into conservation breeding programmes. For additional guidance on the use of molecular markers in conservation breeding programmes and conservation in general, see www.ConGRESSgenetics.eu

Table 1. Summary of the software referred to in the review: the software name and a short description on how it is useful in integrating molecular data into captive breeding programmes. Websites where the software is freely available can be found by following the hyperlink attached to each program's name.

Software	Description
GenotypeChecker (Paterson & Law 2011)	Gives a transparent overview of inconsistencies between pedigree and molecular data and allows the user to determine which of the two is erroneous.
COANCESTRY (Wang 2011)	Simulates genotype data for selection of the best estimator out of seven in a particular situation. Estimates the (molecular) relatedness between individuals.
COLONY (Wang & Santure 2009)	Implements full likelihood methods to determine the most likely pedigree for a population based on molecular data.
MOL COANC (Fernández and Toro 2006)	Provides a hypothetical pedigree for a population's founders based on molecular information.
KIN INFOR (Wang 2006)	Estimates information value of molecular data based on four values for each marker, both for estimates of pairwise relationship and estimates of relatedness.
MULTIV (Pillar 1997)	Flexibly combines different types of data, such as qualitative and quantitative data.
LDSO (Ytournel et al. 2012)	Simulates the forces resulting in certain genotypes (genetic drift, mutation etc.) and resulting in certain phenotypes (dominance, epistasis etc.)
PMx (Lacy et al. 2012)	Analyses pedigrees and provides the option to use molecular relatedness values in the kinship matrix to determine individual, subpopulation and population genetic parameters and select breeding pairs.
METAPOP (Pérez-Figueroa et al. 2008)	Software that determines ideal breeding pairs within sub-populations and migrants between them based on a kinship matrix to optimise genetic diversity maintained in the meta-population. Restrictions on e.g. migration can be made.
Uncertain Paternity Coancestry (Pérez-Enciso 1995)	Analyses a part of the pedigree, also compatible with uncertain paternities.
GENCONT (Meeuwissen 2002)	Assists in selecting individuals to increase the frequency of a trait, while selecting a set of breeders with minimum coancestry to maintain genetic diversity. Freely available by request: jack.windig@wur.nl
EVA (Berg et al. 2006)	Assists in selecting individuals to increase the frequency of a trait, while selecting a set of breeders with minimum coancestry to maintain genetic diversity.

Table 2. Summary of the methods described in the review, ordered by their use in breeding programmes. In the left column, the data used for each method (with reference to a case study), and in the right column, a short description of how these data can be used in conservation breeding programmes.

Data used	Method
	Breeding to maintain genetic diversity, (based on relatedness)
Three DNA studbooks (Henkel et al. 2011)	Breeding based on MK which is lower than average in three different studbooks differing in estimator used for founder relatedness (no founder relatedness, estimated with Q&G and allelic similarity).
Combined molecular and pedigree data (Bomcke and Gengler 2009)	Provides one value of relatedness by combining molecular and pedigree data. Pedigree data is weighed by the depth of the pedigree and molecular data by the polymorphism information content (PIC) of the markers.
Combined molecular and pedigree data (Bömcke et al. 2011)	Provides one value of relatedness by combining molecular and pedigree data. Relatedness is based on molecular information for the part measured by markers and on the pedigree for the rest of the genome.
Molecular data (de Cara et al. 2011)	Using genome-wide molecular data to determine pairs to maintain genetic diversity and heterozygosity.
Molecular and pedigree data (Engelsma et al. 2011)	Maintaining diversity by selecting a relatively small number of individuals from a population with minimum coancestry. This selection can be used for a gene-bank but also to use these individuals as founders for the next generation when fecundity is very high (see also software Gencont and EVA). Both pedigree and molecular data can be used. Molecular data contributes most by revealing when certain important diversity is missing in the selection.
	Breeding to alter allele frequencies
Pedigree (Amador et al. 2011)	Prioritises individuals for breeding on the amount of native alleles they contain based on pedigrees. With decreasing number of animals allowed to breed, the proportion of exogenous alleles removed increases, but this is accompanied by a decrease in genetic diversity.
Molecular data (Amador et al. 2012)	Similar to above, but prioritisation based on molecular data instead of pedigrees. Efficiency increases if the alleles selected for are exclusive to the native species.
Molecular data (Saura et al. 2008)	Method to determine the number of offspring from each individual needed to achieve or maintain a certain balance of allele frequencies.
	Breeding to alter quantitative traits
Combination of pedigree and molecular data (De los Campos et al. 2009)	Predicting quantitative traits in the next generation based on molecular and pedigree data separately and combined.
Combination of pedigree, morphological and molecular data (Fernández et al. 2012)	Combined strategy to increase the frequency of a qualitative trait based on morphological data while maintaining genetic diversity based on molecular and pedigree data.
Combining pedigree, molecular and performance data (Hasler et al. 2011)	Commercial method to breed for performance, while maintaining genetic diversity based on pedigree and molecular data. Also estimates genetic drift from molecular data.
	Evaluation of the breeding programme
Pedigree and neutral and adaptive molecular data	Evaluation of breeding programme based on pedigree data and both adaptive and neutral molecular data. Used to determine whether certain (MHC) genes

(Marsden et al. 2013)	were selected for, possibly because of adaption to captivity, and to produce a management plan.
Molecular data (McGreevy et al. 2010)	Evaluating the ability of a captive population to maintain genetic diversity by comparing with the wild population through molecular analysis.
	Other
Molecular data (Miller et al. 2010)	**Selection of founders or individuals for reintroduction:** Technique to determine a set of animals best fitting a certain genetic profile based on molecular data. This set of animals can be founders that capture the diversity of the wild population or animals fit for reintroduction.
Pedigree, demographic and molecular data (Boettcher et al. 2010)	**Prioritisation of subspecies:** Guidelines of the FAO to prioritise endangered livestock breeds based on demographic and genetic stability and uniqueness of the breed. Principles also apply to (sub)species.
Combed morphological and molecular data (Benin et al. 2012)	**Genetic distance between populations and sub-species:** Using a combined value of molecular and morphological data to determine genetic distance. Developed for strains of wheat to determine combining abilities between different lines; can potentially assist in genetic rescue.
Group-pedigree and demographic data (Wang 2004)	**Group genetic management:** Determining immigration rates in group-managed species based on population size, historical immigration rates and founder number.

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Research article

Changes in microbial diversity associated with two coral species recovering from a stressed state in a public aquarium system

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Abstract

Coral diseases are a major factor in the decline of coral reefs worldwide, and a large proportion of studies focusing on disease causation use aquaria to control variables that affect disease occurrence and development. Public aquaria can therefore provide an invaluable resource to study the factors contributing to health and disease. In November 2010 the corals within the main display tank at the Horniman Museum and Gardens, London, UK, underwent a severe stress event due to reduced water quality, which resulted in death of a large number of coral colonies. Three separate colonies of two species of reef coral, *Seritopora hystrix* and *Montipora capricornis* showing signs of stress and acute tissue loss were removed from the display tank and placed in a research tank with improved water quality. Both coral species showed a significant difference in 16S rRNA gene bacterial diversity between healthy and stressed states (*S. hystrix*; ANOSIM, R=0.44, p=0.02 and *M. capricornis*; ANOSIM, R=0.33, p=0.01), and between the stressed state and the recovering corals. After four months the bacterial communities had returned to a similar state to that seen in healthy corals of the same species. The bacterial communities associated with the two coral species were distinct, despite them being reared under identical environmental conditions. Despite the environmental perturbation being identical different visual signs were seen in each species and distinctly different bacterial communities associated with the stressed state occurred within them. Recovery of the visually healthy state was associated with a return of the bacterial community, within two months, to the pre-disturbance state. These observations suggest that coral-associated microbial communities are remarkably resilient and return to a very similar stable state following disturbance.

Introduction

All coral species have a diverse community of microorganisms living within healthy tissues, the skeleton and the surface mucus layer. These bacterial communities have been shown to differ among and within species within healthy corals (Rohwer et al. 2002; Rosenberg et al. 2007). The physiochemical properties of the coral microhabitats (skeleton, tissues and mucus) appear to have the ability to select certain species from environmental pools, allowing some to grow and develop and eliminating others that initially colonise (Sweet et al. 2011a). Furthermore, certain properties of the coral or its healthy microbial associates, such as antimicrobial activity, have been shown to have a significant effect on the microbial communities within the holobiont (Ritchie and Smith 1995; Kooperman et al. 2007; Sharon and Rosenberg 2008). These microbial communities have been shown to vary between healthy and diseased or stressed tissues (Frias-Lopez et al. 2003; Pantos et al. 2003; Jokiel and Coles,2004; Williams and Miller 2005; Gil-Agudelo et al. 2007; Mydlarz et al. 2009). Specifically bacterial communities have been shown to change in advance of the visible disease signs, highlighting the importance of microbial communities and their role in coral disease (Pantos et al. 2003; Croquer et al. 2012).

Corals have a variety of mechanisms for defense against invasive pathogens, including a physical barrier protecting the epithelium in the form of the surface mucus layer (reviewed by (Brown and Bythell 2005; Bythell and Wild 2011), and the pro-duction of antimicrobial compounds by the host and/or other microbial associates (Ritchie 2006). This latter process is thought to be mediated to a large extent by the coral's natural microbial community, with over 20–30% of bacterial isolates in healthy corals possessing antibiotic activity (Gunthorpe and Cameron 1990; Kim 1994; Castillo et al. 2001; Rohwer and Kelley 2004; Ritchie 2006; Geffen et al. 2009; Rypien et al. 2010). If the bac-terial community associated with the coral is the primary source of this defense (via antibiotic production), then a disturbance of the healthy coral microbiota may allow opportunistic infection and the onset of specific disease signs

(Lesser et al. 2007a). Sweet et al. (2011b) and Garren et al. (2009) showed that ma-nipulation of the natural microbiota in healthy corals (through experimental manipulation and transplantation respectively), allowed opportunistic, potentially pathogenic bacteria to colo-nize the tissue. However, interestingly in both cases, the healthy bacterial community reverted to the natural state after the stress event had subsided and there were no visual signs of stress or disease in either of these manipulations. These results suggest that other factors must be at play to cause the onset of disease other than the availability of potential pathogens or the opening of available niches due to disturbance of the natural associated microbial communities and loss of potentially probiotic strains.

Several studies have shown a general trend towards *Vibrio* domi-nation under stressful conditions (Kushmaro et al. 1997; Cervino et al. 2008; Luna et al. 2010) and therefore it may be expected that corals exposed to identical environmental perturbations may become more similar with their microbial communities. To date few studies have been able to monitor bacterial communities associated with corals recovering from symptoms of stress in controlled environments. In this study we were able to opportunistically sample the bacterial community associated with two species of coral as they recovered from a serious stress event over a period of four months in a public aquarium.

Methods

Sample collection

Corals at the Horniman Museum and Gardens public aquarium underwent a severe stress event in 2010. In total all nine species of scleractinian coral within the display where affected, with mortality ranging from 100% (in species such as *Hydnophora rigida*) to approximately 5% (in *Acropora formosa*). This trend was observed in multiple tanks within the system ruling out a single tank effect. Three colonies of two different coral species, *Seriatopora hystrix* and *Montipora capricornis* which were showing visual signs of stress and subsequently developed lesions were followed over four months and sampled throughout this time period. The three colonies of each species were originally from a single genotype, generated via asexual fragmentation. *S. hystrix* colonies showed signs of acute tissue loss whilst *M. capricornis* showed signs of bleaching and acute tissue loss (Fig 1). Stressed corals were removed from the display tank and placed in an experimental research aquarium for the period of the experiment. Here the water parameters were optimised and the corals recovered and began to regrow over a period of four months. Three approximately 1 cm^2 coral fragments were taken from the disease lesion from each colony initially, and once a month the same size samples were taken at the same part of the coral, where tissue was shown to be recovering and/or regrowing. It must be noted that there were no samples collected for *M. capricornis* at Month 1. Three healthy samples of a coral not affected by the disease were also taken at the start of the experiment for comparison. Each coral was photographed before removal from the aquarium and the subsequent sample taken. These samples were all then placed in sterile 50 ml falcon tubes and stored in 100% EtOH at -20° C until further analysis. Samples were then centrifuged at 13,000 g for 20 min to concentrate the tissue slurry, 1000 µl of which was subsequently used for DNA extraction using QIAGEN DNeasy Blood and Tissue kits with an added step to concentrate the lysate using a vacuum centrifuge for 2 h at 24° C.

Bacterial diversity, DNA extraction, amplification and DGGE analysis

Bacterial partial 16S rRNA gene fragments were amplified using standard prokaryotic primers (357F) (5´-CCTACGGGAGGCAGCAG-3´) and (518R) (5'-ATTACCGCGGCTGCTGG-3'). The GC-rich sequence 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA GCA CGG GGG G-3' was incorporated in the forward primer 357 at its 5' end to prevent complete disassociation of the DNA fragments during DGGE. Thirty PCR cycles were performed at 94°C for 30 seconds, 53° C for 30 seconds and 72° C for 1 min and a final extension at 72° C for 10 min (Sanchez et al. 2007). Three independent 10 µl PCR reactions were used, each containing 1.5 mM $MgCl_2$, 0.2 mM dNTP (PROMEGA), bovine serum albumin (BSA, 400 ng/µl), 0.5 µM of each primer, 2.5 U of Taq DNA polymerase (QBiogene), incubation buffer, and 20 ng of template DNA (Siboni et al. 2007). These replicate PCR's for each sample were then combined and cleaned using QIAGEN QIAquick PCR purification kits, reducing the final volume to 15 µl in Sigma molecular grade H_2O. All reactions were performed using a Hybraid PCR Express thermal cycler. PCR products were verified by agarose gel electrophoresis (1.6% (w/v) agarose) with ethidium bromide staining and visualized using a UV transilluminator.

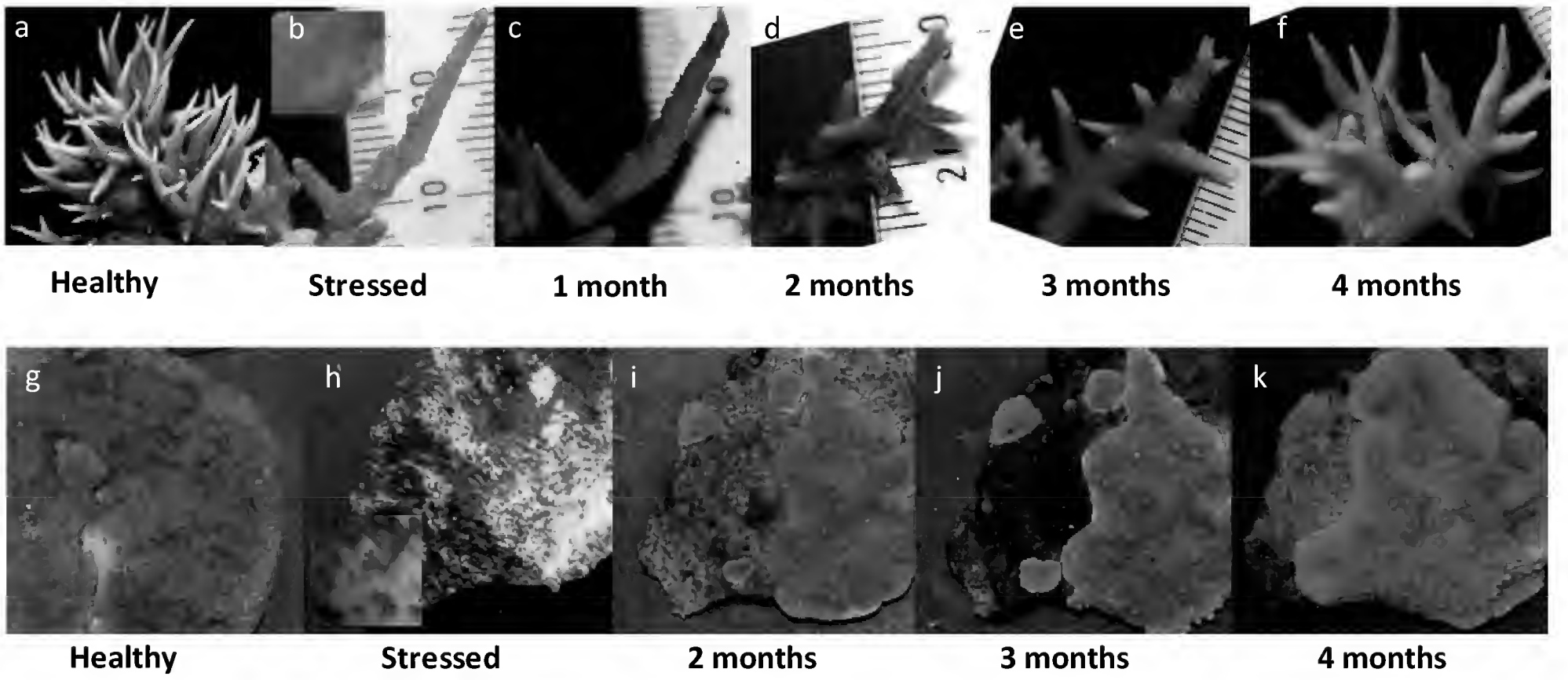

Figure 1. Corals showing signs of stress and subsequent recovery; *Seriatopora hystrix* (a-e), *Montipora capricornis* (f-k). The remaining live tissue in *M. capricornis* was severely bleached (white patches in stressed state, h).

DGGE was performed using the D-Code universal mutation detection system (Bio-Rad). Bacterial PCR products were resolved on 10% (w/v) polyacrylamide gels that contained a 30–60% denaturant gradient for 13 h at 60° C and a constant voltage of 50 V. Gels were stained with a concentrated solution of 9 µl Sybr® Gold (Sigma) in 50 µl of 1X TAE poured directly onto the gel surface, covered and left in the dark for 20 min then further washed in 500 ml 1X TAE for 30 min and visualised using a UV transilluminator. Bacterial operational taxonomic units (OTUs), were defined from DGGE band-matching analysis using Bionumerics 3.5 (Applied Maths BVBA) following methods described by (Guppy and Bythell 2006). Standard internal marker lanes were used to allow for gel-to-gel comparisons. Tolerance and optimisation for band-matching was set at 1%. OTUs of interest (those which explained the greatest differences/similarities between samples), were identified by sequence analysis. Bands were excised from DGGE gels, left overnight in Sigma molecular grade water, vacuum centrifuged, re-amplified with the appropriate primer set, labelled using Big Dye (Applied Biosystems) transformation sequence kits and sent to Gene-vision (Newcastle University, UK) for sequencing.

Total bacterial abundance

To estimate bacterial abundance, three filters per time period were sampled, similar to that for microbial analysis. 1000 µl of tissue slurry was collected, lyophilised to remove the ethanol and weighed to standardise the amount of tissue sampled between replicates. 100mg of lyophilised tissue extract was used for each sample to account for varying amounts of tissue remaining on the samples at time of collection. This was then resuspended in sterile filtered sea water and filtered through a 0.22 µm black polycarbonate filter and fixed with 100 µl of paraformaldehyde until analysis (Fuhrman et al. 2008). The filters were then stained with 100 µl DAPI solution (final concentration 5 µg/ml) for 10 mins rinsed with Phosphate Buffer Solution (Yu et al. 1995; Weinbauer et al. 1998; Yamaguchi et al. 2007), and viewed under epifluorescence microscopy using a DAPI-specific filter set. For each filter, 50 fields of view (FOV) were taken at X1000 magnification. These were then scaled up to the total area of the filter and calculated to give total bacterial abundance per cm_3 of stressed tissue. Total amount of stressed tissue rather than complete coral nubbin surface area was used to account for the varying amount of tissue on the stressed samples as this could not be standardised at time of collection. All images were analysed using an automatic cell counter (Cell C; Selinummi *et al.* 2005). The parameters were set to exclude any objects smaller than 0.0314 μm^2 and anything larger than 0.7 μm^2. Counts of three tissue sub-samples were taken from each coral and averaged to provide a cell density per sample.

Antimicrobial assays from aqueous coral extracts

Bacterial inhibition growth was determined from aqueous extracts of each sample similar to that reported by Mydlarz et al. (2009). For this, the ethanol used to preserve the coral fragments was transferred into new pre-weighted falcon tubes and these were lyophilized to determine the amount of coral extracts in each sample (weighed and standardised as above). Samples were then resuspended in 100% ethanol to reach a standard concentration of 100 mg/ml as a primary stock solution for all samples. This stock extract was further diluted to 3 mg/ml in 0.1 M phosphate buffer, pH 7.8 following the protocol by Mydlarz et al. (2009). In a 96-well microtitre plate, 10 µl of each extract were added to 105 µl of marine broth and 15 µl of bacteria culture. Positive controls using 0.05 mg/ml of tetracycline were utilised along with negative controls using 100% EtOH. The kinetic of bacterial growth was determined by reading the OD every 5 min for 24 h, in a Biotek power wave HT plate reader. The rate of bacterial growth during exponential phase was calculated by plotting the OD against time giving a growth curve. The gradient was then calculated from the linear part of the growth curve, all experimental samples could then be compared to the growth rate of the control sample (ethanol control), giving the relative growth rate.

To obtain the pure cultures of a bacterial pathogen known to affect corals, crushed coral sand from an aquarium where the corals had recently died from an outbreak of coral disease were suspended in filtered sea water and spread onto Thiosulfate Citrate Bile Sucrose (TCBS) Agar media in duplicates. Plates were incubated at 28° C for 24 h. Resulting bacterial colonies were isolated based on colony morphology, size, and shape. These were then picked and spread on individual plates to produce pure colonies. Representatives of each plate were sequenced using universal bacterial primers pA and pH. Gen-Bank BLAST searches of the 16S rRNA gene sequences were performed to determine the percentage of isolate relatedness to known bacteria. Each isolate was stored at -80°C in cryovials containing 30% glycerol and 70% TCBS media. Only one pure culture showed similarities to any known coral pathogen referenced in the literature and this was a ribotype with 100% match to *Vibrio harveyi*. An aliquot of the pure freezer stock from this isolate was streaked on TCBS agar and incubated at 26° C for 24 h. A single distinct colony was removed, put into sterilised marine broth and incubated again in a shaker at 26° C for 24 h. To standardise the bacterial cell density in this assay, the culture was adjusted to an optical density of 0.2 at 600 nm (5 X 107 cells/ml) using a spectrophotometer in a Biotek power wave HT plate reader (Mydlarz et al. 2009).

Water quality

The display and research tanks were monitored before, during and after the stress event, on a weekly basis. Parameters such as NH_3, NO_2, NO_3, (Hach DR890 colorimeter) PO_4 (D&D – The Aquarium Solutions High sensitivity test kit) Ca, Mg and Alkalinity (Salifert) were monitored. pH was monitored using a HQ11d Portable pH/ORP Meter with IntelliCAL™ PHC101 Standard Gel Filled pH Electrode.

Table 1. Tests of metals and other elements present within the water systems at the Horniman Museum and Gardens Aquarium. (i) Analysed by Cheshire Scientific, (ii) analysed by Horniman Museum and Gardens.*Copper readings can be masked by other elements such as aluminium, magnesium, iron and calcium. This problem with readings can be overcome using a calibration buffer (CuVer 2 Copper Reagent) these readings when done in house using a Hach DR/890 Colorimeter reported a higher amount of copper within the system equaling 0.11 mg/l in the display tank.

