

Appendices to:

Genetic frontiers for conservation

An assessment of synthetic biology and biodiversity conservation

Technical assessment (<https://doi.org/10.2305/IUCN.CH.2019.05.en>)

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Appendices

(for detailed reference information please see the bibliography of the main report: *Genetic frontiers for conservation: an assessment of synthetic biology and biodiversity conservation*.

Technical assessment <https://doi.org/10.2305/IUCN.CH.2019.05.en>)

Appendix 1: An introduction to genetics (expanded from Box 1)

DNA

DNA (Deoxyribonucleic acid) is a complex molecule that carries all of the information necessary to build and maintain an organism. All living things contain DNA within their cells – and pass on this information from parents to offspring.

In the nucleus of each cell the DNA molecule is packaged into dense structures called chromosomes.

Each strand of DNA is a long chain of smaller units called nucleotides. Each nucleotide contains one of four organic bases -- adenine (A), guanine (G), cytosine (C) and thymine (T) -- sometimes known as the ‘alphabet of life’. All DNA is essentially the same in every organism on the planet; it is the specific ordering of these universal chemical letters that is responsible for all the variation in life on Earth.

Segments of DNA that encode the information for a specific protein are known as genes (see below) – all organisms within a species contain the same set of genes, however within a gene the ordering of the nucleotides can have subtle variations. For example, most genes are the same in all humans, but a small number of genes are slightly different between individuals (these variations are known as alleles). The combined effect of all these subtle differences are responsible for each person’s unique features.

DNA structure

The DNA molecule itself is in the shape of a double helix: it is composed of two long strands of polynucleotides that run in parallel while winding around each other to resemble a twisted ladder. The bases along these two strands link to each other in a specific manner– A will only pair with T on the opposing strand, and C will only pair with G.

The double helix accounts for two of the most important features of DNA:

- 1) **Information storage** - The double helix holds DNA in its linear structure – allowing the storage of information via the nucleotide ordering.
- 2) **Heredity** - The double helix allows DNA to be copied – and the information it contains to be passed on – when cells divide, and when eggs and sperm are produced. Each strand of DNA is a perfect complement of the other – so that a DNA double helix can be separated into two single strands and two perfect replicas of the original molecule can be formed from the original.

DNA can be thought of as the chemical basis of heredity – the stored information in its sequence can be passed on to descendant molecules as the two halves separate. DNA is passed on this way as cells replicate and divide, ultimately being passed on from one generation to the next.

gene expression

A gene can be basically defined as a section of DNA that codes for a single protein. The order of the nucleotides within a gene direct the synthesis of protein – its composition, when, where, and how much protein is made. This process of directing protein synthesis is known as gene expression.

Given the vital importance of genes in making all of the proteins that enable an organism to function they make up a surprisingly small proportion of the total genome. The human genome is made up of approximately 21,000 protein-coding genes – but this accounts for less than 2% of the nucleotides in the total genome.

Proteins are strings of organic molecules called amino acids – the sequence of these amino acids (encoded by the DNA nucleotide sequence), determines the three-dimensional structure of the protein, which in turn dictates its function.

These molecules form the basis of living tissues and play central roles in all biological processes. Each different protein has a specific role, from antibodies to enzymes and structural proteins to hormones.

anatomy of a gene

An individual gene can be described in terms of multiple different structural components – different components have different roles in the control of gene expression:

Regulatory sequences

Enhancers- Regions of DNA upstream of the gene (often many kilobases away) which bind activator molecules which increase the rate of transcription for a gene

Promoters - A region of DNA a few hundred nucleotides upstream of the gene – it does not form part of the mRNA but determines when the mRNA is made, in which tissues it is made, and how much of it is made.

Start site - the triplet sequence (usually AUG) which signals the point along the mRNA at which a ribosome will start translating its sequence into protein.

UTR - These are sections of mRNA that immediately precede and follow the section which will be translated into protein. They provide another level of control for gene expression – called post-transcriptional regulation, as these can affect how quickly or efficiently the mRNA is translated into protein, how stable the mRNA is and how it is transported.

Coding regions

Coding regions or Exons determine the amino acid sequence of the protein product. This will determine the structure and function of the proteins

Non-coding regions

Non-coding regions or Introns are portions of the gene that do not code for the amino acid sequence. These sections are transcribed into the initial mRNA – but removed before translation occurs.