Test	Display tank	Research tank
i - Copper (mg/l)	0.01*	<0.01
i - Aluminum (mg/l)	<0.02	<0.02
i - Chromium (µg/l)	14	11
i - Selenium (µg/l)	2.8	0.8
i - Mercury (mg/l)	<0.01	<0.01
i - Manganese (mg/l)	<0.01	<0.01
i - Strontium (mg/l)	5.2	5.6
ii - NO3 (mg/l)	10.18	2.9
ii - PO4 (mg/l)	0.06	0.039
ii - Ca (mg/l)	473.61	420.55
ii - Alkalinity (dHK)	9.19	9.56
ii - Magnesium (mg/l)	1378	1310
ii - pH	8.03	8.16

Statistical analysis

Analysis of Similarity (ANOSIM) tests based on Bray-Curtis similarities (Clarke and Warwick, 2001) were used to test for differences in the bacterial 16S rRNA gene DGGE profiles associated with stressed and healthy corals. A non-metric multidimensional scaling (MDS) analysis was used to represent each sample type on a 2-D plot (Clarke and Warwick 2001). An analysis of contribution to similarities (SIMPER) was performed to determine which 16S rRNA gene OTUs best explained dissimilarities among sample types that were statistically different, these were then excised from the DGGE and sequenced to give the closest match according to the BLAST database. The abundance of bacteria (total bacterial counts) was compared between healthy and stressed samples. For *Montipora capricornis*, data met the assumptions of normality and equality of variances and a one way ANOVA was used. However for *Seriatopora hystrix*, Levenes test for homogeneity showed heterogeneity of variances and therefore variances were not equal ($F_{4.10} = 4.020$, $p = 0.034$). Therefore the nonparametric test Kruskal-Wallis was used.

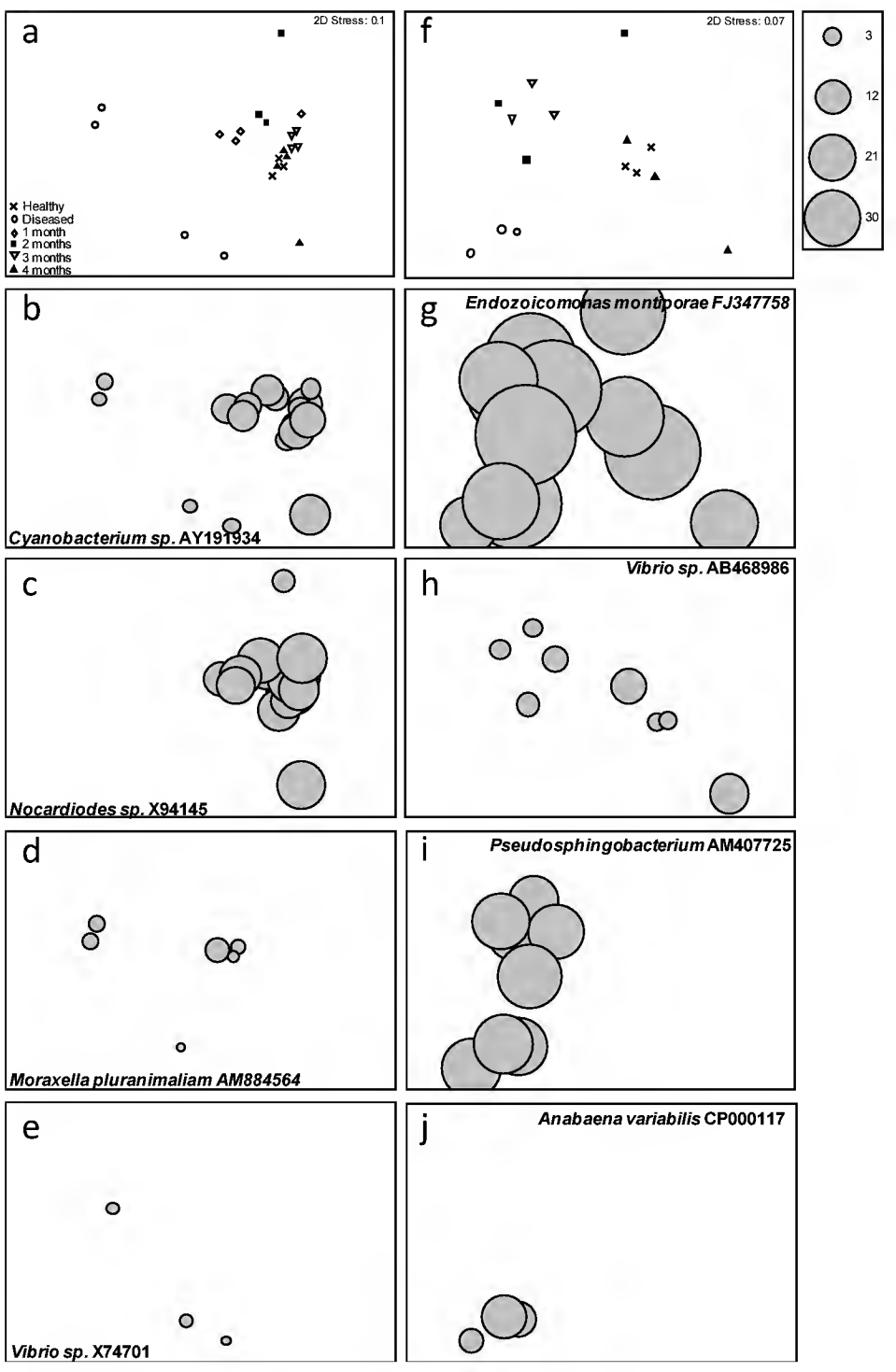

Figure 2. (a) Multidimensional scaling (MDS) plot showing changes in bacterial communities from n = 3 healthy, n = 4 stressed state and n = 3 recovering (4 different time periods) *Seriatopora hystrix* colonies; (b-e) show 16S rRNA gene bacterial ribotypes that caused greatest similarities or differences between sample types (closest relative and best match Table 3) (b) *Cyanobacterium* sp. AY191934 (c) *Nocardiodes* sp. X94145 (d) *Moraxella pluranimaliam* AM884564 and (e) *Vibrio* sp. X74701. (f) MDS plot showing changes in bacterial communities from n = 3 healthy, n = 3 stressed state and n = 3 recovering (4 different time periods) of *Montipora capricornis* colonies (g-j), show 16S rRNA gene bacterial ribotypes that caused greatest similarities or differences between sample types (closest relative and best match Table 2) (g) E*ndozoicomonas montiporae* FJ347758 (h) Vibrio sp. AB468986 (i) *Pseudosphingobacterium* AM407725 (j) *Anabaena variabilis* CP000117. Bubble size represents relative density of denaturing gradient gel electrophore-sis (DGGE) band of that particular sequence within individual samples. There were no samples collected for *M. capricornis* at Month 1.

Results

Water quality

Water quality in the main display tank was below optimal levels prescribed for coral husbandry in aquariums and zoos, with higher than normal readings of various metals and non-metals including copper, iron, aluminium and selenium (Table 1). In contrast, overall water quality was of a higher standard within the research tank behind the scenes. For example; levels of nitrate (NO_3) and phosphate (PO_4) were higher in the display tank compared to the research tank; NO_3; 10.2 mg/l and 2.9 mg/l; PO_4; 0.06 mg/l and 0.039 mg/l respectively. Conversely, pH values were lower in the display tank compared to the research tank; 8.03 and 8.16 respectively (Table 1).

There was a significant difference in 16S rRNA gene bacterial diversity (ANOSIM, R = 0.64, p = 0.001) between healthy coral colonies of *Seriatopora* hystrix and the healthy colonies of *Montipora capricornis*. Although there was a shift in bacterial diversity between healthy and stressed states, a significant difference in 16S rRNA gene bacterial diversity remained between the two species in this stressed state (ANOSIM, R = 0.56, p = 0.001).

Seriatopora hystrix

Bacterial communities were diverse in all samples of *S. hystrix*, with pairwise comparisons showing 16S rRNA gene bacterial diversity being significantly different between stressed and healthy corals and between the stressed corals and all the recovering time periods (ANOSIM, R > 0.44, p < 0.02) (Fig. 2a). However, there was no significant difference in 16S rRNA gene bacterial diversity between the communities associated with healthy *S. hystrix* and those in recovering colonies after four months (ANOSIM, R = 0. 57, p = 0.12). Two bacterial ribotypes dominated the community profile in the DGGE, these included a ribotype similar to a known nitrogen fixing *Cyanobacterium* sp. (AY191934) (Fig. 2b; Table 2) and a *Nocardioides* sp. (X94145) (Fig. 2c; Table 2). The *Cyanobacterium* sp. didn't vary between states and remained a dominant ribotype throughout, however interestingly the *Nocariodides* sp. was completely absent from the DGGE profile in stressed samples and only detectable in healthy corals and those recovering from the disease. Only three species of bacterium were detected in stressed corals and absent in healthy corals, these includes ribtoypes similar to previously identified potential pathogens from the genus's *Moraxella* (NR042666) (Fig. 2d), *Vibrio* (X74701) (Fig. 2e), and *Cyanobacterium* (EU780252) (Table 2).

Bacterial abundance

Although bacterial abundance did not increase significantly in stressed corals compared to healthy corals (Kruskal Wallis $\chi^2_{(4)} = 1.433$, $p = 0.838$). The average bacterial abundance increased in healthy and stressed corals (from $1.7 \times 10^7 \pm 0.76$ cells/cm_3 to $3.4 \times 10^7 \pm 0.15$ cells/cm_3 respectively). Over four months this abundance gradually reduced back to healthy levels (Fig. 3). After an initial drop in abundance after one month, it began to rise again in month 2 but remained below the total abundance of the stressed samples.

Antimicrobial activity

All coral tissues showed significant antimicrobial activity ($F_{6, 33} = 5.665$, $p < 0.008$), suppressing growth of the tested bacterium

Table 2. Heatmap derived from relative 16S rRNA gene DGGE operation taxonomic units (colors arranged by 0, 0.1-1.0, 1.1-2.0, 2.1-3.0, 3.1-4.0, 4.1-5.0, 5+), for *Seriatopora hystrix*. Replicate samples (n = 3 or 4 per time period) were combined to give an overall average for clarity within the display. The larger the number under each sample column the stronger the DGGE band intensity for that specific 16S rRNA gene ribotype.

Closest relative	Accession no.	GenBank	Class	Healthy	Stressed	1 month	2 months	3 months	4 months
Nocardioides sp.	X94145	98%	Actinobacteria	5.43	0	6.3	5.99	6.61	5.37
Unknown	NA	NA	NA	0.67	3.81	0	0	0	0.56
Desulfurococcus sp.	AY264344	100%	Archaea	1.21	3.33	2.7	4.46	0.3	1.05
Unknown	NA	NA	NA	0.62	2.53	0.73	1.26	0	0.32
Lactobacillus sp.	AB326350	99%	Firmicutes	2.61	0	3.5	1.16	2.56	2.41
Coral DNA	NA	NA	NA	2.54	0.52	0	0	1.21	2.7
Coral DNA	NA	NA	NA	2.67	0	0	1.17	2.22	2.27
Moraxella sp.	NR042666	100%	Gammaproteobacteria	0	2	1.84	0	0	0
Actinobacillus sp.	AY362899	98%	Gammaproteobacteria	1.56	1.71	0.63	0.34	1.64	1.09
Methanococcus sp.	DQ195164	96%	Archaea	1.12	0	0	0	0	1.4
Prochlorococcus sp.	BX548174	98%	Cyanobacterium	1.09	0.38	0.83	0	0.63	1.49
Pyrobaculum sp.	AJ277124	96%	Archaea	1.67	0.37	0	0.34	0.95	1.4
Methanocaldococcus sp.	AF547621	98%	Archaea	2.15	1.65	2.12	1.51	2.61	2.31
Ascoparia sp.	FR837678	100%	Ascopariidae - flat worm	0.98	1.07	0	0	0	0.44
Cyanobacterium sp.	EU780252	100%	cyanobacterium	0	1.11	0	0	0	0
Pseudomonas sp.	AM293567	98%	Gammaproteobacteria	0.88	0.65	1.61	2.13	2.19	0.76
Flagellimonas sp.	DQ191180	98%	Flavobacteriia	1.33	0.94	0.46	0.34	0.97	1.35
Cyanobacterium	AY191934	100%	Cyanobacterium	4.08	2.93	4.06	2.38	4.05	3.74
Vibrio sp.	X74701	98%	Gammaproteobacteria	0	0.94	0	0	0	0
Unknown	NA	NA	NA	0	0	0	2.37	1.81	0
Unknown	NA	NA	NA	0.86	0	0	3.16	1.25	0.76

Vibrio harveyi when compared to the EtOH control (Fig. 4). There was no significant difference (p = 1.0) in antimicrobial activity between stressed tissues and those in healthy corals. Interestingly, growth rate of *V. harveyi* was higher in some instances during the recovering periods than in the stressed coral, despite visual coral health appearing to return to normal and tissues beginning to re-grow (Fig. 1 d, e). Growth rate after 4 months was the same as that in healthy tissues (Fig. 4).

Montipora capricornis

Bacterial communities were again diverse throughout all samples (Table 3). Furthermore, there was a significant difference between time periods (ANOSIM, R = 0.707, p = 0.001), with pairwise comparisons showing that the 16S rRNA gene bacterial diversity remained significantly different between stressed corals and healthy corals and between the stressed corals and all the recovering time periods (ANOSIM, R > 0.33, p < 0.01) (Fig. 2f). There was again no significant difference in 16S rRNA gene bacterial diversity between the communities associated with healthy *M. capricornis* and those in recovering corals after a period of 4 months (ANOSIM, R = 0. 78, p = 0.10) (Fig. 2f). Bacterial diversity was higher overall in *M. capricornis* corals compared to *S. hystrix* (Table 2 and 3). Eight ribotypes were dominant in relative 16S rRNA gene abundance throughout all sample points including the coral symbiotic bacterium *Endozoicomonas* sp. (FJ347758) (Fig. 2g; Table 3), two potential coral pathogens from the genus *Thalassomonas* (AY194066 and AY643537), and an opportunistic pathogen from the genus *Bacteroidetes* (GQ204958). Similarly to *S. hystrix,* nitrogen fixing bacteria were also present from the genus *Paenibacillus* (X60625), along with a *Amycolatopsis* sp. (AJ293757) known to produce a weak antibiotic and a *Sphingomonas sp.* (JQ027711) a strong biodegrading species of bacteria (Table 3). A further eight ribotypes were only detected in stressed samples including; the nitrogen fixing filamentous cyanobacterium *Anabaena* (CP000117) (Fig. 2j) and a nitrite reducing *Paracoccus sp.* (HQ538757), two sulphur reducing bacterium, *Deferribacteres sp.* (GQ204938) and *Sulfurisphaera sp.* (D85507), and a potentially pathogenic Vibrio sp. (AB470931). Similar to *S. hystrix,* certain ribotypes were detected in healthy tissues, absent in the stressed lesion and present in the subsequent recovering samples (Table 3). These included ribotypes related to; a bacterium previously associated with marine macroalgae *Kiloniella sp.* (AM749667), a bacterium capable of degrading organic matter from the genus *Actinomyces* (AM084228), and a further *Vibrio sp.* (AB468986) (Fig 2h).

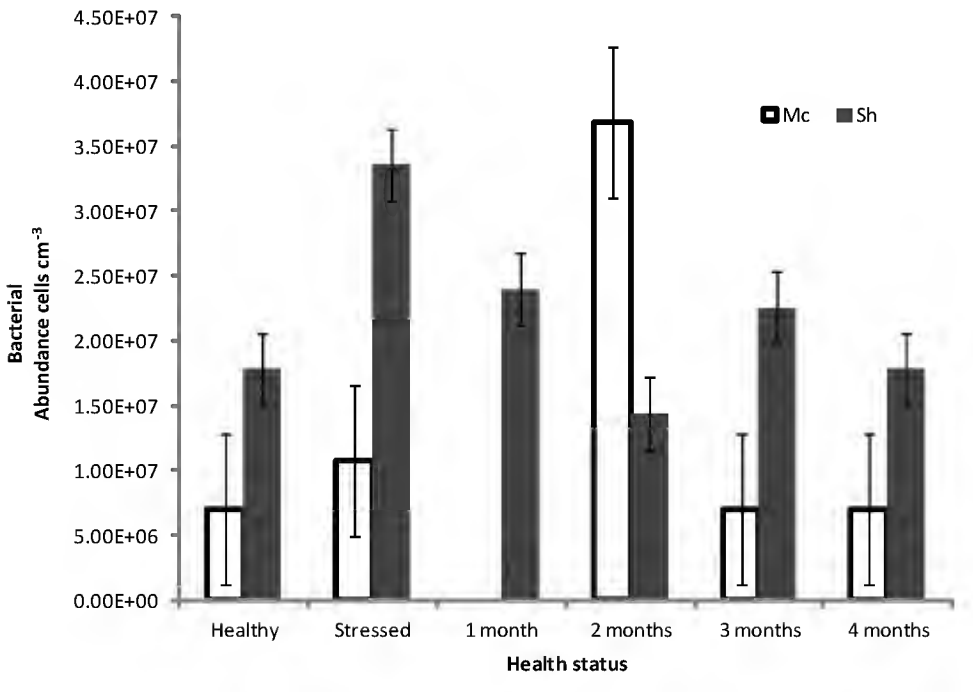

Figure 3. Mean bacterial abundance (cm-3) of n = 3 replicates of filtered coral tissue taken at all time periods. Mc = *Montipora capricornis*; Sh = *Seriatopora hystrix*. Error bars show standard error from collective mean. There were no samples collected for *Montipora capricornis* at Month 1.

Bacterial abundance

Bacterial abundance increased in stressed corals compared to healthy corals similarly to that seen in *S. hystrix*, however the increase again was not significantly different (ANOVA F = 2.259, p = 0.186). Total bacteria abundance in healthy corals was lower

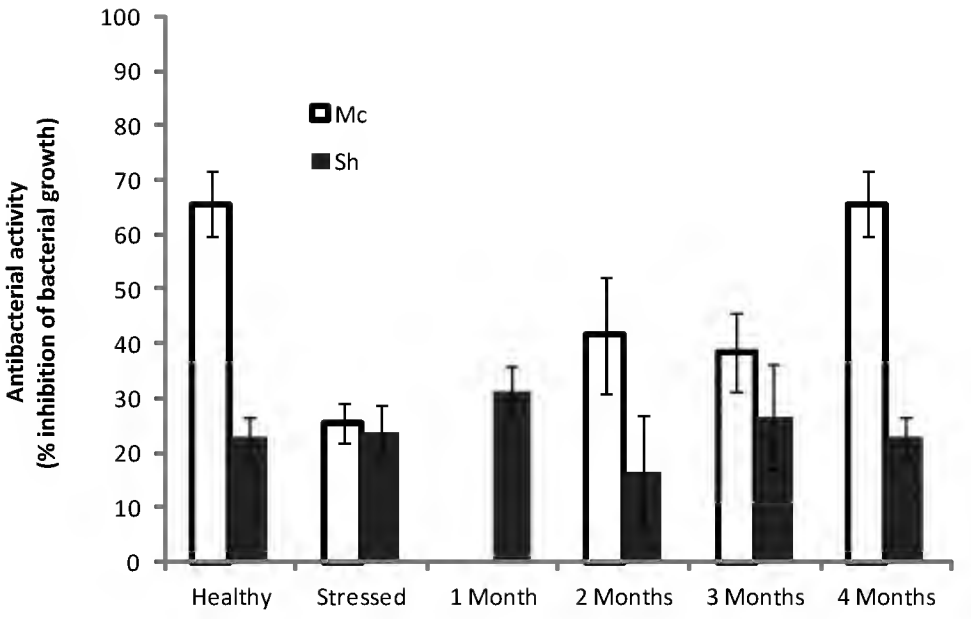

Figure 4. Antimicrobial assays, showing suppression of growth rate of the proposed coral pathogen *Vibrio harveyi* in different coral health states. The coral tissue samples show natural antimicrobial activity of the coral tissues during healthy state, stressed state and during recovery. Replicate samples were combined to give an average mean for each sample period. Error bars show standard error from collective mean. Mc = *Montipora capricornis*; Sh = *Seriatopora hystrix*. There were no samples collected for *M. capricornis* at Month 1.

than that found in *S. hystrix* (Fig. 3), however there was large variation between replicates (7.1 X 10_6 ± 1.11 cells/cm_3). Bacterial abundance increased to 1.1 X 10_7 ± 0.70 cells/cm_3 in stressed corals and in contrast to that of *S. hystrix* abundance carried on increasing after the coral started to recover (Fig 3). After two months, by which time the tissue appeared healthy, the bacterial load was on average three times greater than in the stressed state (3.7 X 10_7 ± 1.73 cells/cm_3), however these replicate samples again showed high amounts of variation within this time period (Fig 3). Furthermore although the tissue appeared to be of a healthy state, growth rate was relatively slow (Fig 1a). After 3 months, bacterial abundance reduced to similar levels seen in the healthy tissues (7.1 X 10_6 ± 1.11 cell/cm_3) and growth rate increased to pre-disturbance levels (Fig 1a).

Antimicrobial activity

Similar to S. hystrix, all coral tissues showed significant antimicrobial activity ($F_{5, 33}$ = 26.731, $p < 0.007$) (Fig 4). In contrast to S. *hystrix, M. capricornis* showed a significant increase ($p < 0.001$) in growth rate of the pathogenic bacterium *V. harveyi* in stressed tissue slurry's compared to healthy samples. Healthy corals inhibited

Table 3. Heatmap derived from relative 16S rRNA gene DGGE operation taxonomic units (colours arranged by 0, 0.1-1.0, 1.1-2.0, 2.1-3.0, 3.1-4.0, 4.1-5.0, 5+) for *Montipora capricornis*. Replicate samples (n = 3 or 4 per time period) were combined to give an overall average for clarity within the display. The larger the number under each sample column the stronger the DGGE band intensity for that specific 16S rRNA gene ribotype. There were no samples collected for *M. capricornis* at Month 1.

Closest relative	Accession no.	GenBank	Class	Healthy	Stressed	2 month	3 month	4 month
Pseudosphingobacterium sp.	AM407725	97%	Sphingobacteriia	0	3.01	2.03	2.82	0
Kiloniella sp.	AM749667	100%	Alphaproteobacteria	1.67	0	0.72	0.73	1.92
Anabaena sp.	CP000117	100%	Cyanobacterium	0	1.77	0	0	0
Cyanobacterium	AY191934	100%	Cyanobacterium	0	1.59	0	0	0
Deferribacteres sp.	GQ204938	100%	Deferribacteres	0	1.51	0	0	0
Streptomyces sp.	DQ462649	98%	Actinobacteria	2.13	0.92	0.82	0	2.33
Sulfolobus sp.	D85519	90%	Archaea	1.12	2.58	2.67	1.7	1.21
Thalassomonas sp.	AY194066	98%	Gammaproteobacteria	0.68	2.1	1.48	1.69	0.8
Marinobacterium sp.	AB006765	96%	Actinobacteria	2.41	1.01	0.12	1.25	2.21
Vibrio sp.	AB468986	100%	Gammaproteobacteria	1.38	0	1	1	1.28
Bacteroidetes sp.	GQ204958	98%	Bacteroidia	2.21	1	0.84	1.14	1.99
Paracoccus sp.	HQ538757	100%	Alphaproteobacteria	0	1.16	0	0	0
Mesorhizobium sp.	DQ310707	98%	Alphaproteobacteria	1.12	0.37	1	1.8	1.24
Pseudoalteromonas sp.	AB544013	100%	Gammaproteobacteria	0.34	1.57	0	0	0.47
Sphingomonas sp.	JQ027711	98%	Alphaproteobacteria	0.77	1.77	1.72	1.81	0.67
Sulfurisphaera sp.	D85507	100%	Archaea	0	1.1	0	0	0
Actinomyces sp.	AM084228	98%	Actinobacteria	1.1	0	0.38	1	1.08
Amycolatopsis sp.	AJ293757	100%	Actinobacteria	2.25	3.46	2.07	2.6	2.45
Endozoicomonas sp.	FJ347758	100%	Gammaproteobacteria	4.67	3.87	4.54	5.09	4.83
Halorubrum sp.	AY149598	98%	Archaea	0.52	0.69	0	0	0.67
Thalassomonas sp.	AY643537	97%	Gammaproteobacteria	2.45	1.87	2.51	2.54	2.77
Paenibacillus sp.	X60625	100%	Firmicutes	0.91	0.65	0.84	0.72	0.79
Unknown	NA	NA	NA	0.58	0.21	0.21	0.3	0.96
Methanococcus sp.	DQ195164	96%	Archaea	0.43	0	0.63	0.81	0.79
Cyanothece sp.	CP002198	100%	Cyanobacterium	0.98	1.67	0	0	0.9
Unknown	NA	NA	NA	0	0.77	0	0	0
Cyanobacterium	JN166514	97%	Cyanobacterium	1.54	1.2	1.52	0	1.82
Vibrio sp.	AB470931	100%	Gammaproteobacteria	0	0.66	0	0	0
Clostridium sp.	GQ204966	100%	Firmicutes	0.44	0	0	0	0.57
Corynebacterium sp.	AY226509	99%	Actinobacteria	0	0.55	0	0	0
Desulfovibrio sp.	AB353727	100%	Deltaproteobacteria	0.46	0	0	0	0.55

growth rate of the bacterium by 65%, whilst in the stressed state inhibition was as low as 25% (Fig. 4).

Discussion

The corals in this study underwent a severe stress event likely caused by changes in the water quality perturbation within the main aquarium display tank at the public aquarium. Increases in both heavy metals and nutrients such as selenium, chromium, copper, NO3 and PO4 were observed in the display tank. One or more of these changes in water quality variables was thought to have caused the onset of a severe stress event, leading to many of the coral species showing signs of stress such as bleaching and/or tissue loss.

Two dominant corals within the system; *Montipora capricornis* and *Seriatopora hystrix* showed dramatic declines in health. *M. capricornis* showed bleached tissues and large areas of tissue loss whilst *S. hystrix* showed an advancing band of tissue loss followed by denuded skeleton similar to that reported for WS seen in many corals in the Indo-Pacific (Willis *et al.* 2004). Once moved to more optimal conditions, the corals gradually showed improvement in their visible health status and by 1–2 months were showing signs of new tissue growth over bare skeleton. Microbial diversity was analysed during these periods to see if bacterial populations changed over time. There was a significant difference in bacterial communities between stressed and healthy individuals in both coral species, similar to that found in many previous studies on coral diseases in the natural environment (Pantos *et al.* 2003; Sekar *et al.* 2006; Sunagawa *et al.* 2009; Sweet and Bythell 2012). Similarly, there were differences between stressed bacterial associates and those present on corals showing signs of recovery.

A large proportion of bacterial ribotypes detected in this study are associated in some way with nitrogen fixation. These include ribotypes related to; *Mesorhizobium* sp, *Cyanobacteria* sp, *Desulfovibrio* sp, *Vibrio sp, Anabaena sp, Actinobacillus sp* and *Paenibacillus* sp. The presence and importance of nitrogen-fixing bacteria has been shown with regard to corals and their symbiotic dinoflagellates (Lesser *et al.* 2004; Lesser *et al.* 2007b; Olson *et al.* 2009; Lema *et al.* 2012). Specifically, studies have claimed that certain diazoptrophic bacteria provide the main nitrogen source to the corals algal symbionts (Muscatine and Kaplan 1994; Olson *et al.* 2009). Eight of the eleven nitrogen fixing bacteria in this study are diazotrophic and have been associated with corals in natural systems, these include three *Cyanobacteria* sp, three *Vibrio* sp, a *Desulfovibrio* sp and a *Mesorhizobium* sp. Although all of these species have been associated with fixing nitrogen compounds within the coral holobiont (Lesser *et al.* 2004; Lesser *et al.* 2007b; Olson *et al.*,2009; Lema *et al.* 2012), there is conflicting evidence as to which genus and/or species are more fundamental in this role.

Three other ribotypes, belonging to diazotrophic bacteria, including *Anabaena sp, Actinobacillus* sp and *Paenibacillus* sp, have not been associated with nitrogen fixation in corals before and thus could pertain to the aquarium system alone (a potential artefact brought about by the high nitrate levels in this system). Furthermore, another interesting note is that a significantly high proportion of the ribotypes sequenced in this study belong to *Archaea*, another group of organisms which have been associated with the recycling of ammonia into nitrite (Siboni *et al.* 2008).

This study is the first of its kind whereby microbial diversity has been followed over time in relation to recovery after a major stress event. Here we show how a shift in the bacterial community structure, one which includes potentially harmful bacterial associates can be reverted to that of healthy communities by improving environmental conditions. Similar results were found by Garren *et al.* (2009) where corals transplanted into waters exposed to fish farm effluents incorporated potentially pathogenic bacteria present within the water column at the new site. Although the corals in their study failed to show field signs of any disease during this period, they showed that the original bacterial community shifted from that of the natural micorbiota and subsequently recovered when returned to non polluted waters after a period of 22 days (Garren *et al.* 2009). One further study by Sweet *et al.* (2011b), showed a similar pattern of bacterial disturbance and recovery yet on a shorter time scale (over 96 h). In that study the antibiotic Ciprofloxacin was utilized to disrupt the natural microbiota on the coral simulating that observed in natural stress events. The treatment showed a significant shift in bacterial diversity between healthy and treated samples with an increase in relative abundance of potentially pathogenic bacteria such as *Clostridium* (Sweet *et al.* 2011b).

Approximately 30% of the natural microbiota in corals has been shown to possess some form of antimicrobial activity. Interestingly, several of the bacteria that were reduced in the stressed state in both coral species in this study are known to be antibiotic producers which target specific microbial species (Attia *et al.* 2009; Wiese *et al.* 2009). Bacterial ribotypes related to *Kiloniella sp.* and *Streptomyces sp.* in *M. capricornis* reduced in dominance or were completely absent in stressed samples, whilst *Moraxella sp.,* in *S. hystrix* is the opposite and increased in disease samples. This perturbation in the corals natural microbial community, may lead to further destabilisation of the community structure as a whole. This may in turn allow potentially pathogenic bacteria to proliferate and cause the onset of specific coral diseases. The two coral species studied in this instance showed that corals and their associated microbes react very differently to stress events despite being within the same environment. In the case of *M. capricornis* the corals natural antimicrobial capabilities are hampered during the disease state, whereby the corals ability of inhibiting potentially harmful bacteria are decreased during this time period. It is likely that the coral in this stressed state is also more susceptible to other potential microbial pathogens such as viruses and ciliates (Sweet and Bythell 2012) although further work analysing these microorganisms would need to be conducted. The antimicrobial capabilities were high during the recovery period but reduced to healthy levels after four months. These results suggest that either; 1) the coral itself has reduced capabilities to fight off potential pathogens during its stressed state or alternatively 2) specific bacterial associates which usually have antimicrobial capabilities are effectively knocked back and allow other potentially pathogenic bacteria to proliferate in their absence. In contrast, this doesn't appear to be the case with *S. hystrix*. The antimicrobial activity, although significantly different than that of the ethanol control, did not vary between healthy and stressed states. This result suggests that antimicrobial capabilities are varied between coral species and affected in different ways depending on species and/or type of disease. As the diseases could not be characterised any further, it is difficult to conclude more form these results, but further studies utilising the techniques in this study should be conducted when similar situations arise within aquariums in the future. Furthermore, we report the presence of potentially pathogenic bacteria such as two *Vibrio sp.* and an *Actinomyces sp.* in healthy tissues, yet absent in diseased tissues confirming many studies showing corals harbour these pathogenic bacteria in their healthy microbial communities (Luna *et al.* 2007; Luna *et al.* 2010; Sweet *et al.* 2011c; Sweet and Bythell 2012). Two further ribotypes, which were closely related to *Thalassomonas ioyana*, the reported causal agent of White Plague-like disease in the Caribbean (Thompson *et al.* 2006), were found in *M. capricornis* corals in all samples, one ribotype did increase in relative 16S rRNA gene diversity during the disease state, however was not a dominant member of the stressed bacterial community. This raises interesting questions in the role of all these bacteria within the healthy corals natural microbial associates.

In conclusion this study shows that distinct bacterial communities develop in different species under identical aquarium conditions, confirming many previous studies which showed that coral species are associated with unique bacterial communities. Contrary to expectation, identical environmental stresses produced different community responses and visual signs of stress between the two species. Interestingly, the bacterial communities associated with the coral show high resilience, returning to a virtually identical state once visibly healthy signs returned. In this case, removal of the environmental stress allowed recovery to a normal health status and normal bacterial communities within two months. However, whether bacterial community shifts are a contributing cause or a simple effect of the visible signs of stress cannot be determined.

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Hand-reared common swifts (*Apus apus*) in a wildlife rehabilitation centre: assessment of growth rates using different diets

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Abstract

Common swift (*Apus apus*) orphans represent an important number of admissions to wildlife rehabilitation centres in Europe. Rehabilitation centres may encounter difficulties in the hand-rearing of large numbers of insectivore chicks if they use commercially available insects, which are usually expensive and nutritionally incomplete. These constraints have created the necessity for alternative diets; however, these may not be optimal for hand-rearing purely insectivorous species. In this study, 116 orphan common swift nestlings were hand-reared during June and July 2008 and 2009 in the Torreferrusa Wildlife Rehabilitation Centre (Catalonia, northern Spain). We assessed growth rates and final fledgling weight under four different diets, comparing the results to those of wild parent-raised common swifts. Clinical condition at admission was the main variable predicted to influence the results. The four diets were (1) rat mince diet, a specific pathogen-free laboratory rat mince; (2) kibble diet, a formula based on a high-protein–low-carbohydrate cat food; (3) cricket diet, based on house crickets (*Acheta domesticus*) and wax moth larvae (*Galleria mellonella*); and (4) mealworm diet, based on mealworm larvae (*Tenebrio molitor*). Reference adult weights of wild animals were obtained from the literature (41.5g ± 2.42 SD). The results showed significant differences in final weights, which were considerably lower for hand-reared animals on the non-insect diets (rat mince diet: 32.8g ± 2.7; kibble diet: 32.5g ± 3.7). The final weights in both insect diet groups were satisfactory, with values close to those observed in the wild (cricket diet: 40.1g ± 4.0; mealworm diet: 40.3g ± 3.1). The results of this research highlight the need to implement changes in diet protocols when using non-insect-based diets.

Introduction

Many orphaned birds are transferred to wildlife rehabilitation centres for attention every year. Common swift (*Apus apus*) orphans represent an important number of admissions in rehabilitation centres in Europe. In 2009, Torreferrusa Wildlife Rehabilitation Centre (Catalonia, northern Spain) received 712 young common swifts. As an altricial species, both nestlings and fledglings are dependent on their parents, thus requiring hand-rearing for survival.

The common swift is a migrant insectivorous apodiform bird that spends most of its life on the wing. It is monogamous and commonly nests colonially in urban areas. The breeding biology of common swifts differs from passerine birds of similar size: it has a smaller clutch size; a rather longer incubation and a much longer nestling period; a greater ability to withstand starvation; and a capacity to retard its growth and become poikilothermic when undernourished, and to recover rapidly when conditions improve (Lack 1956; Bernis 1980; O'Connor 1984). The growth curve may be interrupted by sharp drops in weight with poor weather (cold, rainy or windy conditions), when food is scarce. This may affect development; however, brood reduction mostly compensates for these effects, and usually growth rates and fledging weights are not markedly affected in the remaining surviving nestlings (Lack and Lack 1951; Martins and Wright 1993a, b; Martins 1997).

In common swifts, several reasons why nestlings fall out of the nest have been suggested. Accidental causes include parents or siblings pushing the young unintentionally, young suffering in the heat and scrambling to the entrance (Lack 1956), or young jostling for a position near the entrance to monopolise parental attention (Bize and Roulin 2006). Other losses can be attributed to sibling competition and brood reduction. This is a strategy where parents may induce selective removal of the weakest offspring in an attempt to assure breeding success when food is scarce (O'Connor 1979; Martins and Wright 1994; Cucco and Malacarne 1996), with a parental preference for larger (Lotem 1997) or more actively begging nestlings (Leonard et al. 2000).

Wildlife rehabilitation practice guidelines suggest body weight and plumage condition as essential indicators of individual chances of survival to release (Stocker 2000; MacLeod and Perlman 2001; Best and Mullineaux 2003). Low fledgling body weight can lead to low fitness, and thus decreased chances of survival (Perrins 1965; Johnston 1993; Klasing 1998; Schauroth and Becker 2008). Common swifts need to be in exceptional body condition at fledging, with strong flying abilities and therefore a large pectoral mass (O'Connor 1984). Apparently young spend the first night after fledging on the wing (Lack 1956; Tarbuton and Kaiser 2001), and may start on migration shortly after leaving the nest, a long journey crossing the Sahara to their wintering grounds in Africa (Koskimies 1950; Brown and Grice 2005), flying at high altitudes, often above 2000 m (Gustafson et al. 1985; Chantler 2000). Common swifts need to be able to execute fast movements, flying without rest and usually at high altitudes with low oxygen pressure, which involves tremendous energy expenditure (Palomeque et al. 1980). A reduction in body weight slows down flight speed (Martins 1997) and can have negative repercussions for migration as the distance it is possible to travel can be diminished (Alerstam and Lindström 1990) and predation risk can increase (Lima 1986). Dull plumage, which may consist of severely malformed feathers, cannot supply flight performance, insulation or waterproofing. None of these factors – reduced growth, low body weight or poor feather condition – seem compatible with survival in the wild.

The conditions under which birds are maintained while in captivity, their diet and the amount of parental care received have profound influences on the health, growth and development of nestlings (O'Connor 1984; Flammer and Clubb 1999). Husbandry management should aim to simulate conditions in the wild. With diet an essential factor, nestlings in captivity should be fed the same foods the parents would have fed them with in the wild; however, duplicating this is a challenging task. Wildlife rehabilitation centres dealing with insectivorous species may encounter difficulties in the hand-rearing of large numbers of chicks, as there is a limited selection of commercially available insects (and they tend to be expensive). Even when it is possible to use insects to feed insectivores, the diet is often limited to a single insect species. The nutritional composition of commercially produced insects has been studied, and may be incomplete in terms of minerals and other nutrients without appropriate supplementation (Bernard and Allen 1997; Barker et al. 1998; Finke 2002; Finke and Winn 2004). Cost is usually the limiting factor in using insects; it is an important constraint that has created the necessity for using alternative diets, which also take effort and accessibility into account. The formulation of a diet is complex; a balanced diet requires the precise combination of 45 different nutrients (chemical elements and compounds), and a large number of nutrient interactions needs to be evaluated, considering the differing bioavailabilities of these nutrients from different ingredients (Brue 1999). Dietary formulae where the main components are not insects or are combined with insects have been developed, with good results in nestling passerines (MacLeod and Perlman 2001; Winn 2002), and some authors have stated that some of these diets can be used as stand-alone insect substitutes (Winn and Finke 2008).

Avian insectivores, particularly aerial feeders, consume a huge diversity of invertebrate species (Lack and Owen 1955; Bernis 1987), the combination of which, along with the intestinal content of the prey (Hernandez-Divers 2006), presumably supplies a complete diet. Like common swifts, almost all altricial passerine parents feed their young insects, regardless of the adult's diet (O'Connor 1978, 1984; MacLeod and Perlman 2001).

The aim of this research was therefore to investigate growth rates in hand-reared common swifts fed different diets and compare them to those of wild birds. Clinical condition at admission was the main variable predicted to influence final fledging weight.

Table 1. Number of feeds per day and amount supplied for the four diet groups.

	Approximate daily amount (g)			
Age (days)	Rat (5 feeds)	Kibble (8 feeds)	Cricket (5 feeds)	Mealworm (5 feeds)
10–20	18	14	20	20
21–30	15	14	20	20
30–release	10	14	15	15

Methods

Experimental work was carried out in June and July of 2008 and 2009 in the Torreferrusa Wildlife Rehabilitation Centre. We divided 116 common swift nestling orphans into four different diet groups (two insect and two non-insect diets).

Diets

Diet 1: rat meat. A rat mince diet was used in Torreferrusa Wildlife Rehabilitation Centre until 2008. It consisted of specific pathogen-free laboratory rat, without skin and bowels, minced with the flesh and bones. The rat mince was supplemented with multivitamins, mineral and aminoacids added to the drinking water (Nekton S®, Nekton Produkte, Germany). Rat mince was administered in the form of small balls, with a few drops of water given afterwards to facilitate swallowing.

Diet 2: kibble. This diet formula was based on the formula for nestling songbirds (FoNS©) (Winn and Finke 2008), substituting the original Evo® dry cat and kitten food (Natura Pet Products Inc., USA), which was not available on the European market, with Orijen® (Champions Petfoods Ltd, Canada). The formula also included dried egg white, active-culture plain yogurt and vitamins (Avi-Era™, Lafeber Company, USA). The dry components were pre-soaked in water and blended in a food processor. The mixture, with a cream-yogurt like texture, was administered by syringe tip deep into the oesophagus.

Diet 3: cricket. The cricket diet is used in the Mauersegler Klinik (Frankfurt, Germany), a rehabilitation centre specialising in common swifts (Haupt 2009). It was composed of 90% house crickets (*Acheta domesticus*) and 10% wax moth larvae (*Galleria mellonella*). The insects were 200–300 mm in length. Insects were frozen alive straight from the supplier and thawed before feeding. For one feed a day, the insects were dusted with a vitamin and mineral supplement (Korvimin ZVT®, Firma WDT, Germany). Entire insects, including legs, were administered using rounded-end tweezers.

Diet 4: mealworm. The mealworm diet was based on the formula used to hand-rear chimney swifts (*Chaetura pelagica*) in the USA by Kyle and Kyle (2007). It was composed entirely of mealworms (*Tenebrio molitor*) varying from 100 to 300 mm in length. Larvae were kept at 5° C on a wheat bran substrate. On alternate days the mealworms were either soaked alive in a supplement mixture dissolved in water (Sera Mineral Plus V®, Sera, Germany; Avi-Era™), or soaked just in water and at one feed dusted with supplements

Table 2. Definitions of clinical conditions used in Torreferrussa Wildlife Rehabilitation Centre protocols for orphan birds, and number of animals that completed hand-rearing in each sample group. Survival under non-insect diets has proved very low and will be analysed further (Fusté, in preparation). Data from young common swifts that did not complete the hand-rearing process were omitted from the samples.

Clinical condition	Body condition	Other clinical signs	Rat *n*=34	Cricket *n*=29	Mealworm *n*=28	Kibble *n*=25
1	Apparently normal		4	4	2	9
2	Weight loss	Slight dehydration	18	7	5	5
3	Emaciation	Severe dehydration, weakness	12**	7	9	9*
4	Severe emaciation	Severe dehydration, severe haemorrhage, shock	0**	11	12	2*

** Four birds from the Rat mince group in clinical condition 4 and two in clinical condition 3 died a few days after admission. * Five birds from the Kibble group in clinical condition 4 and three in clinical condition 3 were moved to insect diets to avoid risk of death once poor progress was observed.

(Korvimin ZVT®) and nutritional yeast (Marigold Engevita®, DSM Food Specialties, Netherlands). Insects were administered using rounded-end tweezers.

In addition to the supplementation, vitamin B complex (Complejo B8 Inyectable®, Laboratorios Calier SA, Spain) was administered subcutaneously in the inguinal fold every 10 days in both insect diet groups.

Food intakes were closely related to the begging behaviour of the chicks. Feeding amounts for each diet are summarised in Table 1.

Admission and housing protocol

All nestlings received a physical examination upon arrival and were identified with a numbered ring. Initial care and stabilisation consisted of oral fluid therapy in the non-insect diet groups (Glucolyte®, B. Braun VetCare SA, Spain) and subcutaneous fluids in the inguinal fold on the insect diet groups (Lactate's Ringers Solution®, B. Braun VetCare SA, Spain). Because cestode parasitism is a common finding in common swifts, all birds were treated with an antiparasitic drug, praziquantel, at a dose of 10mg/kg (Droncit®, Bayer AG, Germany). Three birds were housed in each plastic container, with absorbent paper as a substrate, which was changed after each feed. Young were considered ready for release when all primaries were sheathed and the wing length was about 165 mm, extending at least 35 mm beyond the tail feathers. Fledglings were released during the afternoon in areas with some height up off the ground and an abundance of other wild common swifts.

Sample groups

Sample groups were distributed among the different diets, and classified by clinical condition, a number ranging from 1 to 4 as defined in Table 2. Maximum age for sample nestlings was 24 days. Age was estimated by comparing the new arrivals to a set of photographs of the age-specific developmental sequence of well-fed wild nestlings, taking into consideration feather growth characteristics (Jongsomjit et al. 2007; Tigges 2008). Prior to the first daily feed (0800), nestlings were weighed to the nearest 0.1g with an electronic scale (MS500). Plumage condition was assessed during the hand-rearing process, paying special attention to feather loss, fault-bars, feather dirtiness and broken quills.

Body weights at fledging are normally higher than average adult weights (Lack and Lack 1951; Gladwin and Nau 1964; Collins and Bull 1996; Cramp 1998; Chantler 2000). For the purposes of this study, a sample obtained by Gadwin and Nau (1964) in the UK was used as a final body weight reference: n = 208, body weight = 41.5g (range 36.3–49.4, SD ±2.42); these final weights are similar to those observed by Lack and Lack (1951) in the UK and Rodriguez-Teijeiro (1980) in Spain (Table 3).

Internal organ evaluation

In order to evaluate the suitability of the mealworm diet further, three animals that received this diet for 20 days were selected for biochemical and histopathological studies. The birds were not releasable due to poor feather condition at admission.

Blood samples were collected from the right jugular vein and placed in serum separating tubes for biochemical analysis. Total protein, uric acid, calcium, phosphorus, aspartate aminotransferase (AST), bile acids, creatine kinase (CK), total cholesterol and HDL cholesterol were determined. Animals were humanely euthanased and a complete postmortem investigation was performed immediately.

Statistical tests were conducted using R software, version 2.11.1.

Table 3. Common swift body weights in different locations.