Alternative splicing

Why have sections of non-coding DNA in the middle of an mRNA sequence? We do not why this occurs in many genes, however there are some genes which can undergo alternative splicing.

Alternative splicing is where more than one final protein can be produced from a single gene. By alternatively treating segments as exons in one version of mRNA but introns in another it can be possible to produce two different proteins from the same gene.

An example is the *Drosophila melanogaster* gene *dsx*, a gene involved in sex determination. This gene contains 6 exons. In males exon 4 is treated as an intron, while in females exons 5 and 6 are treated as introns while exon 4 is included. This allows the same gene to produce a different protein in males to females and is important in regulating the proper formation of the sexes.

DNA sequencing

DNA sequencing is the process of determining or reading the precise order of nucleotides within a DNA molecule. The rapid advent of technological developments in this field has opened many avenues of research and novel applications, the most well-known are summarized below:

PCR detection – If the specific nucleotide sequence of a target is known then short artificially synthesized nucleotides known as primers can be used to detect presence/absence of genes or alleles.

De novo genome assembly – creating full length reads of a novel genome from scratch.

Genome resequencing – Cataloguing genetic variation within a species. Using a pre-developed genome assembly against which variants (mutations and alleles) can be compared.

RNA sequencing – Allows the quantification of gene expression. Essentially molecular level counting of RNA molecules, this allows the precise measurement of when, where and how much a gene is being expressed by comparing different tissue samples.

Metagenome sequencing – Usually referred to in the context of sampling bacterial communities. Sequencing of DNA taken from environmental samples can quantify the abundance and variety of different species present.

DNA/gene editing

DNA changes may be engineered through a large number of different techniques, mutations may affect the control elements of a gene or the gene product (protein) itself. These effects can be achieved with a varying degree of precision and control.

Random mutagenesis – many of the oldest techniques rely on methods that produce entirely random mutations. This may be achieved through the use of physical or chemical agents – the resultant mutants are screened and selectively bred for desirable characteristics

Site directed mutagenesis – specific and intentional changes to a known DNA sequence is referred to as site-directed mutagenesis. There are numerous methods for achieving this, the development of CRISPR-Cas9 technology rapidly becoming a preferred option for its high efficiency and ease of use. CRISPR-Cas9 genome editing uses the Cas9 protein and a specially designed RNA (guide RNA or gRNA) that directs the protein to cut DNA at a specific nucleotide sequence.

Transgenics – the introduction and expression of new genetic material into a living organism. The universal genetic code of DNA means that transgenic organisms can express genes that originate from different species. Organisms with genes introduced from very closely related species, that might otherwise be produced by breeding are sometimes referred to as Cisgenics. Multiple techniques exist for producing transgenics, including the use of transposable elements for random integration of DNA into the genome, or the use of template-based repair through CRISPR-Cas9 for site-specific integration.

Appendix 2: Gene drive

Gene drive is a naturally occurring phenomenon in which a genetic element reliably spreads through a population even if it does not help an organism reproduce. Most such elements (“gene drive systems”) function by distorting inheritance in their favor (Burt & Trivers, 2009). Gene drive is ubiquitous in nature, with active or broken elements found in virtually every species. Such sequences collectively comprise over half the total DNA in the human genome. Gene drive elements spread from parents to offspring over many generations (“vertical transmission”), which results in slow spread relative to “horizontal transmission” (as generally employed by infectious agents such as viruses), but can lead to substantive increases in frequency in just a few generations. For example, the *P* element transposon was absent from all wild-caught *Drosophila melanogaster* fruit flies in 1950, but was present in every wild population worldwide by 2000.

Scientists have long sought to use gene drive to engineer wild populations of insect disease vectors, starting with experiments in tsetse flies in the 1940s (Vanderplank, 1944). In the 1990s, an Australian-led project began field trials in several countries with a *Wolbachia*-based gene drive for mosquitoes, aiming to impede transmission of dengue virus. *Wolbachia* is a naturally occurring intracellular bacterium that is maternally inherited and blocks transmission of other pathogens; it qualifies as a gene drive element because it distorts inheritance in its favor: infected males are sterile if mating uninfected females, but fertile with infected females, thus giving infected females a reproductive advantage. *Wolbachia* is naturally present in many insect species, though not in the mosquito species that transmits dengue virus, *Aedes aegypti*. A specific strain of *Wolbachia*, isolated from *Drosophila melanogaster*, has now been shown to reduce the ability of infected *Aedes aegypti* to transmit the virus (Moreira et al., 2009; Souta-Maior et al., 2018; Carrington et al., 2018).