Reference	Location	N	Mean weight (g) (range)
Fledglings			
Weitnauer (1947)	Switzerland	30	53.5 (48.0–56.0)
Lack and Lack (1951)	UK	73	41.4 (34.0–52.0)
Rodriguez-Teijeiro (1980)	Spain	30	41.4 (34.5–51.5)
Pellantová (1981)	Czech Republic	31	Not given (45.6–49.5)
Bernis (1987)	Spain	14	Not given (43.0–52.0)
Pellinger (2006)	Hungary	16	53.6 (Not given)
Adults			
Lack and Lack (1951)	UK	102	42.7 (35.9–52.2)
Cramp (1998)	Gibraltar	24	44.9 (Not given)
Pellinger (2006)	Hungary	15	48.4 (Not given)

Table 4. Final fledgling weight and other variables, expressed as mean (range) ±SD.

Sample group	Fledging weight (g)	Admission weight (g)	Weight increase (g)	Estimated age at admission	Days of hand-rearing
Rat diet	32.8 (26.0–36.4) ±2.7	27.8 (21.5–41.3) ±4.9	4.9 (–7.0–11.5) ±4.6	17.0 (10.0–23.0) ±3.8	23.0 (17.0–30.0) ±3.8
Kibble diet	32.5 (27.5–38.0) ±3.7	36.2 (22.8–52.8) ±8.3	-3.8 (-16.0–10.0) ±6.5	18.8 (10.0–24.0) ±0.4	21.3 (16.0–30.0) ±4.0
Cricket diet	40.1 (33.5–48.7) ±4.0	26.8 (17.0–42.0) ±7.0	13.3 (-6.3–26.0) ±7.8	17.1 (10.0–23.0) ±4.0	23.9 (17.0–30.0) ±4.8
Mealworm diet	40.3 (33.0– 46.5) ±3.1	27.0 (11.9–37.8) ±5.9	13.3 (-2.7–25.9) ±6.2	16.8 (9.0–22.0) ±3.6	23.2 (18.0–31.0) ±3.6

Results

Fledging weight

The target variable was the difference between the final weight of hand-reared fledglings (Table 4) and the weight reference (41.5g) for a wild parent-raised fledgling. A two-way analysis of variance (ANOVA) was performed, with two factors: diet (four levels: Rat mince diet, Kibble diet, Cricket diet and Mealworm diet) and clinical condition at admission (three levels: clinical condition 1, clinical condition 2 and clinical conditions 3+4). Clinical conditions 3 and 4 were grouped as birds of clinical condition 4 did not complete the hand-rearing process on the two non-insect diets. Interactions between diet and clinical group were insignificant (F=0.659, df=109,103, p=0.683) when comparing both models. However, clinical condition groups and diet groups were found to be highly significant (F = 74.09, df = 6,109, p < 0.0001, adjusted R^2 = 0.79) (Table 5). The cricket and mealworm diets produced similar results and final weights for both were comparable to those observed in the wild. The non-insect diets were generally inferior, fledglings having a final weight 7 g below the wild reference weight. Clinical condition had some effect on final weight, but this was substantially smaller than the effect of diet. There were no significant differences in the effect of diet in the clinical condition groups, with parallel effects in the three conditions (Fig. 1).

A t-test with a Welch correction for unequal variances was conducted to compare the final weights of birds in two groups: 13 apparently normal young (clinical condition 1) from both non-insect diets (*n* = 4 on the rat mince diet, *n* = 49 on the kibble diet) and 23 birds with severe emaciation (clinical condition 4) on both insect diets (*n* = 411 on the cricket diet, *n* = 412 on the mealworm diet). The means of 34.87g and 39.71g, respectively, for these two groups were highly significantly different (t = -4.05, df = 24.88, p=0.0004). This result is notable, as even though we compared the worst cases in the insect groups with the best in the non-insect groups, the insect group nevertheless achieved the better result.

Internal organ evaluation

The lack of reference values in the literature for common swifts made the interpretation of the results difficult. The results seemed to be within the normal range despite the fact that cholesterol levels were high in all animals (591, 517 and 640 mg/dL) compared

Table 5. The estimated coefficients for the different diet groups, reflecting variation with respect to the wild weight reference. Estimates by clinical condition reflect results for clinical condition 2 and clinical conditions 3+4 with respect to clinical condition 1, which was considered the reference value.

Group	Estimate coefficient	Standard error	t	p
Cricket diet	0.5629	0.966	0.583	0.5613
Kibble diet	-7.5817	0.8845	-8.572	7.55e-14 **
Mealworm diet	0.9961	1.0231	0.974	0.3324
Rat mince diet	-7.1975	0.9628	-7.476	2.05e-11 **
Clinical condition 2 (all diet groups)	-1.1702	0.9871	-1.186	0.2384
Clinical condition 3+4 (all diet groups)	-2.6352	0.9083	-2.901	0.0045 *

**p < 0.0001; *p < 0.001

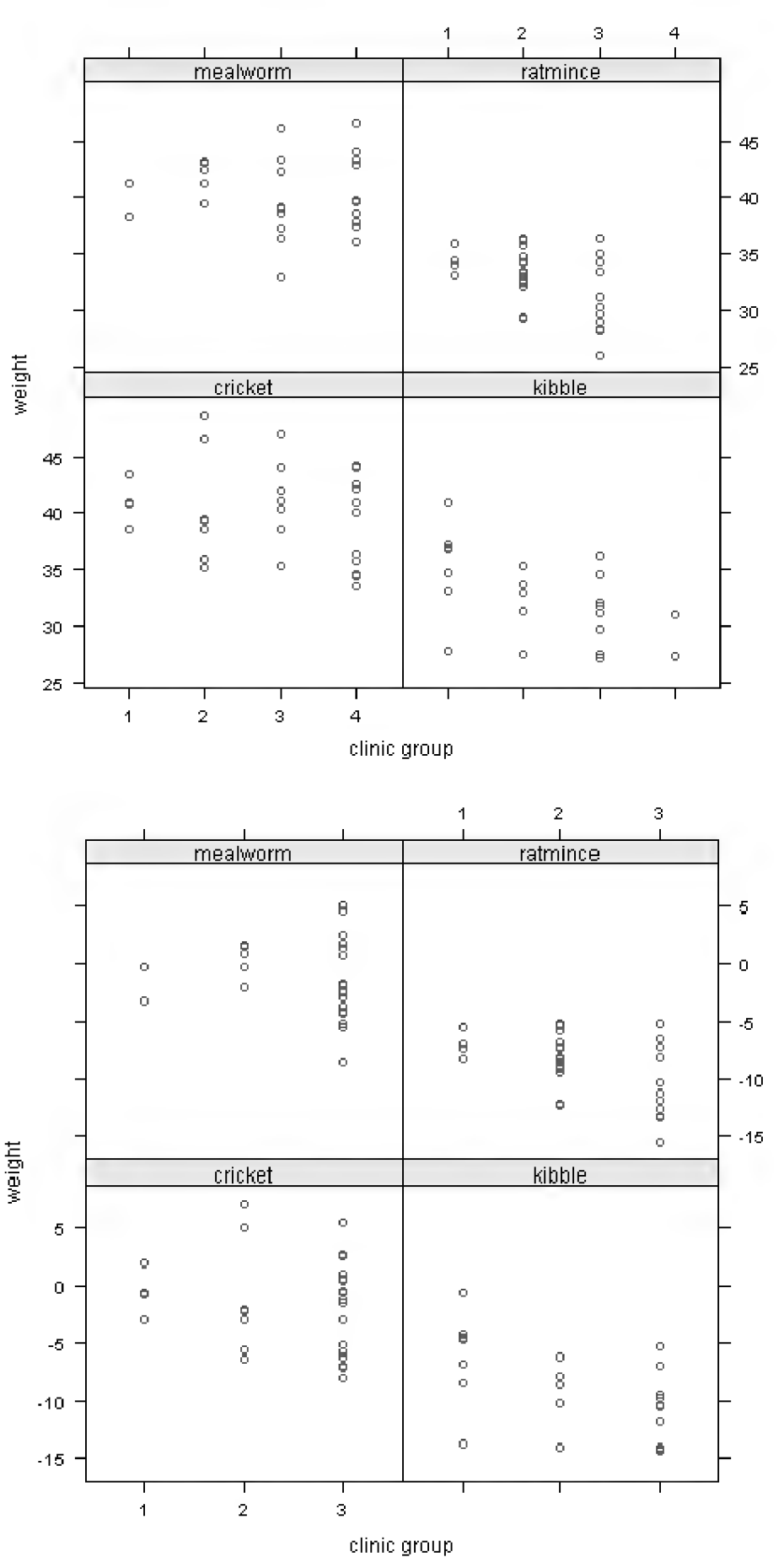

Figure 1. Top: All diet groups and clinical conditions (clinic group) with final weights presented as absolute values. Bottom: The four diet groups and clinical conditions (3+4 grouped into clinic group 3) with weight scales presented as differences compared to the wild reference weight (41.5g).

to reference values for other species. Histopathological studies revealed no lesions in any tissues analysed, which included liver, spleen, kidney, proventriculus, gizzard, duodenum, pancreas, lungs, heart, adrenal gland, ovary and oviduct.

Discussion

The results of this study draw the practice of hand-rearing common swifts with non-insect diets into question; weights of fledglings on insect diets showed a much greater similarity to weights attained in the wild. In interpreting the results, it is important to take into account the reasons for which orphans came to the rehabilitation centre and their recovery options. Clinical conditions 1 and 2 could have represented simply undernourished young, good candidates to recover weight rapidly: possibly nestlings that fell off the nest accidentally in good condition and endured a short period without food. On the other hand, young in clinical conditions 3 and 4 may have suffered sibling competition and been ejected from the nest, resulting in starving, emaciated nestlings (Martins and Wright 1993a). It was assumed that such emaciated birds, which would have endured a critical fasting period, were poor candidates for recovery. The results in both insect diet groups, however, showed optimal final weights in all clinical conditions, even in severely emaciated birds. Conversely, in both non-insect diet groups even the good candidates performed poorly. In addition, many birds in extreme clinical conditions (3 and 4) on the non-insect diet did not complete the hand-rearing process (i.e. they died or were moved to an insect diet), whereas on insect diets, the survival rate was high (Fusté, in preparation).

A compensatory strategy observed in common swifts, where individuals that have suffered retarded growth enter a phase of growth acceleration when conditions improve (Metcalfe and Monaghan 2001), was observed only in the insect diet groups. When individuals are unable to undergo compensatory growth, they become stunted, with a smaller body weight and size (Bize et al. 2003). Thus we have shown that the poor growth observed in the non-insect diet groups was related specifically to the diet: almost all birds on a non-insect diet had a stunted appearance when compared to their conspecifics on the insect diet groups.

Low body weight or poor growth may be caused by any factor that interferes with the homoeostasis of the nestling: improper feeding (insufficient energy, unbalanced nutrition or inappropriate diet), poor environmental conditions in early development or subclinical diseases that cause the nestling to expend energy fighting the disease instead of using it for growth (O'Connor 1984; Macwhirter 1999; Flammer and Clubb 1999). Nestling nutrition is the most obvious mechanism that may influence growth and body size (Ricklefs 1979; Johnston 1993), and it is a major factor in the husbandry management of any species (Best and Mullineaux 2003). This is particularly true of nestlings, as growth is the period during which most nutrients need to be at their maximum levels (Brue 1999). In young altricial nestlings, the energetic cost of growth is often more than 50 per cent of the daily metabolisable energy requirements (Bryant and Gardiner 1979; O'Connor 1984).

Birds are very sensitive to acute deficiencies in some nutrients (Klasing 1998; Brue 1999). The non-insect hand-reared groups, with the poorest growth rates, were fed on a diet that differed significantly from their natural food. The nutritional status of a growing bird is based on its ability to assimilate and metabolise the food supplied (O'Connor 1984). Insectivores, like other faunivorous birds, rely on a very competent digestive enzymatic capacity. Animal food prey is high in protein with a balance of essential amino acids similar to the bird's requirements (Klasing 1998). In terms of nutritional components, insects are high in proteins and lipids, with the amino acid balance almost as good as vertebrate prey, with good sources of phosphorus, vitamins and trace minerals, but low in calcium (Finke 2002; Hernandez-Divers 2006).

The rat mince diet and kibble diet were complete in terms of macro-nutrients, with protein and lipid contents similar to those observed in crickets and mealworms. However, nutritional strategies determine the types of food that may be consumed without digestive or metabolic complications: species are adapted to foods that are attainable and can be metabolised appropriately by an adapted digestive tract (Snyder and Terry 1986). Insectivorous birds have a moderate rate of passage, with an efficiency of digestion that approaches 100 per cent of the non-chitin components of insects. On the other hand, carnivorous birds have a slow rate of passage, an adaptation to efficient digestion of vertebrate prey (Klasing 1998). Common swifts fed on a carnivorous diet may therefore have less opportunity to assimilate and metabolise the food completely. A theoretically balanced diet may also appear to have all the required nutrients, but in fact be nutritionally inadequate due to the interaction of specific nutrients. This imbalance may be caused by excess of one nutrient impairing the metabolism of another functionally similar nutrient, causing a decrease in its absorption or increasing its catabolism or excretion (Klasing 1998; Brue 1999).

MacLeod and Perlman (2001) reported observations on nestling passerines fed commercial dog food. Birds matured at a slower rate than in the wild, they were stunted, and the plumage was not glossy and keratinised as in their wild conspecifics at the same age. In the present study, rat mince produced poor plumage and caused dirtiness on feathers, and thus many birds required a bath during the hand-rearing process. Flight performance at release, assessed in subjective terms by observation, was questionable (very few birds in the rat mince group managed to fly high). Plumage condition on the kibble diet was more acceptable, as was flight performance. Numerous birds on both non-insect diet groups, particularly on the rat mince diet, had retained feather sheaths during the hand-rearing process and needed manual preening. Even with this, fault-bars at the spot where the sheath constricted the feather left a weakened structure. In the insect diet groups, feather condition and flight performance was optimal when compared to those wild fledglings arriving at the rehabilitation centre and released the same day.

Nestlings show a form of sigmoidal growth (Ricklefs 1968); initially weight increases gradually, then speeds up, reaching a peak of 20–30% over the average adult, and finally falls again, with asymptotes that tend to exceed or be similar to adult weights (Lack and Lack 1951; O'Connor 1984). Birds rely on two major sources of energy, lipids from fat stores and proteins (O'Connor 1977; 1984). If they do not have enough lipids, they may start protein catabolism at a stage when proteins are fundamental for the development of vital organs and muscles (Ricklefs 1979). Adipose tissue was not observed in the non-insect diet group during development, in contrast to the birds in both insect diet groups. Fat deposits are important to avoid the formation of fault-bars, defective barbule formations that may represent predilection sites for breakage in the feathers (O'Connor 1977). If fat stores are depleted, birds start to compensate by using protein, catabolising muscle tissue (Snyder and Terry 1986). This effect can cause the release of endogenous corticosterone, detrimental while feathers are developing (Macwhirter 1999; Flammer and Clubb 1999). Desrocher et al. (2009) observed how endogenous corticosterone in passerines released under physical stressors (food restriction) resulted in greater inter-barb distances in primaries, secondaries and rectrices, fewer barbules and weaker feathers when compared to control birds.

During the hand rearing process, begging behaviour was recorded for the birds in the different diet groups. Begging behaviour is essential in a healthy nestling to get the parents'

attention (Lotem 1997; Leonard et al. 2000; Bize and Roulin 2006), and was observed in all birds in both insect diet groups; even in severe cases, where initially the chicks had to be force fed, they started begging for food within a short period of time. Conversely, in nestlings in the non-insect diet groups, begging behaviour was infrequent, particularly in the kibble diet group, thus requiring force feeding throughout the hand-rearing process. One important concern in the non-insect diet groups was how even the few nestlings that were begging refused repeatedly to swallow a house cricket.

Klasing (1998) described how birds adapted to soft foods are typically unwilling to consume significant quantities of hard foods if they suddenly become available, although this occurs in species adapted to such changes in diet (e.g. granivores). Piersma et al. (1993) summarised some studies that show how birds consuming soft foods have smaller gizzards than when they eat harder foods. Captive shorebirds, acclimatised to soft foods, initially did not consume their introduced natural hard-shelled food until a period that appeared to correspond with the enlargement and adaptation of the gizzard to the new hard food. Kasarov (1996) reviewed how digestive features are influenced by factors such as diet quality and quantity. Plasticity of the digestive tract includes changes in the size and musculature of the different organs, changes in pancreatic enzyme levels, and changes in absorption rates and retention times. Given these considerations, common swifts may be affected physiologically when changing from a soft rat mince or kibble with a yogurt-like texture to the new air-borne diet. For instance, they may need to strengthen the gizzard to assimilate the exoskeletons of the new natural diet. These are physical adaptations that may require several weeks for completion (Kasarov 1996; Klasing 1998) and this possibility is therefore of some concern.

Focusing now on some aspects of the individual diets, we noticed that the daily amount we were able to administer with the kibble formula was lower than for the other three diets (Table 1). The creator of the FoNS© formula (Winn, pers. comm.) suggested that low weight progression observed on the kibble diet could be attributable to a lower overall caloric intake rather than the composition of the diet per se. In addition, she observed that the cat food brand we used (Orijen®) was different from the original (Evo®), and might perform differently. Winn explained the success of the original formula when hand-rearing chimney swifts, describing how they were fed as much as they would eat every hour for 12 hours a day. However, although we increased feeding frequency to eight times per day versus five for the other three diets, weight gain was not achieved. Birds seemed unable to digest the food during those shorter intervals, as they presented hard gizzards and a distended digestive tract.

The use of mealworms has been somewhat controversial among the common swift rehabilitation community in Europe. Some claim that the chitin of mealworms may contain substances that cause liver and kidney intoxication in common swifts when fed for long periods, although we found no published data on such incidents. A negative point of mealworms, although this is shared with all commercially produced insects, is the unbalanced composition of vitamins and minerals. We proved that the mealworm diet did not cause histological lesions in major internal organs when administered for a period of 20 days in three animals. All animals had excellent body condition with fat stores that could possibly provide the energy necessary for the migration. All three animals had high cholesterol levels, even though no reference values were found for the species. It was assumed that the animals were starved for at least 8h prior to sampling but this could not be totally confirmed. Further work is needed to establish normal reference values for the species and thus permit the interpretation of biochemistry results from animals receiving different artificial diets.

Barker et al. (1998) described how chitin, measured as neutral detergent fibre, comprised about 15% of dry matter in many cultured insect species, with a higher content in house crickets (19.1%) when compared to mealworms (14.5%). Few studies related to chitin digestibility have been conducted on wild birds (Weiser et al. 1997; Akaki and Duke 1999) or on poultry (Hossain and Blair 2007), although none exposed any adverse health effects. When comparing mealworm and cricket nutrients, we observed that crude protein was similar in both insect species (about 19%). Larval stages, as in mealworms, have a higher fat content than adult insects (e.g. dry matter: mealworm 31.1% vs adult cricket 22.8%) (Baker et al. 1998). Fat has a higher calorific content than protein, providing a more concentrated energy source. Fat content also has an influence on the rate of food passage – as the fat content increases, the rate of passage is slowed. This effect improves digestibility of most nutrients, increasing exposure to digestive enzymes and time for absorption (Brue 1999). Fat provides the essential unsaturated fatty acids such as linoleic acid required for good growth (Snyder and Terry 1986), with mealworms an especially rich source of linoleic acid (Finke and Winn 2004).

In general terms, any hand-rearing formula needs a balance of calcium and phosphorus between 1.5:1 and 2:1 in order to avoid the development of metabolic bone disease and contribute to proper growth and health (Brue 1999; Duerr 2007). Baker et al. (1998) noted that most cultured insects, including mealworms and house crickets, were a poor source of calcium, with inverse calcium: phosphorus ratios. Copper, iron, magnesium and zinc, though not manganese, were adequate in terms of dietary requirements. As far as vitamins are concerned, most cultured insects but also free-ranging insects are low, particularly in vitamin A. Supplements of those nutrients most likely to be missing in commercially-supplied invertebrates, especially calcium and vitamin A, should therefore be used, though levels should be carefully controlled. Excessive calcium intake (when combined with vitamin D) in birds may cause kidney damage and secondary visceral or articular gout, while excess vitamin A can interfere with bone growth and disrupt epithelial cells, causing lesions in the mouth, nares and eyes (Klassing 1998; Brue 1999). Interestingly, Kyle and Kyle (2007) noted that metabolic bone disease in chimney swifts was rare, and thus low calcium concentrations in their diet might not be of particular concern.

Overall, we can recommend the use of mealworms, as long as a supplementation regime is strictly followed, when the cricket diet (Haupt 2009) cannot be provided for economic reasons. Possibly, a combination of both insects and even adding other species (cockroaches, wax worms or fruit flies) would enrich the diet. Kyle and Kyle (2007) have also stressed the importance of diet in the successful hand-rearing of chimney swifts, measured as high survival and release rates of raised birds, as well as extensive post-release breeding success, and post-migration data on many hand-reared individuals studied over a 20-year period.

Probably only a small percentage of even wild-raised nestlings survive to reproduce. We cannot provide for the needs of orphaned common swifts as their parents do, but we must emulate them as closely as possible if we want to give them any chance at all of survival, initially for the long migration journey, and then to reproductive age.

Conclusions

1. Final fledgling weights, feather condition and flight performance on both non-insect diets, rat mince and kibble diet, were questionable when compared to wild-raised birds, while the same variables were considered optimal in all clinical conditions for the insect diets. We anticipate that final weight could even be increased on both insect diets if feeding intake had been improved.

2. The results demonstrated the success of both insect diets when recovering nestlings in poor condition, even if severely emaciated, and highlighted the fact that all birds, regardless of condition, had a high possibility of survival, making a sacrifice protocol based on poor clinical condition at admission superfluous.
3. There are concerns about the use of mealworms in hand-rearing common swifts, even though they have proved successful when hand-rearing chimney swifts. This should be scientifically investigated.
4. Research on post-release survival should be encouraged. Knowing if hand-reared young have managed to forage and survive for a while would mean an initial success. Other research should also include morphological features, such as plumage quality compared to the wild.
5. The authors recommend that rehabilitators who have created their own diet analyse it thoroughly, observing carefully the final results when compared to wild conspecifics. Even birds that appear to be healthy may be undernourished on rehabilitation diets, leading to animals with weak bones, delayed growth and consequent lack of biological fitness.
6. The authors recommend discontinuing the use of the non-insect diets analysed in this study, and switching to an insect diet. Mealworms, which are considerable cheaper than house crickets, seem to be an excellent alternative as a base diet for hand-rearing common swifts when the established cricket diet cannot be provided for economic reasons.

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Research article

Response of predatory birds to varying levels of difficulty in obtaining food

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Abstract

This study investigated the development of the ability to obtain food in predatory wild birds (*Ardea cinerea*, *Accipiter gentilis*, *Milvus migrans*, *Athene noctua*, *Strix aluco* and *Bubo bubo*) in a wildlife rehabilitation centre. Birds were stimulated to find/capture food, dead or alive, at five levels of increasing difficulty, and compared with controls. Each level of difficulty lasted about a week for each animal. They were only moved to the next level if they did not lose weight and did not show signs of loss of physical abilities. Weights were obtained about once every seven days. The mean time taken to reach the highest level of difficulty was shorter for Strigiformes (16.6 ± 8.8 days) – especially *Athene noctua* – when compared to Falconiformes (54.1 ± 10.2 days). When compared to control birds, the animals tested did not show significant weight loss. The time to reach prey competence (number of days a bird needed to attain the last level and stay there at least a week) ranged between 10 and 47 days. The time that the test birds needed to reach the last level (and full recovery) was not significantly different from the time controls took to recover. The number of weights (the total number of times a bird was weighed until it reached the final level and stayed there, reflecting the number of manipulations to which individuals were subjected during the recovery process) ranged from five to 10, while the average for controls was 5.9 ± 1.87. We conclude that adding some degree of difficulty to the feeding of captive birds of prey recovering at wildlife centres does not seem to have negative effects on the recovery process itself or on the centre's routine work, and can contribute to improving birds' physical and psychological health as it stimulates movements and skills that, apart from the recovery process itself, may increase the chances of survival when the birds are released.

Introduction

Animal welfare can be defined in various ways according to the emphasis that is placed on the different characteristics of animals (Duncan & Fraser, 1997). However, the fundamental goal of welfare is to keep animals in good health, both physical and psychological (Young 2003). An approach that brings together both components of animal welfare is environmental enrichment (Forthman-Quick 1984). Within the five sub-types of environmental enrichment (Bloomsmith et al. 1991), emphasis is often put on food enrichment that takes into account the ecological characteristics of species in relation to their diet and feeding habits (Arent 2007).

Birds of prey are a highly ecologically sensitive group with an increased risk of extinction, because they occupy the top position in the food chain (Cabral et al. 2005). It is on this group that most rehabilitation efforts for wild animals in the Portuguese countryside are centred (Horta et al. 2009). This research aimed to study responses to varying difficulty in obtaining food by predatory wild birds held in rehabilitation centres, with the goal of improving the overall recovery process.

Methods

The work was done with birds held at a recovery centre for wildlife (CERVAS – Centro de Ecologia Recuperação e Vigilância de Animais Selvagens), located in Gouveia, Portugal. The methodology used was based on stimulating the birds to overcome each of five levels of difficulty in getting food (see Table I) and to find/capture food supplied dead and alive in a variety of ways (Table 2). The tests took place between 7 September 2008 and 30 June 2009. Birds were exposed to each level of difficulty for about a week, but only moved to the next level if they did not lose weight and showed no decline in their physical condition. Whenever they did not meet the above criteria, the animals were kept at the same level or put back to lower levels of difficulty. The species studied were grey heron *Ardea cinerea* (1 animal), goshawk *Accipiter gentilis* (1 animal), black kite *Milvus migrans* (6 animals), little owl *Athene noctua* (7 animals), tawny owl *Strix aluco* (7 animals) and eagle owl *Bubo bubo* (2 animals), which were compared with control animals kept in the same conditions but fed in the standard way (food supplied on a single tile placed on the floor of the cage). Each of the 24 birds studied was weighed every

Table 1. Levels of food enrichment and descriptions.

Food enrichment level	Description
Level 1	Dead food (cut into pieces, placed on a single tile)
Level 2	Dead food (cut into pieces) placed on several tiles, at different places and heights
Level 3	Dead food (cut into pieces, placed at different places and heights but not on tiles), at equal amounts with and without fur/scales
Level 4	Equal weights of dead food (cut into pieces, placed at different places and heights but not on tiles, with fur/ scales) and live prey
Level 5	Equal weights of dead food (cut into pieces, placed at different places and heights but not on tiles, with fur/ scales) and live prey hidden with the help of specific structures (e.g. mouse shelters)

7.4 (± 4.94) days. Timing of weight recording depended on the capacity and logistical requirements of the recovery centre, the need to ensure that clinical recovery, either of the animals under study or others, was not called into question, and the balancing of the various tasks.

We calculated number of weighings (the average number of weighings that each species was subjected to up to and including reaching and staying at the last level of difficulty, which reflects the manipulations to which the individuals are subjected and is related to the total recovery time in captivity), and time to prey competence (number of days a bird needed to attain the last level and stay there for at least a week, i.e. the point at which the birds were able to search for and capture live food).

Results

Strigiformes, particularly *Athene noctua*, reached the highest level of difficulty faster than Falconiformes (Table 3). The average number of days that these birds took to reach the highest level of enrichment was significantly lower than the average number of days that Falconiformes took to reach the same level (16.6 ± 8.75 and 54.1 ± 10.20, respectively; $t = 9.01$, $p < 0.001$, $df = 21$). As a consequence the Strigiformes were subjected to fewer manipulations than the Falconiformes (weighings: 3.9 ± 1.0 and 4.9 ± 0.4, respectively; $t = 2.44$, $p = 0.024$, $df = 21$).

The grey heron was also slower than the nocturnal species ($t = 3.83$, $p = 0.002$ and $t = 12.20$, $p < 0.001$, respectively) (Table 3). However, among the diurnal birds, the grey heron proved to be significantly faster than the Falconiformes ($t = 7.56$; $p < 0.001$). Among the birds studied black kites were the slowest, taking about 58 days to reach the highest level (Table 3).

Comparing the controls and experimental birds revealed no major weight differences, and only the grey heron showed a small weight loss in the early stages of the enrichment programme (Fig. 1).

Time to prey competence

Of the test birds, 75% reached the last level and stayed on it, i.e. began to capture live prey that had the opportunity to hide, with no decline in their condition. The time to prey competence was shorter or very close to the average recovery time of the control birds, but not statistically different (Table 4).

The time to prey competence was shorter for the nocturnal birds of prey (29.1 ± 17.90 days) than for the diurnal birds (92.6 ± 24.20 days) ($t = 7.1$, $p < 0.001$). The duration of the period to prey competence ranged between 10 and 47 days. However, black kites never managed to capture live food during the 102 days of the study (Table 4).

Number of weighings

Table 5 shows that the number of weighings of test birds was only significantly higher than the controls in the case of black kites. As also shown in Table 5, black kites never reached the highest level of difficulty. The number of weighings of each individual test bird ranged from five to 10 (average 7.2 ± 1.9) and was not statistically different from the average number of times that the controls were weighed (5.9 ± 1.9) (Table 5).

Discussion

Our results show that it is possible to introduce some degree of difficulty in the feeding of birds of prey kept in wildlife rehabilitation centres without negative consequences for the recovery process or for the birds' condition. Neither the weight nor the condition of the test birds differed from those of controls. However, in some cases the procedure may increase the number of times a bird is handled for weighing, which on the one hand may be harmful because of the increased stress imposed by capture, but on the other hand enables greater control of the clinical and physical condition of the animals.

The results also suggest that Strigiformes responded faster than Falconiformes. This may be due mainly to better physical condition of these birds upon arrival at the centre and to their metabolic needs. On the other hand, black kites never captured live prey; their necrophagous habits may contribute to explaining this (Cramp and Simons 1980).

Table 2. Type of dead food, dead food covering and live food, used to feed the different species in the study.

Species	Dead food	Type of dead food covering	Live food
Ardea cinerea	Small pieces of fish without scales (approx. 6 × 2 × 2 cm)	Scales	Small fish
Accipiter gentilis	Small pieces of rabbit without fur (approx. 4 × 4 × 4 cm)	Fur	Small mice
Milvus migrans	Small pieces of rabbit without fur (approx. 4 × 4 × 4 cm)	Fur	Small mice
Athene noctua	Small pieces of rabbit without fur (approx. 2 × 2 × 2 cm)	Fur	Small mice
Strix aluco	Small pieces of rabbit without fur (approx. 3 × 3 × 3 cm)	Fur	Small mice
Bubo bubo	Small pieces of rabbit without fur (approx. 4 × 4 × 4 cm)	Fur	Quails and rats (1:1 proportion)

Table 3. Number of weighings and time (in days) necessary for individuals of each species to achieve different levels of enrichment.

	Food enrichment level					
	3		4		5	
	Days	Weights	Days	Weights	Days	Weights
A. cinerea	4	3	7	4	25	7
A. gentilis	1	1	16	3	31	4
M. migrans	27	2	49	4	58	5
A. noctua	2	1	4	2	8	3
S. aluco	6	1	19	4	26	5
Bubo bubo	4	1	9	2	14	3

Table 4. Time to prey competence (Tcomp) for experimental group, and length of conventional recovery process (CRP) for control group (in days).

	Tcomp (test group)	CRP (control group)	Statistical result
Ardea cinerea	31 ± 0.0	49 ± 0.0	$\chi^2_1 = 3.61$; $p > 0.05$
Accipiter gentilis	38 ± 0.0	34 ± 0.0	$\chi^2_1 = 0.13$; $p > 0.05$
Milvus migrans	– (102 ± 0.0)	22.5 ± 6.5	$t = 25.04$; $p < 0.001$
Athene noctua	10 ± 0.0	35.0 ± 20.1	$t = 2.49$; $p = 0.089$
Strix aluco	47 ± 0.0	50.3 ± 14.8	$t = 0.44$; $p = 0.690$
Bubo bubo	33 ± 0.0	30 ± 0.0	$\chi^2_1 =$; $p > 0.05$

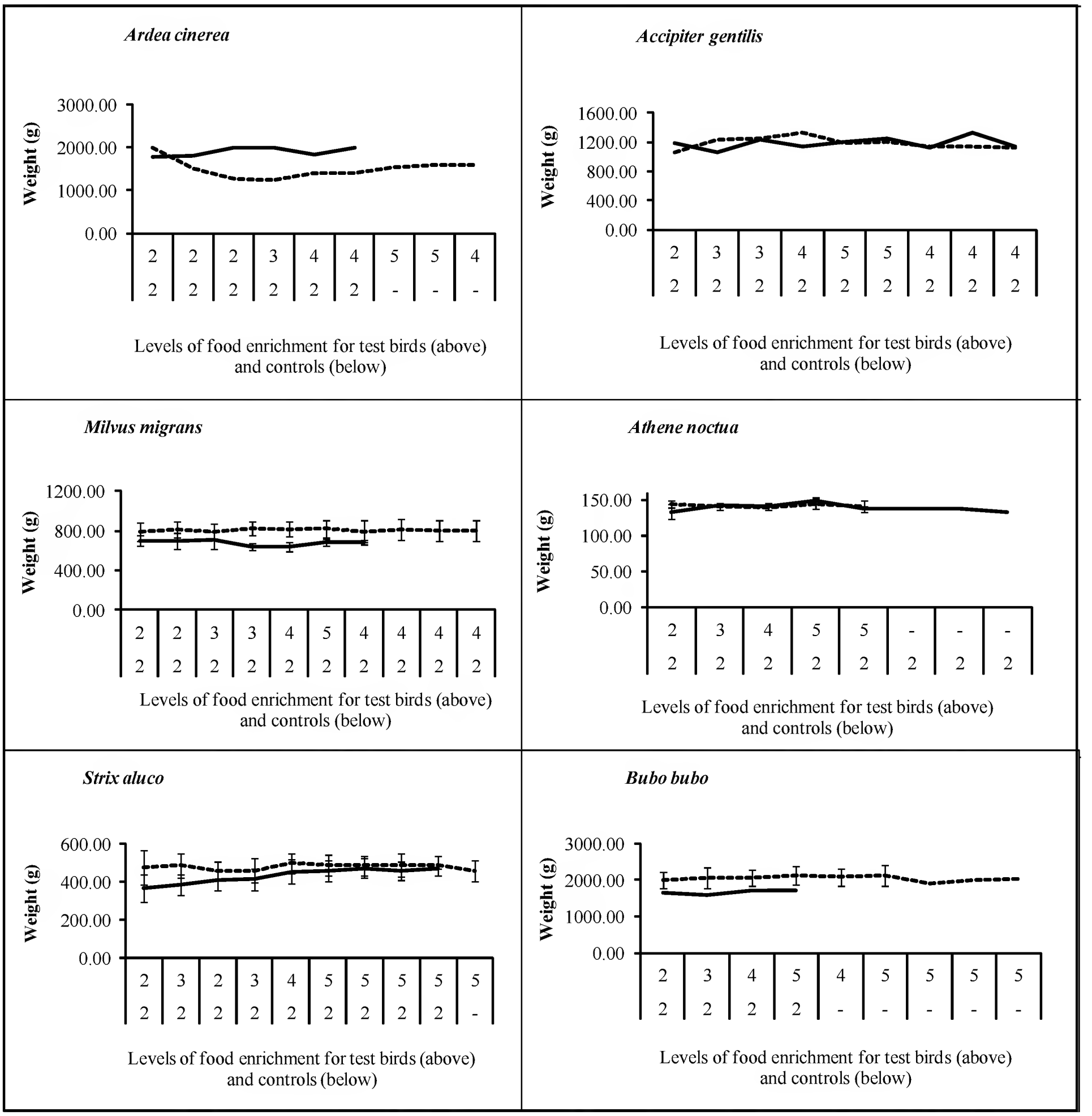

Figure 1. Weight variation in individuals of *A. cinerea*, *A. gentilis*, *M. migrans*, *A. noctua*, *S. aluco* and *B. bubo* over the food enrichment levels compared to controls. Dotted lines represent test birds, solid lines control birds.

Table 5. Number of weighings during the time to prey competence (test birds) and during recovery time (control birds).

	Test birds	Control birds	Statistical result
Ardea cinerea	8	6	$\chi^2_1 = 0.07$; $p > 0.05$
Accipiter gentilis	6	6	$\chi^2_1 = 0.08$; $p > 0.05$
Milvus migrans	– (10)	5.3 ± 2.06	– ($t = 4.61$; $p = 0.019$)
Athene noctua	5	5.3 ± 1.89	$t = 0.26$; $p = 0.809$
Strix aluco	7	7.8 ± 1.25	$t = 1.19$; $p = 0.319$
Bubo bubo	7	4	$\chi^2_1 = 0.36$; $p > 0.05$
Total	7.2 ± 1.90; $n = 24$	5.9 ± 1.87; $n = 15$	$t = 1.98$; $p = 0.055$

Some birds seem to respond faster than others to the difficulties created in response to their conditions, either physical – nutritional status and cause of admission to the rehabilitation centre (fall from nest, electrocution, etc) – or ecological, e.g. predation techniques (Nieuwenhuyse et al. 2008). It is to be expected that animals that suffered less trauma and that started the enrichment process in a good nutritional state would progress better in their ability to obtain food, but conditions in captivity may also influence the fitness of predatory birds. The size of cages relative to the size of the birds can affect prey competence, as can the existence of support structures such as perches and shelters (Schmidt-Nielsen, 1984). The fact that the birds are confined to outside cages means that the climate could also influence the results of food training, and we might expect seasonal variation in the results, particularly for migratory birds such as the black kite.

Overall, this process may contribute to improving rehabilitated birds' physical and psychological health as it stimulates movement and skills and, apart from the recovery process itself, this may increase the changes of survival once the birds are released. The effect of such a feeding regime on actual survival after release should be investigated.

Conclusions

1. Adding some degree of difficulty to the feeding of captive birds of prey recovering in wildlife centres does not seem to have negative effects on the recovery process.
2. The food enrichment strategy adopted here did not result in weight loss and/or decline of the birds' condition.
3. In most species, regular monitoring of weight during the experiment did not indicate an increase in the stress of handling or capture.
4. Some birds seem to respond faster than others to the difficulties created in response to their physical and ecological conditions and the characteristics of captivity.
5. Clearly more work is needed on this subject but the adoption of food enrichment strategies in predatory birds undergoing rehabilitation may prove to be very important in captivity and also after release.

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Research article

Diet review and change for monkeys at Paignton Zoo Environmental Park

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Abstract

Between 2003 and 2010 the diets of all medium-sized monkeys at Paignton Zoo Environmental Park were subjected to a continual process of review and improvement. This resulted in the removal of all fruit, bread, eggs and seeds from the diets and changes to commercial products used for some species. All species are now provided with similar diets consisting of a suitable commercial pelleted feed, a variety of fresh vegetables and small amounts of dog biscuits and cooked brown rice to provide opportunities for scatter feeding. Compared with the 2003 diets the 2010 diets have higher levels of protein (3–47% increase) and fibre (36–77% more NDF) and lower levels of readily digestible carbohydrate (6–14% decrease). Resultant health benefits have been improved dental health and weight loss in some previously overweight individuals. In addition, the 2010 diets are also considerably less expensive than the 2003 diets resulting in an estimated annual cost saving of £9717 based on current prices and animals held.

Introduction

Although primates are traditionally regarded as relatively easy to feed, there is growing recognition that inappropriate diets contribute to several common health problems seen in captive omnivorous primates (e.g. see Oftedal and Allen 1996; Schwitzer et al. 2009). Most common among these is obesity (e.g. Schwitzer and Kaumanns 2001; Videan et al. 2007), which is associated with many other illnesses such as heart disease, cancer, diabetes and reproductive problems (Register and Clarkson 2009).

Obesity can have a number of contributory factors, including low activity levels in captivity, but is largely due to captive diets that are high in energy without the foraging and digestive costs associated with the species' natural nutritive strategies (Schwitzer at al. 2009). This is particularly common in omnivorous primates, which are often fed a wide variety of food items, including fruit, vegetables, insects and vertebrate prey, reflecting assumptions about their food selection in the wild. Traditionally, most tend to be regarded as fruit eaters, at least to some extent, and are therefore fed fruit in captivity. This is even the case for some highly folivorous species. Unfortunately, fruit cultivated for human consumption is very different in terms of nutrient composition to leaves and wild fruits eaten by free-living primates (e.g. Oftedal and Allen 1996). Selective breeding and modern cultivation methods produce fruit that is high in sugars and low in fibre, and therefore high in readily digestible energy. Cultivated fruit also tends to be lower in protein, minerals and vitamins than most foodstuffs consumed by primates in the wild (see Schwitzer et al. 2009 for an excellent review). In addition to contributing to obesity, captive primate diets containing large amounts of cultivated fruit may cause gastrointestinal problems due to low fibre content (Edwards and Ullrey 1999) and poor dental health due to high sugar levels (Johnson-Delaney 2008).

Between 2003 and 2010 we underwent a continual process of review and improvement of diets fed to medium-sized monkeys at Paignton Zoo Environmental Park. These were initiated as a result of a number of different issues of concern that could be related to diet, although most of the individuals were generally healthy. The first of these, and the main trigger for diet review, was the poor dental health of several of the Abyssinian colobus (*Colobus guereza*) and king colobus (*C. polykomos*) monkeys. Following reviews of these diets, increased awareness of potential nutritional problems, particularly obesity, stimulated keepers on the section to instigate similar reviews of the diets for the rest of the monkeys in their care. Additional species present in this section of the zoo (echidna, pygmy slow loris,

Table 1. Monkey species included in diet reviews at Paignton Zoo Environmental Park between 2003 and 2010.

Species	Diet review period	Group size and structure	Main concerns
Abyssinian colobus *Colobus guereza*	March 2003–July 2008	Initially 2 family groups. Gradually phased out; 1 group of 2 males by 2010	Many individuals had serious dental problems including tooth decay and gingivitis
King colobus *Colobus polykomos*	March 2003–July 2008	Single male, multi-female plus young	Dental problems as above, but not so severe
Diana monkey *Cercopithecus diana*	April–September 2007	Adult pair plus young	Adult male overweight
Hamadrayas baboon *Papio hamadrayas*	July 2007	Large multi-male, multi-female group with many young (>50 individuals)	Cost of the diet for such a large group
Variegated spider monkey *Ateles belzebuth hybridus*	September 2007–July 2010	Adult pair, arrived at zoo 2007	Frequent loose faeces and diarrhoea
Sulawesi crested black macaque *Macaca nigra*	September 2007–July 2010	Group of mixed sex and age, 1 dominant adult male (10–15 individuals)	Frequent loose faeces and diarrhoea. Some overweight individuals

African porcupine, meerkat, sloth and red panda) were also subject to similar diet reviews over the same period but are not discussed in this paper.

Methods

Study animals

All animals studied were housed at Paignton Zoo Environmental Park, Devon, UK, and remained in their usual enclosures with normal husbandry throughout the study period. Subjects included all medium-sized monkey species included in the "Monkey Heights" section (Table 1).

Diet review process

Starting in 2003 we conducted collaborative, multi-department, nutrition meetings involving keepers on the Monkey Heights section, veterinary and research staff approximately every three or four months. Issues of concern possibly related to diets were raised by keepers, discussed and if felt necessary investigated further by the research team. A pattern very quickly emerged that the first step in any review was to assess exactly what the animals were currently eating. Dietary intake studies were initially conducted by researchers but all keepers on the section were soon trained in the method and then conducted the trials themselves.

Dietary intake

For all species the daily food intake was measured for at least five days and up to three blocks of five days over three weeks. All food items provided to the monkeys were prepared in the normal way and weighed immediately before presentation. A small amount of the same food items was placed in a desiccation dish at the same time to adjust for water loss in the leftovers. Food was presented as normal, usually in three feeds per day. Any uneaten food remaining the next day was collected and weighed along with food in the desiccation dish. Weight of remaining food was adjusted according to the desiccation rate and deducted from

Table 2. Weight of food (g, as fed) provided per individual per day for six species of monkey at Paignton Zoo Environmental Park prior to (2003) and following diet reviews (2010).

	Abyssinian colobus		King colobus		Diana monkey		Hamadryas baboon		Spider monkey		Sulawesi crested black macaque	
Food type	2003	2010	2003	2010	2003	2010	2003	2010	2007	2010	2003	2010
Primate pellet[1]	24		30		11		160	170	56		35	
Trio Munch[1]	50		50						12			
Leaf eater primate pellet[1]		24		24		40				30		70
Terrier biscuit[2]		16		16		10	33	40		16		30
Brown rice		54		54		10		20		25		35
Bread	30		30		25		40		20		30	
Seed mix[3]	5		6		30		30		20		38	
Dried fruit mix[3]	95		70						2		4	
Egg	3		58		10		11		36		9	
Fruit (apple, banana etc)	320		285		341		263		290		374	
Green leafy veg	293	450	402	450	45	475	71	156	118	325	49	338
Starchy root veg	82	363	91	363	29	75	54	156	106	325	138	150
Other vegetables	23	363	50	363	52	100	51	156	87	325	55	394

[1]Mazuri Zoo Foods, Witham, Essex, UK.
[2]Winalot mixer, Purina, Horley, Surrey, UK.
[3]Seed mix was 5% peanuts, 95% sunflower seed by weight; dried fruit mix was approximately equal weights of raisins and sultanas.

Table 3. Nutrient composition of the diets as consumed by six species of monkey at Paignton Zoo Environmental Park prior to (2003) and following diet reviews (2010). Highly digestible carbohydrate estimated by calculation (Dry matter – protein – fat – NDF – ash). ME = metabolisable energy.