Scientists have also long sought to engineer gene drive elements from scratch (Curtis 1968; Burt 2003), using components or principles from natural gene drive systems to create artificial equivalents (Alphey, 2014 ARE; Burt & Crisanti, 2018). Proofs-of-principle have been established

in *Drosophila* for several designs (Chen et al., 2007 *Science*; Reeves et al., 2014 *PLoS ONE*; Buchman et al., 2018 *ACS Syn Bio*; Gantz & Bier, 2015 *Science*; Chan et al., 2011 *Genetics*), of which the highest-profile and perhaps most rapidly advancing are those employing CRISPR nucleases (Esvelt et al., 2014). The best-known such project is Target Malaria, an effort launched in 2005 that seeks to contribute to eradication of the disease by targeting and reducing populations of three closely-related African malaria vector mosquitoes in order to break the parasite transmission cycle (Burt 2003; Target Malaria 2018). This project originally used homing endonuclease genes, but progress accelerated in 2014 with the emergence of the more versatile CRISPR nuclease system (Esvelt et al., 2014). Within little more than a year of the initial description of this new system, functional proof-of-principle was established in yeast, fruit flies, and two species of mosquito (DiCarlo et al., 2015; Gantz & Bier, 2015; Gantz et al., 2015; Hammond et al., 2016). This rapid progress has stimulated considerable discussion concerning CRISPR-based drives.

Some natural gene drive elements distort the sex ratio as they spread, which can impair the fitness of a population. In 2003 researchers speculated that ‘homing endonuclease’ gene drive systems might be used to suppress populations by distorting the sex ratio or by ensuring that organisms inheriting a copy of the drive from each parent are nonviable, sterile or have reduced fertility; these ideas are now being developed in several projects including Target Malaria, now primarily using CRISPR-based designs (Hammond et al., 2016; Galizi et al. 2014). Models have predicted that this approach can cause populations to crash, even leading to extirpation (local extinction) in some areas. Popular media coverage suggests that suppression drives could result in global extinction (need citation here). However, research has not supported this contention; models suggest that suppression drives will not result in extinction unless assisted by continued human monitoring and intervention (Eckhoff et al., 2017).

Gene drive might also be used to spread an unrelated trait through a target population or species, for example by physically encoding that trait on DNA attached to the gene drive so that the two spread together. This has also been termed “population replacement”. Potential

applications include spreading a trait through a mosquito population that makes it less able to transmit a particular pathogen (Gantz et al., 2015). The *Wolbachia*-based gene drive discussed above is a population alteration drive. In contrast to a suppression drive, the target population or species would not be substantially reduced in number, but rather altered in one or more phenotypic characteristics.

The first implementation of CRISPR-based gene drive in yeast demonstrated that a second gene drive can overwrite and replace an earlier one, restoring a disrupted gene and trait, though leaving behind engineered sequences (Di Carlo et al., 2015 *Nat Biotechnol*). In principle, overwriting provides a means to prevent substantial unwanted impacts on the traits of populations (and even ecosystems) as long as monitoring swiftly detects problematic gene drive elements (Esvelt et al., 2014)⁹. Because CRISPR is not naturally found in sexually reproducing eukaryotes, sequencing should reliably detect any gene drive present in the genome of such an organism, since this will be recognisably foreign DNA not normally present in that species. Active funding programs seek to develop efficient ways of detecting these signatures from environmental sequencing data (FELIX, 2018).

CRISPR-based gene drive elements that cut only a single sequence are expected to be limited in their ability to achieve high frequency within a target population due to “resistant alleles.” These are variant sequences at that site, either pre-existing or generated by cutting and repair, that are no longer recognised by the CRISPR nuclease - that cannot be cut and replaced, preventing it from affecting every organism within a population and precluding any form of population suppression. However, models predict that using CRISPR to cut multiple sequences within genes important for fitness should overcome pre-existing or drive-generated alleles that could otherwise block cutting (Noble et al., 2017; Marshall, 2017; Prowse et al., 2017). Experiments in fruit flies using two (Champer, 2017) and four (Oberhofer, 2018) guide RNAs support these studies, but are not yet conclusive.