	Abyssinian colobus		King colobus		Diana monkey		Hamadryas baboon		Spider monkey		Sulawesi crested black macaque	
Nutrient	2003	2010	2003	2010	2003	2010	2003	2010	2007	2010	2003	2010
Dry matter (g)	283	296	282	296	146	169	324	295	194	258	222	269
Crude protein (%)	11.7	16.4	15.9	16.4	19.8	21.2	18.9	21.7	16.9	18.4	12.9	19.0
Crude fat (%)	3.9	2.6	6.2	2.6	13.4	6.3	10.1	5.5	10.3	2.7	12.2	3.4
NDF (%)	12.6	17.2	9.7	17.2	11.5	17.9	11.2	15.8	10.5	17.6	11.9	18.2
ADF (%)	8.1	11.2	6.2	11.2	7.6	12.3	6.1	10.6	7.1	11.5	8.4	11.7
Ca (%)	0.44	0.48	0.67	0.48	0.46	0.75	1.20	1.5	0.90	0.57	0.48	0.60
P (%)	0.39	0.43	0.57	0.43	0.55	0.50	0.80	0.90	0.72	0.44	0.43	0.44
Highly digestible carbohydrate (%)	68.1	58.4	63.2	58.4	51.2	48.5	52.4	48.3	55.8	55.8	58.8	53.3
ME (kJ per day)	3765	3850	3680	3850	2385	2300	5650	4730	3180	3430	3600	3390

the provisioned weight to calculate weight of each food type consumed. Data were collected on a group basis and divided by the number of individuals to give a mean intake per individual. Following dietary changes food intake was recalculated as above.

Nutrient analysis and composition

To minimise time and cost, standard nutrient values given in Zootrition (Zootrition™, version 2.6) were used for most food items. Where these were not available food samples were subjected to laboratory analysis by an external laboratory (Eurofins, Wolverhampton). Mean daily intake of each food type per individual was entered into Zootrition and a full diet analysis performed. Readily digestible carbohydrate (sugars and starch) was estimated by calculation: 100% dry matter minus crude protein, crude fat, NDF and ash.

Recommended dietary changes

Following nutrient analysis of existing diets, some changes were recommended based on available information in the literature for the species, any issues in the group and recommended nutrient requirements (NRC 2003). Dietary changes evolved over a period of years starting with the removal of all fruit, then bread and eggs and lastly sunflower seeds. For some species the commercial pellets used were changed to increase fibre content. Fruit and dried fruit were replaced with vegetables. Vegetables were divided into three types: Group A, 'green leafy', such as cabbage, lettuce, spinach; Group B, 'other', such as celery, cucumber, peas, peppers, fennel; and Group C, 'starchy root', such as carrots, beetroot, swede, sweet potato. To make diet preparation easier the total amount of each vegetable group was stated rather than each particular type of vegetable.

Results

Dietary intake

Prior to the review process, most of the monkeys were fed a similar diet of commercial pellets, fruit and vegetables, a seed mix (peanuts and sunflower seed), a dried fruit mix (raisins and sultanas), bread and eggs (Table 2). Very little food was left uneaten by any species, so the amounts of provisioned food are similar to those actually consumed in most cases. During the diet review process all fruit and dried fruit was removed from the diets. Bread was included in the original diets because it had traditionally been donated free by local supermarkets when nearing its sell-by date. However, this had stopped and it was now being purchased at a much higher cost than providing similar grain-based nutrients via pelleted feeds; bread was therefore removed. The seed and dried fruit mixes were considered important to provide a hard-to-find scatter feed and promote foraging behaviour but are high in energy and sugar. These were replaced with alternative dry feeds; terrier biscuit and cooked brown rice. Most species were switched from primate pellet to leaf eater primate pellet to further increase fibre levels.

Nutrient composition

By 2010 the diets of all six species were higher in protein, with increases of 3–47% compared with their 2003 diet (Table 3). Fibre levels were also higher, with NDF increasing by 36–77%. Estimated levels of readily digestible carbohydrate decreased by 6–14%. With the exception of the two colobus species, fat levels also decreased substantially, largely due to the removal of sunflower seeds from the diets.

Dental health

Following dietary adjustments the frequency of dental treatment required decreased rapidly (Fig. 1). Between 1998 and 2003 there were 17 instances of treatment for dental problems including gum disease, gingivitis, tartar build up, the removal of several teeth and even one euthanasia due to extremely poor dental condition. These involved 12 different individual monkeys, all but one of which were Abyssinian colobus (Fig. 1). Following the initial changes to the diet to reduce sugar levels, the frequency of dental treatment

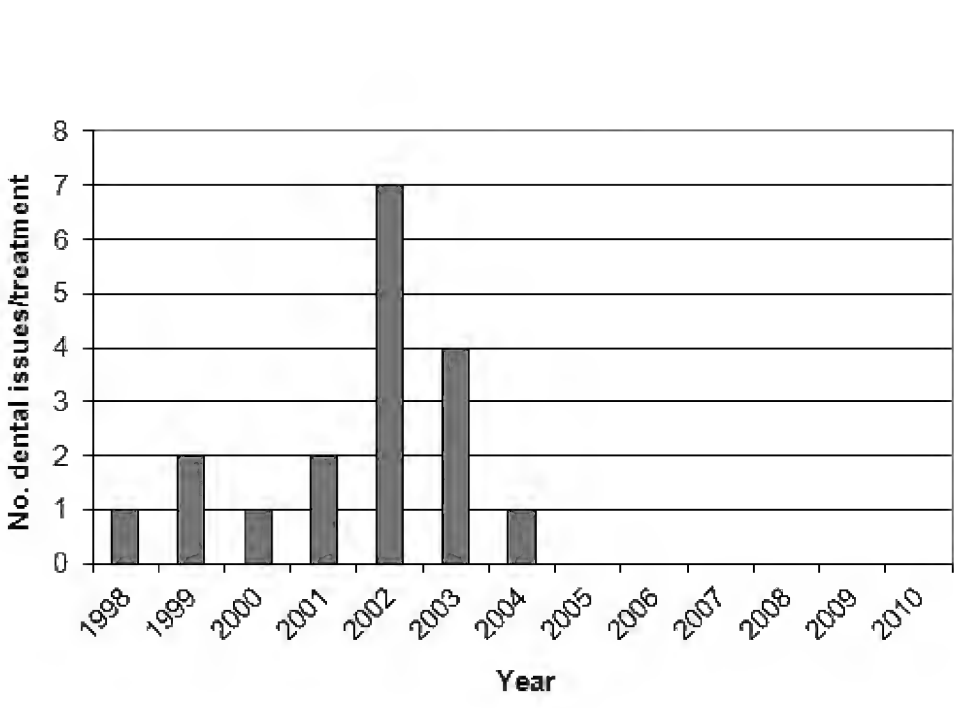

Figure 1. Frequency of dental issues identified or treatment required for six species of primate at Paignton Zoo Environmental Park before and after the initiation of diet improvements to reduce dietary sugar (March 2003).

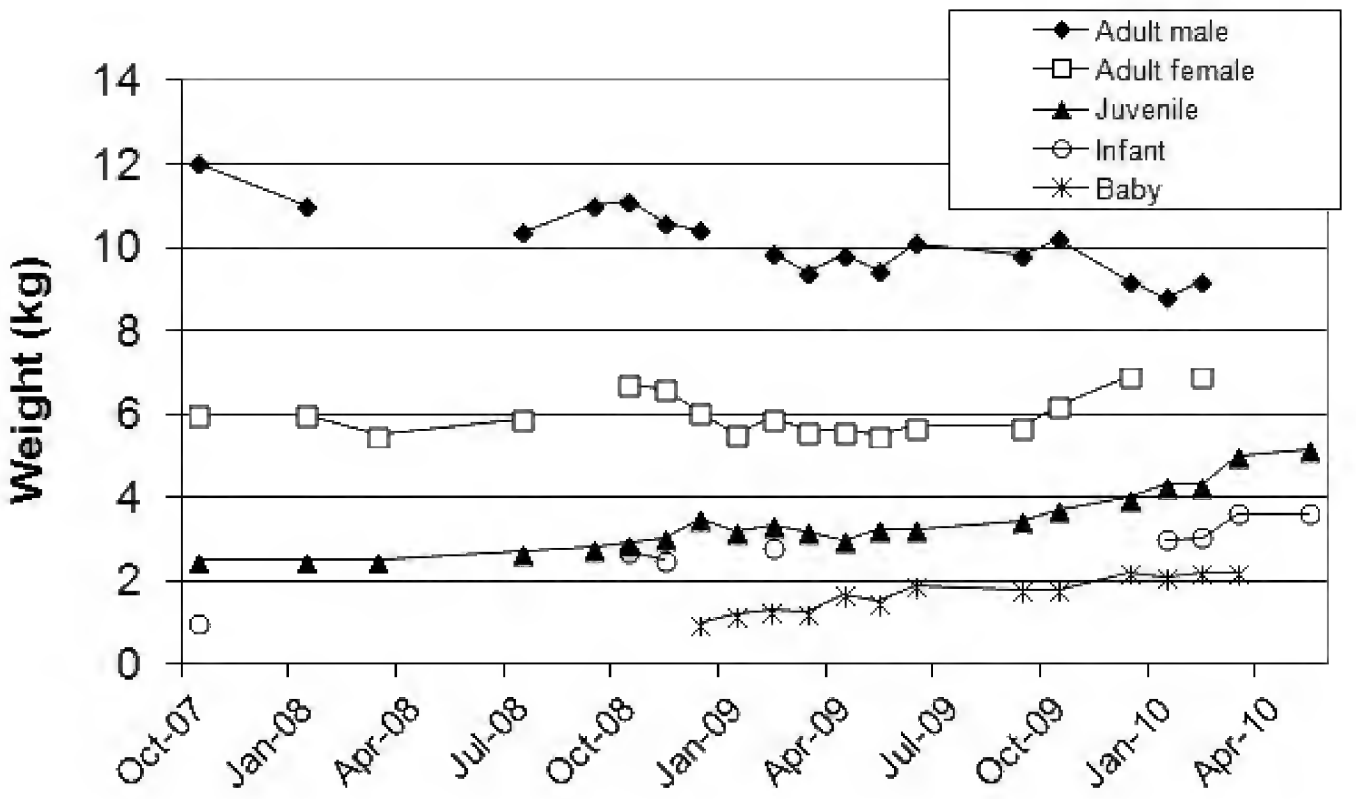

Figure 2. Body weights of a group of Diana monkeys at Paignton Zoo Environmental Park following a diet review that prompted a change from a fruit-based to a vegetable-based diet. The removal of all fruit occurred in April 2007; other changes were completed in September 2007 immediately before the first weights were obtained.

declined markedly, to the extent that veterinary intervention specifically for dental treatment has not been necessary since 2004. Dental health continues to be monitored opportunistically when monkeys are restrained for other reasons and is generally very good.

Body weights

In order to monitor the effects of diet reviews over this period, keepers on the section began to train the animals to station on weighing scales and have since been able to obtain body weights on a regular basis for most individuals. The diet changes implemented resulted in gradual but sustained weight loss of some overweight individuals within a group, whilst others maintained stable healthy weights. This was particularly noticeable in the Diana monkey group, where the overweight adult male decreased in weight from 12.0 kg in October 2007 (immediately after the first diet changes were made) to 9.2 kg in early 2010, and appeared to be stable at this weight prior to his departure to another zoo in late 2010 (Fig. 2). A similar effect was also seen in the Sulawesi crested black macaques, where three overweight individuals also showed slow, sustained weight loss whilst other members of the group maintained steady weights throughout. These were the adult male (from 17.0 kg to 14.5 kg) and two adult females, both 9.0 kg before diet changes and now 7.5 kg and 6.9 kg. These three individuals also now appear to be at a stable weight.

Faecal quality

Faeces was not formally monitored, but anecdotally keepers report that for all species, with the exception of Sulawesi crested black macaques, it has generally improved in consistency with far fewer instances of loose faeces. The Sulawesi crested macaques still have frequent diarrhoea that does not appear to be related to diet and to date remains unexplained despite extensive veterinary investigation.

Diet costs

The cost of the 2003 diet for each species was calculated using 2010 prices for each ingredient and compared with the cost of the current diet (Table 4). The 2010 diets are substantially cheaper than the 2003 diets for all species. For the total number of individuals held in 2010 these reductions in cost represent an overall saving of £9717 per year as a result of using the 2010 diets rather than the 2003 diets.

Discussion

The diet review process in the Monkey Heights section started in 2003 and has resulted in changes to most of the medium-sized monkey diets such that they now all receive a very similar diet that is higher in fibre and protein and lower in readily digestible sugars and starch than previously. During the review process, low fibre and

Table 4. Cost comparisons of the diets fed in 2003 and 2010 to six species of monkey at Paignton Zoo Environmental Park; all costs based on 2010 prices (UK £ sterling).

		Abyssinian colobus	King colobus	Diana monkey	Hamadryas baboon	Spider monkey	Sulawesi crested black macaque
Cost per monkey per year	2003 diet	441	453	291	359	331	362
	2010 diet	296	296	162	222	241	267
Group size in 2010		2	3	4	50	5	12
Total annual cost	2003 diet	882	1359	1164	17950	1655	4344
	2010 diet	592	888	648	11100	1205	3204

high sugar in largely fruit-based diets was concluded to be the most likely cause of a variety of issues of concern. All fruit was therefore removed from the diets and the amount of vegetables increased. However, some vegetables, particularly starchy root vegetables also contain high levels of readily digestible carbohydrate (e.g. Clauss et al. 2010). In order to prevent overfeeding of these vegetables types, the 'three veg group' system was devised. These three groups are green, leafy vegetables (e.g. cabbage, spinach, lettuce), starchy root vegetables (e.g. carrots, swede, squashes) and others (e.g. peas, onions, fennel). The total weight of each group is specified but keepers are free to use any vegetable type within the group to make up that weight. This, along with the fact that diets across the section are now so similar, has reduced preparation time.

In addition, because we have replaced relatively expensive fresh and dried fruit with cheaper vegetables, and increased scope for flexibility to take advantage of seasonally cheap produce and special offers, we have made substantial costs savings, estimated to be on average £129 per year per monkey, resulting in an overall saving of nearly £10,000 per year.

Although it has increased since 2003, the fibre content of the 2010 diets is still not as high as that found in items consumed by free-living monkeys (typically over 50% NDF; Oftedal and Allen 1996). However, the dietary analysis presented here does not include browse, which is very high in fibre and provided to most of the target species on a regular basis.

The main impetus for beginning diet reviews on this section was poor dental health of the colobus monkeys, which had necessitated 17 instances of restraint and anaesthetic for treatment within the previous five years. The removal of sugary fruit from their diet had an almost immediate effect, drastically reducing the need for treatment. Since 2003 there has only been one case in which an individual of the species concerned has been specifically restrained for a dental condition. The vast majority of dental issues affected the Abyssinian colobus monkeys so we would expect fewer instances now we have fewer individuals in the collection. However, until about 2007 the numbers of animals was still similar to the situation pre-2003, so there was a genuine reduction in treatment rate, at least from 2004–2007.

An unexpected result of the dietary changes was weight loss of overweight individuals within a group, whilst other animals in that group maintained healthy weights. This was achieved without taking steps to limit food intake of particular individuals, such as separating them at feeding times. These overweight individuals are the more dominant animals in the group and so are able to select and consume much more than their fair share of their preferred food items (Smith et al. 1989). When the diet included many highly desirable, sweet items, this ability to select preferred items and prevent access for other group members presumably resulted in an over-consumption of high energy items. It appears that when these highly preferred items were removed from the diet, there was much less motivation for the dominant animals to monopolise certain food types, and thus they no longer consumed more than their fair share of the group diet. Within the macaque group, the most subordinate individual is now the heaviest adult female, possibly due to higher voluntary energy intake in response to the chronic stress of being subordinate (Wilson et al. 2008).

During the process of diet review for the monkeys only very minimal human resistance to changing the diets was encountered, mostly towards the initial removal of all fruit. Possible factors limiting resistance to change could include the gradual nature of the changes over a period of time. It was also beneficial that support for review and change was generated across all relevant departments early on in the process through collaborative and consensus-building meetings, where keepers were the main drivers in determining priorities for dietary review.

Conclusions

A continual process of diet review and adjustment for all medium sized monkeys over several years has resulted in less expensive diets that better meet the nutritional needs of the animals. The most significant change made to the diets was the removal of all fruit, including dried fruit, in order to reduce the levels of readily available energy and particularly sugar in the diets.

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Dietary management of an obese kinkajou (*Potos flavus*) with congestive heart failure secondary to hypertrophic cardiomyopathy

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Abstract

An adult castrated male pet kinkajou (*Potos flavus*) with a history of bilateral stifle osteoarthritis was diagnosed and successfully treated for congestive heart failure secondary to hypertrophic cardiomyopathy. The animal was markedly obese and with a presenting body weight of 8.8 kg. The kinkajou's home diet was not balanced and there was no energy restriction. Dietary modification was directed as part of the patient's clinical management, and owners were given guidance on content and proportions of the recommended diet. Dietary changes were implemented over several months with some adaptations based on the kinkajou's food preferences and clinical improvement. The maintenance diet was calculated to about 1047 KJ/day and mainly consisted of an assortment of fruit and sugar-free juice. The kinkajou responded well to its new diet, and over a 6-month period its weight decreased by almost 40%. Obesity is considered a major risk factor for developing cardiac disease in many mammalian species and may contribute to the high prevalence of cardiomyopathy in this particular species. Proper diet composition, adhering to what is known about diets in the wild, along with controlled energy intake, can reduce the health risks commonly related to obesity, increase longevity, and offer better care for captive kinkajous.

Background

Kinkajous (*Potos flavus*), medium-sized mammals, belong to the family Procyonidae, related to raccoons, coatis, ringtails and olingos (Ford and Hoffmann 1988). Kinkajous inhabit the canopies of neotropical primary rainforests throughout Central America and northern South America (Ford and Hoffmann 1988). Kinkajous are often found in zoological collections and also as privately -owned pets (Wright and Edwards 2009).

In this case, a 14.5-yr-old castrated male kinkajou was presented for severe respiratory distress. Prior history included castration at 3 years of age, bilateral stifle osteoarthritis, and intermittent bronchitis for the past 5 years treated with unreported antimicrobials. The animal was diagnosed with congestive heart failure (CHF) secondary to hypertrophic cardiomyopathy (HCM) and suspected pulmonary arterial hypertension (Eshar et al. 2010). Diagnosis was based on history, clinical signs, clinical pathology, radiographs, abdominal ultrasonography, abdominal fluid analysis, electrocardiography, and echocardiogram (Eshar et al. 2010). An undetermined hepatopathy was also found at presentation and resolved following metronidazole antimicrobial treatment.

Figure 1. Picture of the kinkajou taken at the initial presentation. The animal appears markedly obese, the abdomen is severely distended and with no ability to move. There is loss of body line curvatures and the animal has a BCS of 5. The abdomen was shaved for the sonographic evaluation.

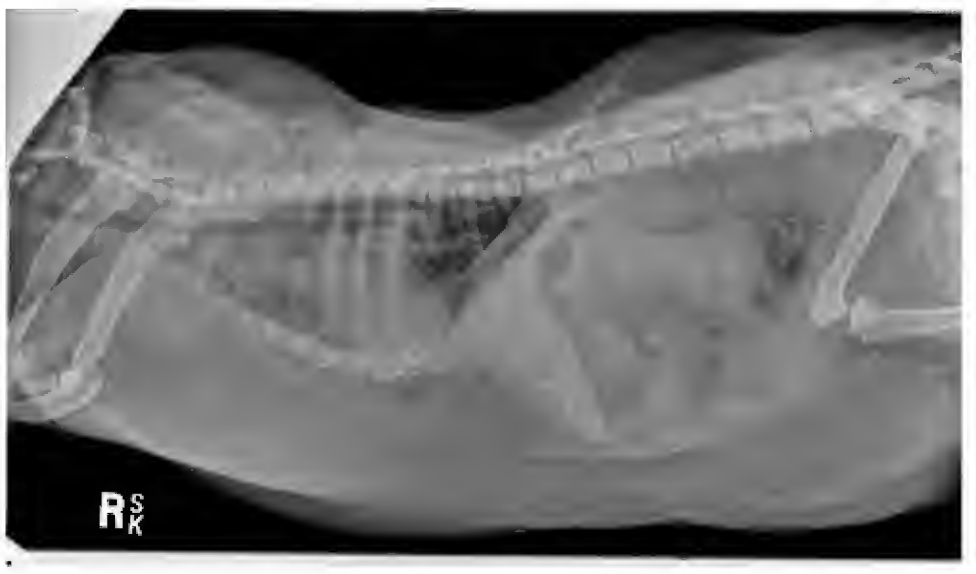

Figure 2. Right lateral radiograph of the kinkajou on presentation. There is bi-cavitary and subcutaneous effusion, cardiomegaly, hepatomegaly with multiple foci of mineralisation and stifle osteoarthritis. There is a marked subcutaneous adipose layer over the thoracic and abdominal regions.

Cardiopulmonary medical treatment, including a loop diuretic, an ACE-inhibitor, a beta-adrenergic receptor blocker and a bronchodilator provided improvement of the clinical signs (Eshar et al. 2010).

On presentation the kinkajou's weight was 8.8 kg (Figs 1–2), almost 200% of the reported upper range for captive male kinkajous (1.9–4.75 kg) (Wright and Edwards 2009). Using a body condition score (BCS) of 1 to 5, with 1 representing an emaciated kinkajou, 3 being a healthy weight, and 5 being an obese animal, a healthy kinkajou should appear sleek from all sides, similar to the domestic cat (Wright and Edwards 2009). As a result of its medical condition the animal was almost completely sedentary with no ability to freely lift itself or move around. The owner obtained the animal as a weaned baby at 4 months of age, and since then, the home diet had no calorie limits and comprised table scraps, rice porridge baby formula, high calorie sports drinks, candy and select fruits.

An adequate kinkajou diet is a matter of debate in the literature. Some resources describe kinkajous as omnivorous, feeding on insects, small reptiles, rodents and a diverse range of wild fruits (Nowak and Paradiso 1991) with recommendations for keepers to provide animals with up to 50% live protein in their diet (Pernalete 1997). Scientific observations from the field suggest that although within the order Carnivora, and without having obvious anatomic–physiological adaption, kinkajous are primarily frugivorous, feeding almost exclusively on fruits (90%) supplemented with nectar, pollen and leaves (Charles-Dominique et al. 1981; Bisbal 1986; Julien-Laferriere 1999; Kays 1999).

Action

Once the cardiac disease was under control, and since weight loss can have a positive impact on longevity and ability to cope with the cardiac and joints diseases, a long-term weight loss plan was part of the clinical management of this patient. The weight loss strategy aimed to address two key points: first, to normalise animal–owner food interaction, where the owner needed to recognise the problem and be willing to take corrective steps, and second, to supplement an adequate (i.e. balanced and energy restricted) and palatable diet.

The weight loss protocol was designed to put the kinkajou in a 'negative energy balance'. The resting energy requirement (RER) was calculated using the following formula for animals weighing 3–25 kg: RER = (30 x current body weight in kg) + 70 = 334 kcal/day (Chan and Freeman 2006). Kinkajous are thought to have a basal metabolic rate of approximately 0.316 mL O_2/g/h, which is only 65% of the predicted mammalian metabolic rate calculated by the Kleiber (1961) formula: M (kcal/h) = $3W^{0.75}$. Therefore, a kinkajou's diet should contain 209 KJ/day less than the one of an equivalent size placental mammal, and in this case the kinkajou's RER was calculated to be 1189 kJ/day. Due to its obesity and decreased ability to exercise, the daily energy requirement (DER) was calculated as DER = 0.8 x 1189 = 963 kJ/day.

Nutrient requirements of kinkajous have not been studied clearly or defined under controlled conditions (Wright and Edwards 2009). As a result, practical diets are extrapolated from the nutrient requirements of domestic dogs, and a detailed description of such diets and their nutritional contents can be found in the literature (Wright and Edwards 2009). However, in this author's experience not all kinkajous can accept the composition of the canine diet; some animals either eat too much of the dog kibble or completely ignore it for the lack of palatability. In this case, the kinkajou was given a diet based on its favoured items with a combination of commercial fruits and vegetables. Bananas, apples, oranges, kiwi, melons, pears and berries were given in moderation to prevent sugar overload. Favoured vegetables included cucumbers, corn, snow peas and sweet potatoes, which have a nutrient content similar to many wild fruits. The initial amounts to be given were calculated to be about 3 cups (921 kJ) per day. Dried fruits (raisins, dates, prunes, papaya, pineapple, apricots) were given as single treats to maintain the owner–pet relationship.

Fruit consumed by wild kinkajous has lower sugar, starch and moisture content, higher protein and fibre content, and more concentrated minerals and vitamins than domestically available fruits (Edwards 2006; Wright and Edwards 2009). Dietary protein was provided by offering beans, peas and low-fat non-dairy peanut butter (10 g), which was also another positive reinforcement treat. The undetermined liver disease of this kinkajou was taken into consideration, and protein requirements were adjusted based on those recommended for dogs with hepatic disease that need 2–3g/419 kJ/day (Chan and Freeman 2006). Dietary fibre was included by giving cooked pumpkin and sweet potatoes. Multivitamin (5 drops/day: Enfamil Poly-Vi-Sol: multivitamin drops, manufactured by Mead Johnson & Company, Glenview, IL, USA) was given because of the concern about hypovitaminosis from offering an incomplete diet. Although some dried figs (relatively high calcium content) were given as treats, the kinkajou was also given a calcium supplement (1 ml/day: Calcionate: Ca-glubionate syrup, manufactured by Rugby Laboratories, Inc. Duluth, Georgia, USA).

Consequences

The kinkajou was checked on a monthly basis. After one month the kinkajou lost 1.3 kg from its presenting body weight (15%). It is also possible that the diuretic drugs had an initial weight loss effect as internal body effusions and subcutaneous oedema started to resolve. Clinically, the kinkajou started to move around, and water with natural sweetener (SteviaPlus Fiber™: artificial sweetener, manufactured by Wisdom Natural Brands, Gilbert, AZ 85233, USA) was provided. Since fluids are important for weight reduction diets because of the low calorie:volume ratio, water-based fruit shakes (with flax seeds and natural sweetener) were also given.

After two months the kinkajou's weight dropped by 2.8 kg (32%) to 6.0 kg. The animal was now moving more and behaving as if hungry because of the increased energy requirements, and the daily rations were increased by another 0.5 cup/day (approximately 150 kJ) mixed fruits. Environmental enrichment was needed to increase physical activity, and food was offered in

a covered bucket with holes in it. After six months the kinkajou's weight was 5.7 kg (35% less). The owner reported that the animal was now able to climb up and down stairs, run around the house and jump from ground level up to table height. Exercise is the best and practical means of increasing energy expenditure, and it also benefits obese patients by lessening the loss of lean body mass and maintaining or improving resting energy rate (RER); therefore, further physical enrichment was provided by placing dried fruit in a Wiffle Ball (hollow toy ball, manufactured by The Wiffle Ball, inc., Shelton, CT, USA) and ferret Kong toys (Kong Ferret Treasure: rubber toy, manufactured by Kong Company, Golden, CO, USA).

After 11 months the kinkajou's weight was 5.3 kg, a drop of 3.5 kg (39%) from the initial presentation. Clinical evaluation, including blood tests, abdominal ultrasonography, radiology and cardiography showed marked improvement of the cardiac and hepatic disease.

This case emphasises the need for weight management and diet considerations to be part of the clinical management of every medical case. It also demonstrates the challenges in clinical management of a severe chronic disease parallel to dietary management of an exotic species with our limited knowledge of its nutritional requirements.

Kinkajous are commonly kept in small cages, not allowing for enough energy expenditure, and food is usually offered with no caloric restriction and few foraging opportunities (Wright and Edwards 2009). Neutering can be another contributing factor to obesity, with the absence of the catabolic effect of testosterone. Captive kinkajous are often obese and commonly present with associated medical conditions such as diabetes mellitus, pancreatitis, arthritis and cardiovascular diseases (Wright and Edwards 2009). Since prevention is the best way to fight obesity, it is our hope that kinkajous in captivity receive better nutrition and thus evade many of their common dietary-related illnesses.

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Evidence-based practice

Bear weight management: a diet reduction plan for an obese spectacled bear (*Tremarctos ornatus*)

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Abstract

Spectacled bears (*Tremarctos ornatus*) are agile climbers and many aspects of behaviour in the wild are related to procuring food. In captive situations obesity can result from reduced activity and offering food in excess of energy requirements if food is not presented in a way that encourages increased foraging time. In December 2006, a fifteen-year-old male spectacled bear was received into quarantine at the Smithsonian Institution's National Zoological Park. During the quarantine examination, nutrition staff manually assessed body condition and at 222.5 kg, the animal was deemed grossly obese with a body condition score of 9 on a 1–9 point scale. A weight reduction plan was developed with a goal of gradual and continual weight loss at a rate of 1.0% of initial body weight (BW) per week, with a maximum of 2.0% and a minimum of 0.5% of initial BW/week to reach an initial summer target BW of 170 kg in 6–8 months. Once the initial goal was achieved, seasonal target BW ranges were further refined over the following 12 months, resulting in a total weight loss of 77 kg (35% of initial BW). Throughout the process keepers noted a marked positive increase in physical activity and associated behaviours. The male was successfully introduced to a newly acquired female spectacled bear and mating occurred during June 2009, with two genetically valuable cubs born in January 2010.

Introduction

The last of the lineage of short-faced bears (subfamily *Tremarctinae*), the Andean or spectacled bear (*Tremarctos ornatus*), is endemic to the tropical Andes and the sole extant bear species in South America. Much of spectacled bear behaviour is related to food selection and abundance, and this species is known to move significant latitudinal and altitudinal distances and across several types of vegetation in search of food and in response to seasonal ripening (Nowak 1999; Rios-Uzeda et al. 2006). In the wild, spectacled bears demonstrate a preference for terrestrial and epiphytic bromeliads; these plants are available year round and thus provide a staple diet item when ripe fruit is unavailable (Nowak 1999; Troya et al. 2004). Spectacled bears are also the main mammalian fruit and berry consumer within their range and serve as extremely valuable seed dispersers (Clark 2004; Rios-Uzeda et al. 2006).

Spectacled bears are agile climbers with non-retractable, thick, hook-like claws to assist in digging up and tearing apart food items. Similar to other bear species, spectacled bears rely heavily on an acute sense of smell to find food (Peyton, 1998). Highly developed radial sesamoid bones, or 'false thumbs', aide in manipulating vegetation and arboreal locomotion (Salesa 2006) while enlarged molar grinding areas and strong jaw muscles assist in mastication of vegetation (Sacco and Van Valkenburgh 2004). In addition to a wide range of plant material, an estimated 4% of the diet consists of animal matter (Peyton 1980). Spectacled bears do not hibernate due to year-round food availability throughout their range (Paisley and Garshelis 2006).

Diet formulation in zoological collections involves an understanding of general feeding ecology, gastrointestinal tract anatomy, nutrient content of foods consumed in the wild, nutrient requirements, husbandry and behavioural factors, and assessment of food items and ingredients that are available. Obesity can result from a number of factors, including (1) offering food in excess of an individual's energy requirements; (2) offering food items of poor nutrient quality and/or items

Table 1. Smithsonian National Zoological Park (NZP) Department of Nutrition clinical record: male spectacled bear quarantine examination.

History

1. Specimen received into NZP quarantine, December 2006.

Subjective

1. Immobilized for physical exam.
2. Body condition evaluated in both lateral and sternal recumbency.
3. Smooth transition from neck into shoulder.
4. Visual evidence of any skeletal landmark lacking throughout.
5. Ribs palpable with heavy pressure. Unable to palpate spine of scapula, spinous processes, tuber ischii, or tuber coxae due to thick layer of subcutaneous adipose tissue.
6. Head of tail difficult to locate due heavy fat layer adjacent to this anatomical landmark.
7. Oral health is compromised, and follow-up procedures are indicated to address.

Objective

1. Tip of nose to base of skull = 34 cm; base of skull to head of tail = 152 cm; tail = 9.5 cm.
2. Right fore leg circumference at elbow = 52.5 cm.
3. Right fore foot length = 19 cm; right hind foot length = 23 cm.
4. Neck girth = 76.5 cm; chest girth = 132 cm; abdominal girth = 177 cm.
5. Axial skin fold thickness = 11.8 mm; inguinal skin fold thickness = 7.5 mm.
6. Bioelectrical impedence analysis (BIA) was measured: Resistance = 122.
7. Total body fat, % = 48.2.
8. Body weight recorded = 222.5 kg.

Assessment

1. Specimen is grossly obese (30% over target BW) (BCS = 9 on a 1–9 scale).
2. Total body fat measurements (48.2%) exceed those of temperate species bears (*Ursus americanus*) during early winter (31–45%) (Farley and Robbins 1994, 1995; Lundbery et al. 1976).

Plan

1. Diet modifications required to facilitate weight loss, as well as address oral health issues.
2. Initial target BW = 170 kg.

inappropriate for an animal's gastrointestinal tract anatomy; and (3) lack of emphasis on diet presentation – it is very beneficial to present diet and enrichment items in ways that encourage natural feeding behaviours and increase activity level through increased foraging time.

Case report

In December 2006, a fifteen-year-old male spectacled bear (*Tremarctos ornatus*) was received into quarantine at the Smithsonian Institution's National Zoological Park (NZP). As part

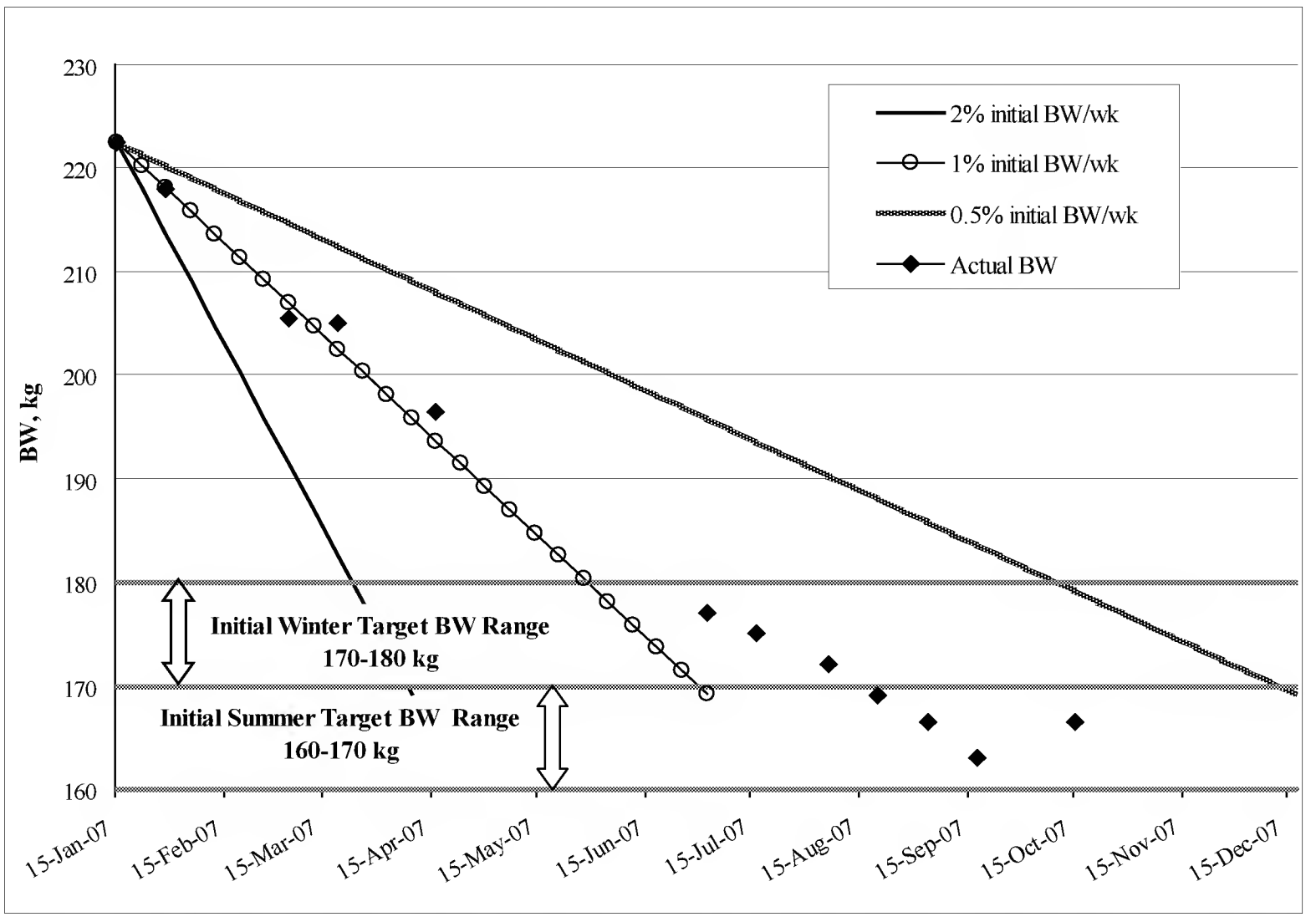

Figure 1. Weight reduction plan for male spectacled bear, with initial seasonal target body weight ranges.

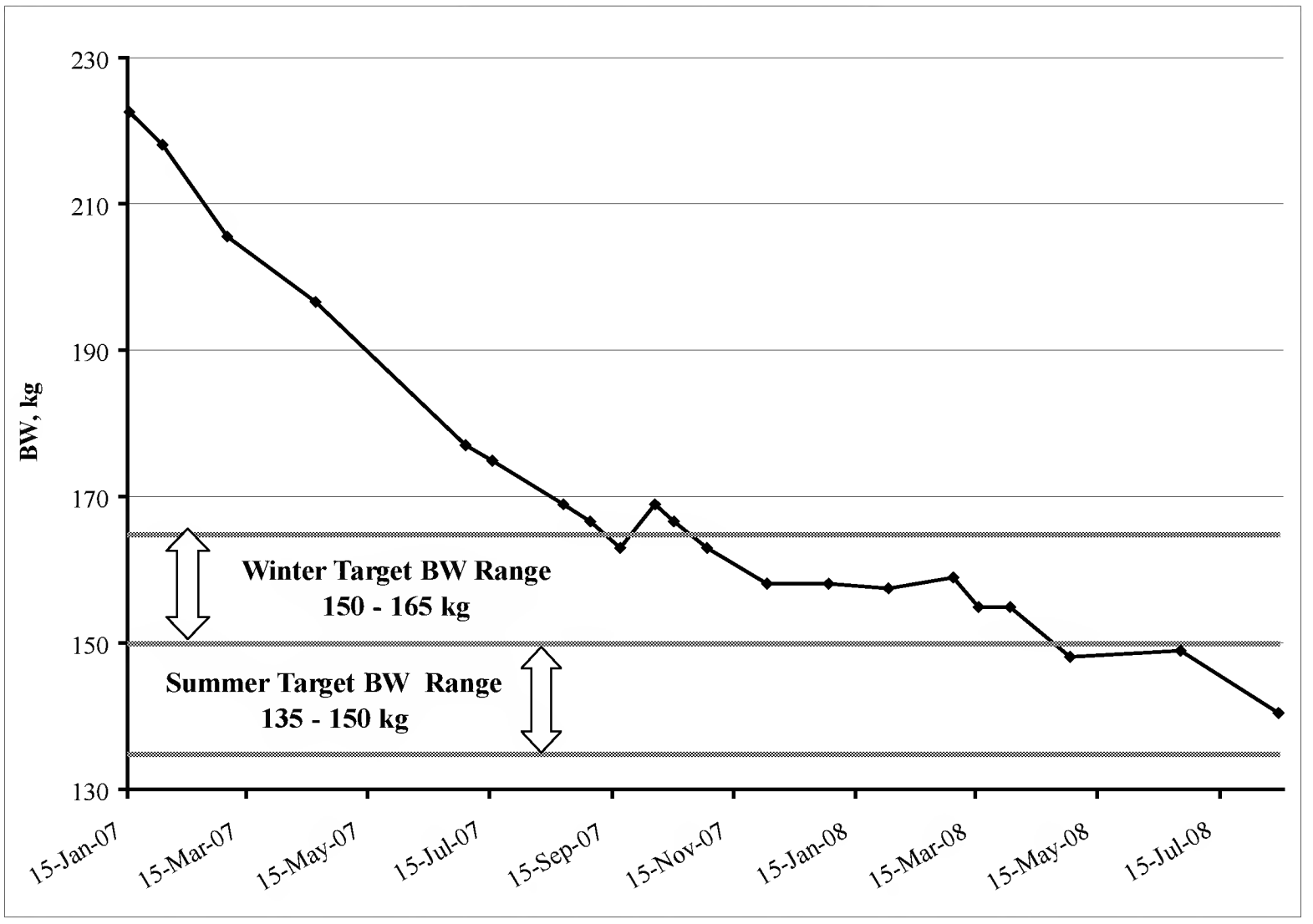

Figure 2. Revised seasonal target body weight ranges for male spectacled bear.

of collection planning and species management the animal was transferred to NZP for breeding purposes. Diet information from the sending institution was analysed, and although components and nutrient content of the base diet were appropriate for the species, the quantity of food was excessive, providing 31,694 kJ metabolisable energy (ME) per day. This determination was based on historic diet and body weight (BW) records for male spectacled bears, as well as the estimated ME requirement of adult inactive dogs: kJ/d = 397.7(BW, kg)$^{0.75}$ (NRC 2006). Using this equation, the ME requirement for a 170 kg bear is 18,724 kJ/d. The National Research Council equation provides a frame of reference; however, given species and seasonal variability, the authors do not suggest that it be used as a definitive tool for calculating the ME requirement of bears. Additionally, the above calculations did not include a wide variety of food-based enrichment offered to the animal on a daily basis. Furthermore, due to poor oral health, the animal had been prescribed long-term medications, which were noted to be well accepted with vehicles such as honey, molasses, syrup and fruit preserves.

As part of the quarantine examination, nutrition staff manually assessed body condition, recorded physical measurements, and determined total body fat content by bioelectrical impedence analysis (Farley and Robbins 1994; Lintzenich et al. 2006). The detailed clinical record is provided in Table 1. At 222.5 kg, the animal was deemed grossly obese, with a body condition score (BCS) of 9 on a 1–9 point scale. Total body fat (48.2%) exceeded that of a temperate species of bear (*Ursus americanus*) during early winter (31–45% body fat) (Lundbery et al. 1976; Farley and Robbins 1994, 1995). As noted previously, *Tremarctos ornatus*, a South American species, does not hibernate.

A weight reduction plan with a specified timeline was developed and presented to NZP veterinarians and animal care staff. The overall goal was gradual and continuous weight loss over a 6–8 month period at 1.0% of initial BW/week, with a maximum of 2.0% and minimum of 0.5% of initial BW/week (Burkholder and Toll 2000), to reach an initial summer target BW = 170 kg (Fig. 1). The plan also coincided with a period of seasonal weight loss characteristic of the species. Fish was found to be an adequate vehicle for medication, and all sugar-based foods were eliminated.

The base diet was reduced sequentially (approximately 10% of average daily kJ at each step) in conjunction with regular weighing, visual assessment of body condition, maintenance of detailed food consumption records and ongoing communication with primary keepers. Food-based enrichment was initially removed from the diet and later reintroduced in defined types and quantities to provide 1250 kJ ME/d. Keepers made valuable use of their knowledge about the natural history and feeding behaviours of this species, and presented the base diet and food enrichment in interesting ways to encourage increased activity. Throughout the series of diet reductions, the animal was monitored for behavioural changes such as aggression, difficulty shifting, and development of stereotypic patterns – none were noted. Seasonal target BW ranges were further refined over the following 12 month period (Fig. 2), resulting in a total weight loss of 77kg (35% of initial BW). Sequential diet records are maintained, providing a framework for standardised seasonal diets for this individual.

Upon arrival into the collection the male spectacled bear reportedly exhibited little interest in daily routines or novel food presentation, and even minor movements were laboured. Health and well-being benefits of weight reduction were clear, but there were also concerns about this animal's ability to breed. Keepers noted a marked positive increase in physical activity and interaction with enrichment items throughout the weight reduction period,

and this activity level has been maintained to the present. NZP has since received a female spectacled bear. The pair was successfully introduced, many mating bouts were observed during June 2009, and two (1.1) genetically valuable cubs were born in January 2010.

Conclusions

1. Communication, education, and setting distinct achievable goals, while meeting the animal's husbandry, behavioural, and nutritional needs, were key components to successful weight reduction in this individual.
2. Detailed record keeping is an integral component of animal care.
3. Prevalence of obesity can be greatly reduced through objective assessment of body condition, setting target body weight ranges, and recording body weights on a regular basis.

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Evidence-based practice

The use of risk analysis methodology to generate evidence-based decision making in zoo animal disease management: using simian immunodeficiency virus (SIV) in De Brazza's monkeys (*Cercopithecus neglectus*) as a model

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Abstract

Difficult decisions regarding the management of disease in zoo animals are faced routinely. These may have a significant impact on the individual animal or a population of animals and therefore the best available evidence must be used. However, in zoos there are many situations where there is a lack of peer-reviewed papers, or significant uncertainty, controversy or confusion means that decision-making is hindered. This paper demonstrates how qualitative risk analysis techniques can be used to aide decision-making in circumstances where there is a lack of other evidence. Simian immunodeficiency virus in the De Brazza's monkey (*Cercopithecus neglectus*) has been diagnosed in the European population. Risk analysis was used to generate management guidelines to address the potential risks to other De Brazza's monkeys, other primates and humans.

Introduction

Risk is the likelihood that a hazard will cause its effects, together with a measure of its impact (MacDiarmid and Pharo 1997). Risk assessment is a tool intended to provide decision makers with an objective, repeatable and documented assessment of the risks posed by a particular course of action (MacDiarmid and Pharo 1997). It is a tool now routinely used to guide policy making and disease control planning by governments and international organisations such as the OIE (World Organisation for Animal Health). Risk assessment is intended to answer the questions:

- What can go wrong ?
- How likely is it to go wrong ?
- What would be the consequences of it going wrong ?
- What can be done to reduce the likelihood or the consequences of its going wrong?

This technique is rarely used to aide decision making in managed zoo captive breeding programmes. However, risk assessment has significant potential to help Taxon Advisory Groups formulate evidence-based policies for issues where there is an element of uncertainty, confusion or controversy, as this technique is designed to present information fully in a structured and transparent way. It is particularly useful as qualitative rather than quantitative techniques can be used where numerical or statistical data are not available or are of limited value – for example, because of small population sizes.

Simian immunodeficiency viruses (SIV) are lentiviruses that infect a wide variety of primate species (Ohta et al. 1988). Cases of SIV infection have been diagnosed in De Brazza's monkeys (*Cercopithecus neglectus*) (Bibollet-Ruche et al. 2004), and there was considerable concern about the risks these animals posed to other primates and humans. Policies thus needed to be developed for the management of these individual animals and the European Studbook (ESB) population as a whole, and this paper describes how risk assessment techniques were used to develop guidelines for the management of SIV in De Brazza's monkeys in European zoos.