Models predict that although resistance and inbreeding can block self-propagating gene drive systems from spreading to every member of a population, they do little to prevent spread from one population to another (Noble et al., 2018). For some gene drive systems, introducing comparatively few organisms into a new population is predicted to typically suffice for invasion (Marshall, 2009), in which case eventually all populations connected by even low levels of gene flow are likely to be impacted (Noble 2018). This efficiency is a major advantage when the goal is to affect most populations of the target species, but such invasiveness makes restricting spread difficult, even from limited field trials. Importantly, while 'standard' CRISPR gene drive systems are predicted to be invasive, several other 'local drive' designs are not, including threshold-dependent, precision and self-exhausting drives (Esvelt & Gemmell, 2017). Scientists have also developed frequency-dependent gene drive systems that only spread above a certain frequency in the population, typically 20-50% (Akbari et al., 2013; Buchman et al., 2018). However, these cannot readily be used for population suppression.

Targeting sequences unique to a specific population with a CRISPR-based suppression drive should substantially confine effects to that population, assuming the drive system is unable to escape. Efforts to sequence invasive mouse populations to identify suitable sequences and build precision drives are underway. If suitable natural variants do not exist, threshold drives might be used to introduce them (Esvelt et al., 2014).

Gene drive systems that lose components each generation will eventually run out of genetic fuel and cease to spread. 'Daisy drive' systems -- in which the CRISPR components are split up and scattered throughout the genome so that none of them can be copied on its own -- are a CRISPR-based example, but have not yet been demonstrated in the laboratory (Noble et al., 2016; Min et al., 2017; Min et al., 2017). Conservation-relevant organisms in which they are under development include the invasive house mouse *Mus musculus* and the mosquito *Culex quinquefasciatus*, a vector of avian malaria.

In terms of biodiversity conservation, gene drive systems may provide a tool for manipulating otherwise hard-to-reach wild populations. Development and funding has largely focused on mosquitoes transmitting human pathogens, but this may be leveraged for conservation purposes (Sections 4.X, 5.X). At the time of writing, all known research efforts focused on conservation applications seek to develop drive systems with inherently localized effects (Esvelt & Gemmell, 2017).

Appendix 3: Expanded discussion of technical points made in Chapter 5.

Potential synthetic biology applications for Invasive Alien Species

The constraints of current Invasive Alien Species (IAS) management tools and approaches are causing scientists and managers to seek additional tools that are more targeted and species-specific, economical, self-sustaining and with lower animal welfare impacts; however no widely-applicable alternative solutions have yet been identified (e.g. Campbell et al. 2015).

One approach that has been successfully applied for non-conservation purposes is the Sterile Insect Technique (SIT) for the eradication of invertebrate vectors of diseases of concern to human health and domestic animals such as the screwworm fly (Dyck et al., 2005). This involves the repeated release of large numbers of males that have been sterilized by irradiation. However, such an approach would not be easily transferable to other IAS due to issues of sterilization approach, releases themselves causing undesired impacts, and the cost of maintaining long-term rearing programmes of sufficient magnitude (Vreysen et al., 2007). Research into a novel spin on the SIT, the 'Trojan Female Technique' (TFT), has identified that naturally occurring male-sterility causing alleles in the mitochondrial DNA can theoretically provide persistent population regulation (Gemmell et al., 2013). However subsequent laboratory development has encountered issues such as mito-nuclear interactions and compensatory breeding effects that greatly reduce population impact (Wolff et al., 2017).