Methods

There are a number of approaches to risk analysis; perhaps the most widely used and flexible is the OIE Risk Analysis Framework (1994). This is composed of four steps: (1) hazard identification, (2) risk assessment, (3) risk management and (4) risk communication.

The risk assessment process is constructed using the following steps:

Table 1. Risk terminology.

Term	Definition
Likelihood	Probability; the state or fact of being likely
Likely	Probable; such as might well happen or be true; to be reasonably expected
Negligible	So rare that it does not merit being considered
Very low	Very rare but cannot be excluded
Low	Rare but does occur
Medium	Occurs regularly
High	Occurs very often

Table 2. Uncertainty definitions.

Level	Definition
Low	Solid and complete data available; strong evidence provided in multiple references; authors report similar conclusions
Medium	Some but no complete data available; evidence provided in small number of references; authors report conclusions that vary from one another
High	Scarce or no data available; evidence not provided in references but rather in unpublished reports or based on observations, or personal communication; authors report conclusions that vary considerably between them

- Define the unwanted outcomes and the relevant risk questions.
- Clarify the steps that are necessary to get from the hazard to the defined unwanted outcomes. This is usually achieved by producing a 'risk pathway'.
- Collect the information necessary to estimate the probability of each event in the pathway.
- Assess the risk.

Risk management is the process by which the risk manager uses the results of the risk assessment, balanced with the 'level of acceptable risk', to determine the risk mitigation measures to be put into place. Levels of acceptable risk are value-based and affected by many factors including costs, culture and perceptions, and will differ between different groups of those who are likely to be affected by the risk.

Risk communication is the exchange of information between risk managers, risk assessors and stakeholders during the development of the risk assessment and certainly before the policy is finalised. This often includes a peer-review process by experts both in risk assessment techniques (to review the methodology) and in the hazard that is being assessed. This is vital, to ensure acceptance of the risk assessment and implementation of the resulting decisions guided by it.

Results

Hazard identification

The first stage in the process is hazard identification, which determines the hazard(s) that are to be assessed. In this case SIV virus infection in De Brazza's monkeys is the hazard of concern.

Simian immunodeficiency viruses (SIV) infect a wide variety of non-human primate species in sub-Saharan Africa. The evolution of the lentiviruses is very complex but there is some evidence to suggest that the viruses are ancient and co-evolved with specific species (Allan et al. 1990; Beer et al. 1999; Hirsch and Johnson 1994). The virus that naturally infects a specific species causes

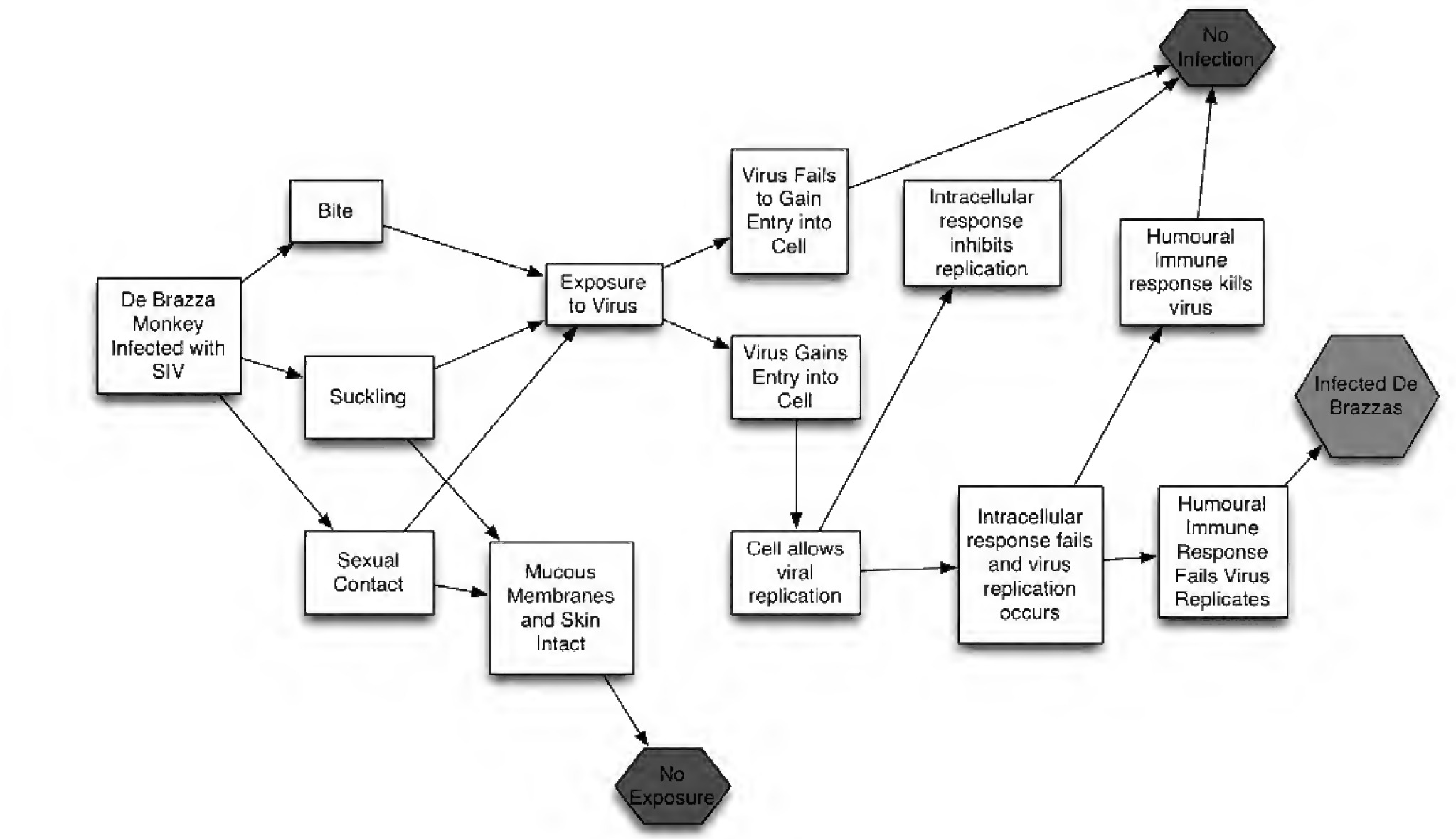

Figure 1. The risk scenario tree – the pathway of transmission between an infected De Brazza's monkey and an uninfected animal.

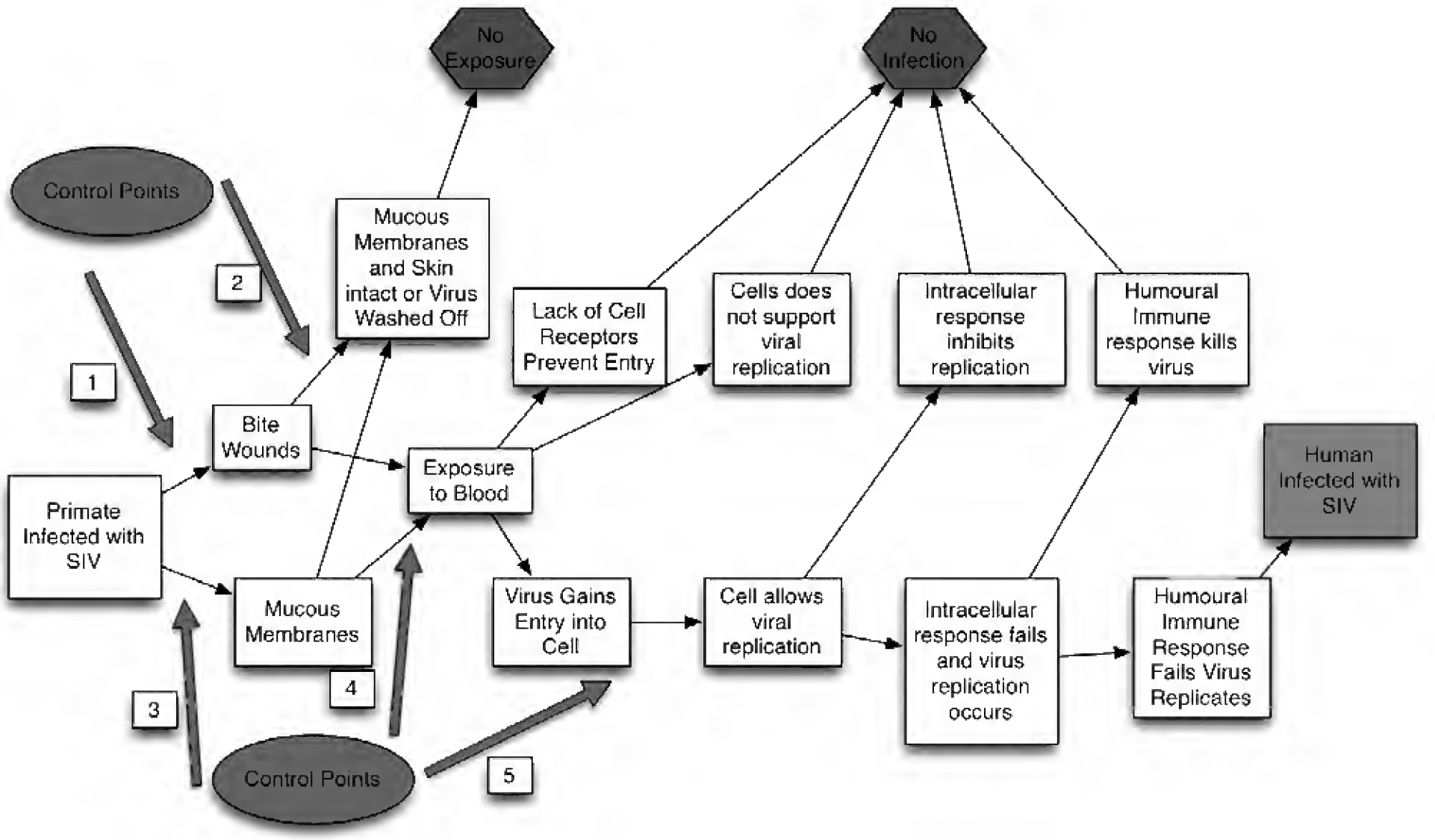

Figure 2. Transmission pathway from SIV-infected De Brazza's monkey to a human.

lifelong unapparent infection but not clinical disease. However, there is significant evidence of multiple cross-species infections (Ohta et al. 1988). In most instances these infections do not cause clinical disease but can on occasion result in immunosuppression, meningioencephalitis and lymphoproliferative disease.

The De Brazza's monkey is naturally infected with its own SIV virus, SIVdeb, which is very distinct from other guenon SIV viruses (Bibollet-Ruche et al. 2004). It is non-pathogenic to De Brazza's monkeys. Research suggests that up to 30% of this species are infected in the wild (Peeters et al. 2002).

Risk questions and pathways

The next stage in the process is to determine the risk questions. In this study these are:

1. What is the risk that an SIV-infected De Brazza's monkey will transmit the virus to another De Brazza's monkey?
2. What is the risk that an SIV-infected DeBrazza's monkey will transmit the virus to another primate that is housed in the zoo?
3. What is the risk that an SIV-infected De Brazza's monkey will transmit the virus to a human (either a keeper or zoo visitor)?

Risk questions 1 and 2

Pathways for the risk questions are then developed. For questions 1 and 2, these are presented in Figures 1 and 2 respectively.

The risk pathway is broken down into its components and the risk for each step is assessed. Risk assessment uses the risk terminology shown in Table 1, while uncertainty is categorised as shown in Table 2.

The virus is primarily transmitted horizontally through bite wounds and less commonly through sexual contact and breast milk. Indeed the virus can rarely be isolated from semen, cervical secretions or breast milk (CDC 1998). This does vary between species, with research suggesting that SIV in sooty mangabeys is definitely spread sexually, whilst this is less frequent in mandrills (George-Coubert et al. 1996). No experimental infections to further investigate transmission of SIV in De Brazza's monkeys have taken place.

Once the virus is transmitted to the new host it must enter the cells via cell receptors. The host's immune system will try to prevent this. There is evidence that intra-species and inter-species exposure does occur but an effective immune response prevents infection, as animals have been found to be serologically positive for SIV infection but not infected with the virus (VandeWoude et al. 2010).

To infect the animal the virus must successfully enter the cells and interact with cell organelles in order to replicate (VandeWoude et al. 2010). As the SIV viruses are very species-specific, it is likely that there will be incompatibility and the virus will not be able to replicate and therefore be unable to infect the animal. Experimental cross-species infection of SIVs among different species of primates has shown that in many cases the virus is harmless or cleared by the new host's immune system.

Table 3 summarises the analysis of questions 1 and 2. The overall risk assessment is of low to medium risk with medium uncertainty.

Risk question 3

A study of people with occupational exposure to primates was conducted by the USA Centers for Disease Control and Prevention Switcher et al. 2004, cited in Weston Murphy et al. 2006). Three thousand samples from people potentially exposed to SIV were tested. Only two demonstrated antibodies cross-reactive to SIV, a prevalence of less than 1%. One of these people handled known (experimentally) SIV-infected material without gloves whilst having a severe dermatitis of the hands and forearms. The second person had suffered from a needle-stick injury whilst handling

Table 3. Analysis of stages in risk pathway for questions 1 and 2.

Stage in risk pathway	Bite wound/suckling/sexual contact	Virus infects cell	Virus replicates in cell	Virus causes active disease in another primate
Mitigating actions	Avoid conflict in groups so that aggression is low. Hand raise infants of infected mothers. Do not allow infected monkeys to mate with uninfected monkeys.	Post-exposure prophylaxis	None possible but the SIV virus are very species-specific and so there is likely to be cell receptor incompatibility.	
Risk	Medium	Medium	Medium in other De Brazza's. Very low in other primates	Low
Uncertainty	Medium	Medium	Medium	Low

known experimentally infected blood. Both of these people had virtually undetectable levels of virus; this explains the lack of AIDS-like symptoms as a high circulating viral load is required for disease and transmission in HIV-infected humans. Evidence of SIV infection in zoo keepers has not been reported (Weston Murphy et al. 2006).

Epidemiological surveys of 1800 people from nine villages in Cameroon suggested very high (>60%) exposure to nonhuman primate blood and body fluids and demonstrated that 1% of exposed individuals were seropositive for SIV with three different nonhuman primate origins (Wolfe et al. 2004). Despite the fact that these events clearly demonstrate that human–primate contact occurs commonly, and can result in nonhuman primate to human retroviral transmission, human exposure to SIVs resulting in patent infections has been extremely rare. Therefore, exposure of humans to SIVs does not *a priori* result in successful cross-species infection; seropositivity merely demonstrates exposure to SIV and a subsequent immune reaction, not infection.

Cross-species infection from the natural host to other species can occur, however, and can result in pathological disease. Cross-species transmission of the specific chimpanzee and sooty mangabey SIV viruses to humans has been linked to the origin of the HIV-1 and HIV-2 virus respectively. It is thought that the SIVs entered human cells and underwent genetic changes, which then allowed human-to-human transmission. This is supported by the fact that humans in Africa have been exposed for centuries to SIVs and yet the HIV epidemic has only apparently emerged in the second half of the last century, which suggests that some other factor influenced the virus. This suggests that viral cross-species transmission is in itself not the only factor required for development of pathological disease (Wolfe et al. 2004).

Despite the large exposure of humans to SIV-infected primates in central and west Africa, through consumption of bushmeat, extensive molecular epidemiological studies have shown only 10 cross-species transmission events during the last century, and only four of these resulted in epidemic transmission (Apetrei et al. 2004).

There are over 40 species-specific SIVs, and only those from chimpanzees (SIVcpz) and sooty managabeys (SIVsm) have been shown to be associated with HIV. Indeed SIVdeb is one of the most genetically distinct viruses and is not similar to these two SIVs (Apetrei et al. 2004). The general experimental approach to determine this is to try and grow virus in human cells (human peripheral blood mononuclear cells, PBMCs) *in vitro*. Although many SIV viruses have been shown to grow in PBMCs, most of the cercopithecine SIVs do not grow in human PBMCs (Apetrei et al. 2004; Grimm et al. 2003) and none of the cercopithecine SIVs has been identified in humans (Apetrei et al. 2004).

Table 4 summarises the risk pathway for human exposure to De Brazza's monkey SIV. The overall risk assessment is very low to negligible with low uncertainty.

Risk management

By using the risk pathways it is possible to identify potential control points at which the risk pathway can be blocked and the likelihood of the pathway being completed reduced. In both pathways there are two control points; the first is preventing transmission and the second is preventing the virus from infecting cells. Once the virus has entered the cell there is little practical intervention possible to prevent infection.

There are several ways that transmission of SIV from an infected De Brazza's monkey could be prevented. SIV-infected animals could be euthanased or they could be housed individually in isolation facilities. In order to prevent infection of young born to SIV-infected mothers, infected animals could be contracepted or the young removed for handraising. Other options include

Table 4. Risk pathway for human exposure to De Brazza's monkey SIV virus.

Stage in risk pathway	Bite wound or mucus membrane exposure	Virus infects cell	Virus replicates in cell	Virus causes active disease in human
Mitigating actions	Handling precautions, gloves, goggles, face mask, washing hands, appropriate wound management	Post-exposure prophylaxis	Cells do not have correct receptors or cellular function to allow virus to replicate	None
Further evidence		In both occupational at-risk workers and bush meat hunters seroprevalence was less than 1%; infection in zoo keepers has not been reported	SIVdeb virus does not replicate in human PMBCs	Despite regular and widespread exposure for centuries, only 10 incidences of cross-species infection have been identified and only 4 of these have resulted in human disease
Risk	Low	Negligible	Negligible	Very low
Uncertainty	Low	Low	Low	Low

managing SIV-infected monkeys in groups composed only of infected animals, and enforcing management guidelines designed to reduce aggression and conflict in De Brazza's monkey groups with known infected animals.

The second control point is attempting to prevent infection in an animal exposed to the virus through the use of prophylactic drugs. This has not been attempted widely in naturally occurring exposure but has been effective in experimental infections.

Transmission to humans can be prevented through the use of protective clothing and management practices that reduce the risk of animal bites and bodily fluid transfer. Following a mucus membrane or bite wound exposure, copious lavage with chlorhexidine, which is virostatic, can be effective. Post-exposure prophylaxis with anti-retroviral drugs may be indicated following potential exposure. Medical intervention should be sought (Weston Murphy et al. 2006).

Risk communication

This risk assessment was reviewed in three ways as part of risk communication. The paper was reviewed by an expert in SIV to ensure technical and scientific completion and accuracy. The paper was also reviewed by members of the Old World Monkey Taxon Advisory Group and presented to this group in a formal meeting for ratification.

Risk mitigation and discussion

This risk assessment allowed a structured and objective evidence base to be presented to the European Association of Zoos and Aquaria (EAZA) Old World Monkey Taxon Advisory Group (TAG) for the development of a management strategy for SIV infection in the European studbook population of De Brazza's monkeys. The risks identified need to be balanced with the requirement to maintain and increase a genetically sound population of this species in European zoos.

The first decision made was that it is essential to identify which animals in the population are SIV positive and which are not. This allows zoos to implement the management protocols devised and actively manage the low but potential risks to humans and other species of primates sharing mixed exhibits with De Brazza's monkeys. Accordingly the TAG has advised that De Brazza's monkeys of SIV positive or unknown status should not be housed in mixed exhibits with other primate species.

The risk assessment provides evidence to allow the following advice to be provided to keepers working with De Brazza's monkeys infected with SIV. The majority of these should be in use for routine contact with non-human primates in a zoo environment.

The risk of transmission from urine and faeces is negligible and SIV is susceptible to household bleach and disinfectants, which should therefore be used routinely for general cleansing.

Blood is the main risk to humans. As with all primates, latex gloves should be used when handing De Brazza's monkeys. Unknown status or SIV-positive De Brazza's should not be handled when conscious to avoid bite injuries, and should not be netted, but should be darted or a put in a crush cage and then examined under anaesthesia only. Should bite injuries occur they should be immediately and thoroughly washed and lavaged with chlorhexidine. During blood collection or other invasive procedures on unknown status or SIV-positive animals, goggles, gloves and face-masks should be worn to prevent mucous membrane contamination. If mucous membranes (eyes, mouth, nose, ears) are contaminated by SIV-infected primate bodily fluids, the area should be immediately washed with chlorhexidine.

The more challenging issue is to decide if the management of the De Brazza's monkey ESB should be changed in light of SIV status when SIV in De Brazza's monkeys is a naturally occurring infection and is non-pathogenic. However, as a result of perceptions and misunderstanding of the risks, some zoos are reluctant to hold SIV-infected animals and in some collections SIV status has contributed to a decision to euthanase animals (Redrobe, pers. comm.). There is also the moral quandary of placing an animal at risk of an infectious disease, albeit a non-pathogenic disease, by knowingly moving it into a group infected with SIV.

It was decided to collect further information on the status of the current ESB population and to avoid increasing the number of SIV-infected animals by not introducing SIV-infected animals to groups that were not infected or of unknown status.

Testing for SIV in primates is well established. However, there have been some problems with interpretation of the results of tests undertaken by different laboratories as the tests have differing sensitivities (i.e. ability to detect a positive result). This has resulted in animals previously testing negative to test positive when tested by a different laboratory. It is important to remember that once animals test positive they cannot revert to being negative. If an animal tests negative if could have been recently infected and the virus not yet replicated to detectable levels. As the amount of virus in the animal is so low it will not yet be able transmit disease. This animal can be considered negative, but at some point that cannot be determined will test positive when the virus reaches detectable levels. This is, however, rare as all the tests can detect virus at very low levels. If an animal tests negative and then at a later date tests positive it has been infected by the virus in the intervening period and the animals with which it has been in contact should be tested for SIV. In order to ensure consistency and expert interpretation of the results obtained, the De Brazza's monkey ESB has recommended using a single laboratory for all testing.

It was decided to undertake testing strategically and focus on groups of monkeys that were involved in movement transactions. This is for two reasons: firstly, these animals have the greatest potential to change the infected status of a group, and secondly, the potential conflict during introductions increases the risk of transmission.

Therefore the ESB instructed that when a movement recommendation has been made, both the animal that is being sent to the new zoo and the entire group the animal is destined to join should be tested for SIV. In this way we can ensure that an SIV-positive animal is not moved into an SIV-negative group or vice versa. Zoos are also being encouraged to submit samples opportunistically.

It was also decided that any animal that did test positive for SIV should not be euthanased but that groups of known positive animals would be established so that these animals could continue to play an important and full role in the ESB. It is important not to presume that offspring born to SIV-positive parents will also be positive, so using contraceptives in infected females was not considered appropriate.

Finally, it was also decided that due to the non-pathogenic nature of the virus, stable family groups do not need to be broken up if one of the animals tests positive. This positive result has no implications for the health of the group and indeed, the risk of SIV transmission will be increased by disrupting the group and increasing the likelihood of fighting. Ultimately, the long-term viability of the ESB could be threatened as a result.

The additional evidence obtained from the risk mitigation processes described above will be fed back into the risk assessment process. Regular review of the risk assessment in the light of new information and evidence ensures that management decisions are still appropriate.

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