Synthetic biology offers the potential of new tools for suppressing and eradicating IAS that are more targeted and species-specific, economical, self-sustaining and with lower animal welfare impacts than traditional management tools such as traps and toxins (Harvey-Samuel et al. 2017, Ricciardi et al. 2017). Several approaches are currently under consideration (Redford et al. 2014, Piaggio et al. 2017), which for risk and benefit assessment are categorized here as "gene-drive" and "non-gene-drive" approaches. "Non-gene-drive" approaches are those where the subsequent spread of any synthetic biology component across pest species generations after an introduction for management purposes is through non-manipulated inheritance (i.e. in general 50/50 Mendelian inheritance for sexual species; Ridley, 2003). "Gene-drive" approaches are those where the subsequent spread of the synthetic biology component across pest species generations after an introduction for management purposes is through synthetically biased inheritance in favour of particular genes or alleles ('cargo genes'; Burt, 2003).

There are several actual and proposed non-gene-drive synthetic biology approaches to IAS. 'Release of Insects carrying Dominant Lethals' (RIDL), for example, is an advanced version of the

SIT, involving genetically engineered males and female insects from which all offspring die even when mating with wild individuals (Black et al. 2011). It has been used for non-conservation purposes in the Grand Cayman Islands, Panama, and Brazil to control mosquitoes (Carvalho et al. 2015) and is being developed for use in diamondback moth, medfly and olive fly (Harvey-Samuel et al. 2015). For similar reasons to the SIT, it is less likely to be applicable to vertebrate pests (Moro et al. 2018).

A second approach is to use genetic editing to cause sex ratio biases in offspring, theoretically leading to population collapse. One such approach in mosquitoes, 'X-shredder', which causes a male bias in offspring through the destruction of X chromosome bearing sperm, has been demonstrated in the laboratory (Galizi et al., 2014, 2016). Another 'daughterless' approach has been developed for application against invasive fish, but is yet to be applied in the field (Thresher 2005, 2007), and similar has been proposed for control of cane toad, bighead carp and sea lampreys (Harvey-Samuel et al., 2017).

A third example is the 'Incompatible Insect Technique' which uses the effects of symbiotic bacteria in insects called Wolbachia whereby cytoplasmic incompatibility at fertilization can be used to control natural populations of insect pests in a way analogous to the SIT (Zabalou et al., 2009); it can also be used in combination with the SIT (Zhang et al. 2015). This approach is currently being applied for the control of agricultural insect pests (Nikolouli, 2018) and vectors of human disease (Flores & O'Neill, 2018) and is in development for the purpose of controlling avian-malaria vectoring mosquitoes in Hawai'i (Box 2).

The application of synthetic biology to produce fertility vaccines for agricultural pests may also have utility for conservation (e.g., the past attempt to develop 'bacterial ghosts' expressing host fertility proteins to trigger auto-immune reductions in fertility; Walcher et al., 2008). Such vaccination approaches would have benefits of 'tactical controllability', but they are generally too logistically difficult and too costly to apply to large wild populations (Kirkpatrick et al., 2011).

Genetic alterations have in the past and are still being considered to improve the efficacy of traditional biocontrol approaches for application to invasive alien species (e.g. Hoddle et al., 2015). Past attempts to develop for application to mammals such as transgenic parasitic nematodes to reduce brushtail possum fertility and transgenic viral agents to do similar for mice have proven unsuccessful (Cowan, 1996; Hardy, 2007). Notably, this approach raises undue risk in the minds of many, due to the non-tactical-controllability of self-disseminating agents of controls and the risk of non-target-population impact (the same concerns as for non-self-limiting gene-drive approaches; see below).

A variety of synthetic gene-drive systems have been proposed (Burt, 2014; Sinkins & Gould, 2006), many of which could theoretically cause IAS suppression or eradication, through the spread of gene cargos for example causing sex-ratio bias or reduced fecundity (Champer et al., 2016; Prowse et al. 2017). Alternatively, it has been proposed that such drives could be used to reverse evolved pest resistance to pesticides or create new pest vulnerabilities to otherwise harmless chemicals (Esvelt et al., 2014). Although attention is currently focused on 'homing-based' drives, that increase their frequency through cleaving DNA at specific genomic locations and then being copied into the cleavage site through 'homology-directed repair', there are other mechanisms such as 'Maternal Effect Dominant Embryonic Arrest' ('Medea'; e.g. Buchman et al., 2018) and 'engineered haploinsufficient underdominance' (e.g. Reeves, 2014), that have been developed to proof-of-concept for invertebrates but with no field application.

Medea systems rely on expression of a toxin–antidote combination. The toxin is inherited by all progeny from a Medea-bearing mother, resulting in suppression of an essential embryonic gene that causes disruption of normal development during embryogenesis. Offspring that inherit Medea receive a tightly linked antidote, consisting of a resistant copy of the targeted essential gene, that allows for restoration of normal development; non-Medea-bearing progeny from Medea-bearing mothers lack this antidote and perish. Due to this biased inheritance, Medea is predicted to rapidly spread itself, and any linked cargo genes, through a target population (Akbari et al., 2014).

Underdominance describes a scenario where a heterozygote is less fit than either parental homozygote, the opposite of the well-known phenomenon of 'hybrid vigour'. Assuming equal fitness of two competing underdominant alleles, an unstable equilibrium is created, with the most common allele tending towards fixation and the other towards extinction. This behaviour has long been recognized as a potential means of spreading genetic traits (Curtis, 1968), including transgenes (Davis et al. 2001), through a pest population. The drive of sex-biasing genes to cause population suppression or eradication is also currently being explored in mice, through linking such genes to a selfish genetic element called the 't-complex' that occurs naturally in mice (Piaggio et al. 2017). However, this approach faces stiff hurdles as such natural elements that can still be found in populations (i.e. they have not gone to fixation) tend to do so because they face barriers to spread (Gemmell & Tompkins 2017).

Homing-based drives are the current choice of exploration for pest management and many other applications requiring self-sustaining strategies that persist or spread, due to their potential relative ease of development using the CRISPR/Cas9 gene-editing toolkit (Jinek et al., 2012; Esvelt, 2014). Such drives for use in manipulating populations, using homing endonuclease genes (HEGs) to cleave DNA at the desired drive site, were first proposed by Burt in 2003 (Burt, 2003);

the first demonstration of homing-drive systems used HEGs was by Windbichler and colleagues in 2011 (Windbichler et al., 2011). Similar to HEGs, Cas9 genes express an endonuclease which binds to and cleaves specific DNA sequences. However, these target cleavage loci are defined not by the DNA binding affinity of the endonuclease (as in HEGs), which may require laborious and complex protein engineering techniques to respecify, but by complementary single-guide RNAs (sgRNAs). sgRNA coding sequences can be linked to the Cas9 gene allowing the endonuclease and targeting system to home as a single unit. The ease with which sgRNAs may be reengineered (and therefore different genomic sequences targeted) constitutes a major advance in broadening the potential genes and species against which homing-drives may be applied (Esvelt, 2014).

Several lines of technical development have been proposed to make self-disseminating drives self-limiting, such that they can be tactically applied to target pest populations with greatly reduced risk of spread to non-target populations. These include approaches such as ‘precision drives’, where the drive only functions in the presence of target-population-specific alleles (Esvelt et al., 2014), ‘daisy-chain’ drives, where a linear series of independent CRISPR–Cas9 elements are configured so that no individual element is autonomous but depends on the element immediately below it in the series for drive (Noble et al., 2016), ‘sensitization drives’, where a drive for example confers toxicity to a normally benign molecule (Esvelt et al., 2014), or ‘frequency threshold’ drives, where mechanisms such as genetic underdominance are employed so that spread only occurs above threshold frequencies (Marshall & Hay, 2012).

It has also been proposed that should a CRISPR/Cas9 homing-drive disperse to a non-target population of the target pest species, ‘reversal drives’ also built on the CRISPR–Cas9 architecture could be designed with sgRNA sequences directed to disrupt or alter sequences of the earlier wayward gene-drive (Esvelt et al., 2014). To date, ‘reversal drives’ have been demonstrated to the proof-of-concept stage in yeast culture (DiCarlo et al., 2015) and fruit flies in the laboratory (Wu et al., 2016), and a ‘precision’ drive has similarly been demonstrated in fruit flies (DiCarlo et al., 2015). Other proposed limitation approaches are currently only theoretical.

Potential synthetic biology solutions for increasing general species viability

Somatic nuclear cell transfer (cloning) is being discussed for many species as a way of reintroducing extinct genetic variation stored in ‘frozen arks’ back into extant populations to increase their genetic diversity and thus general species viability (IUCN SSC, 2016). Cloning is currently most advanced for mammals and amphibians; the first ever clones were of northern leopard frogs in the 1950s. This has been done successfully with many mammal species, with more or less safe results for offspring. For mammals, the nucleus of a somatic cell taken from an

individual of an extinct species is removed and inserted into the enucleated egg cell (cell from which the nucleus has been removed) of a suitable surrogate species. The cell is induced to start to divide and the embryo is implanted into the surrogate host to gestate to term. However, cloning is still a relatively inefficient process even in mammals, with many eggs being required to derive viable embryos, and many embryo implantations being required to achieve even partial gestation and few live births.

For birds, somatic cell nuclear transfer currently seems impossible. The problem with cloning birds is that egg cell contains a large quantity of yolk that makes identification of the nucleus extremely difficult, and the female must be killed to obtain a newly ovulated egg because by the time an egg is laid the embryo has already begun to develop on the yolk. Genomic engineering techniques (such as those discussed above) would be needed if cloning is to be conducted for birds. Cloning techniques have been developed for plants and amphibians, but is not yet clear whether reptiles can be cloned. If the cryo-preserved cells are gametes then in vitro fertilisation might be possible, thus avoiding the considerable challenges of somatic cell nuclear transfer, though the use of gestational surrogates would still be necessary for mammals, with the attendant issues around interspecies cloning (IUCN SSC, 2016).

Potential synthetic biology applications for resilience to specific threats

CRISPR-Cas9 genome editing has accelerated and facilitated synthetic biology: through gene knockouts and allele replacements, researchers have established phenotypes in organisms within a single generation that would take selective breeding or hybridization many generations to achieve. Genome editing is not only faster and more precise than selective breeding, it also bypasses the genetic load problems that that involves, which cannot always unlink deleterious traits from desired traits (Novak et al. 2018). It has been proposed that precise genome editing also provides the potential for facilitated adaptation to benefit whole ecosystems by helping foundation species (i.e. species whose presence and processes disproportionately form the structure of habitats, such as corals and trees; Ellison et al., 2015) adjust to climate change or disease.

Synthetic biology approaches, primarily involving genetic modification, have been proposed to protect many species threatened by disease impact (Redford et al., 2013; Piaggio et al., 2017), such as bats in North America threatened by White-Nose Syndrome (Redford et al., 2013), amphibians and salamanders threatened by chytrid fungus globally, and plague in black-footed ferrets in North America (Box 3).

Potential approaches for disease management fall into three general categories. The first is the modification of disease vectors to reduce their vector competence for disease through the introduction of novel genes (transgenesis) e.g., to block disease transmission (Alphey et al., 2002). Although such an approach has yet to be seriously considered for conservation benefit, proof of principle has been shown for rodent and chicken malaria (Marshall & Taylor 2009), and application for human malaria control is well advanced with mosquitoes genetically modified to resist the parasite shown to transfer the resistance to offspring when mated with wild mosquitoes in laboratory experiments (Pike et al., 2017). It has also been proposed that rodent vectors of disease could be modified to reduce their potential to vector diseases of concern to humans (e.g., lyme disease in mouse reservoirs), although such application is currently only theoretical.

The second is again the reduction of vector competence, but through gene-knockout (e.g., using the CRISPR/Cas9 gene-editing toolkit) rather than transgenesis. To date this approach has only been demonstrated in the laboratory for human malaria in *Anopheles gambiae* (Dong et al., 2018).

The third is to use transgenic approaches to introduce novel genes to confer disease resistance to susceptible host species. This approach has been shown to create American chestnut trees that can tolerate blight infections to which they were previously highly susceptible (BOX 4), by inserting a single gene from wheat, oxalate oxidase (although disease tolerance and tree fitness has yet to be assessed in the wild). It has also been suggested for black-footed ferret threatened by plague, introducing resistant alleles from the domestic ferret (for which plague is not fatal) into the genome of the black-footed ferret (Novak et al., 2018).

Finally, the engineering of avirulent strains to confer resistance to disease impacted hosts, or using genetic editing to 'awaken' innate resistance genes in hosts, has also been proposed for white-nose syndrome in bats. Additionally, using CRISPR/Cas9 gene knock-out has been proposed to give elephants resistance to Elephant Endotheliotropic Herpesvirus (EEHV) by stopping the host cells from creating the proteins that the virus uses to infect its host (Lee, 2018). However, outside of the specific technical details noted above, proposed applications of synthetic biology approaches to diseases impacting biodiversity are currently speculative.

For resilience to climate change, attention is currently focusing on using genetic engineering to edit alleles for resilience from other populations or species into the target species (see Box 5). In the case of coral, genetic editing to improve resilience to ocean warming, genetic engineering methods are poorly developed for the coral host animal and its microbial symbionts. In 1998, a *Symbiodinium* A1 strain was transformed at very low efficiencies (ten Lohuis & Miller, 1998).

After a 17-year gap in the literature, another case of transgene expression in *Symbiodinium* was reported, but transformants were transient and results were not validated through DNA, RNA, or protein analysis (Ortiz-Matamoros et al., 2015). Others have also found it challenging to obtain stable *Symbiodinium* transformants using standard methods (Chen et al. 2018).

Elevated temperatures are known to cause oxidative stress in the coral holobiont (i.e., the coral host animal and all of its associated microbial symbionts); thermal damage of the algal endosymbiont's photosystems leads to an increased production of reactive oxygen species (ROS) which leak into the host tissues (Weis 2008). This triggers a cellular cascade and culminates in the loss of the algal endosymbionts (*Symbiodinium* spp.) from the coral's tissues (i.e., coral bleaching). Augmenting ROS scavenging capacity of the coral animal and/or its microbial symbionts may increase the holobiont's thermal tolerance, and this hypothesis is supported by the enhanced bleaching tolerance of corals treated with an antioxidant (Lesser, 1997; Yudowski et al., 2018). Genetic engineering and gene editing tools may thus be used to insert coral or microbial genes encoding antioxidant enzymes (Levin et al. 2017) or to introduce gene pathways or synthetic microbes able to produce non-enzymatic antioxidants. Other genes involved in the thermal stress response may also prove useful genetic engineering targets to enhance thermal tolerance (van Oppen et al., 2017). Alternative synthetic biology approaches may be developed to prevent algal symbionts from becoming parasitic during heat stress (Baker et al., 2018).

A huge step forward was recently achieved in gene editing by the successful introduction of mutations targeted to three genes of the coral *Acropora millepora* by injection zygotes with CRISPR/Cas9 ribonucleoprotein complexes (Cleves et al., 2018). While considerable technological development is still required before genetic engineering methods can be applied to corals and their microbial symbionts, these early achievements suggest such manipulations are within the realm of possibility.

Where disease resistance, or resilience to other stressors such as climate change, is being conferred to a species, the selective advantage conferred may lead to the resilience genes naturally increasing in frequency in the targeted population. For example, transgenic malaria-resistant mosquitoes have a fitness advantage when feeding on Plasmodium-infected blood (Marrelli et al., 2007). However, requiring impacts to threatened populations to occur for resilience genes to spread will likely not be desirable for most conservation applications; in addition, the time-frame required for such spread to occur would in most cases be undesirably long. Also, resilience genes can be associated with other fitness costs such as lowered competitive ability or fecundity (Hudson & Greenman, 1998), that may inhibit their spread. Thus for most proposed conservation applications, the resilience conferring gene cargo would need to

be linked to a drive mechanism to cause its spread through the targeted population (see above for a discussion of such mechanisms).

In general, with notable exceptions discussed above, most applications to improve resilience are currently speculative as the gene-editing toolkits to allow such manipulations to be undertaken in target species of conservation concern remain to be developed.

Potential synthetic biology applications for reducing wildlife trade pressures

In general, the technology approaches for proposed synthetic biology approaches for reducing wildlife trade pressures are still to be developed. An exception is the technological development conducted thus far to create a squalene replacement for the cosmetic industry, with potential conservation benefits for harvested shark populations (see Case Study in next chapter). Based on public information, the production appears to involve the use of genetic modification approaches to create proprietary yeast strains that convert sugar to produce various hydrocarbons of interest, in this case, squalene. Technological development for other synthetic biology replacements for wildlife-derived items in trade will likely involve similar approaches, at least in the near future.

Potential synthetic biology applications for proxy species development

It has been proposed that synthetic biology in the case of proxy species development will involve i) working out the complete genome of the target extinct species and the nearest living relative; ii) identifying the differences; iii) synthesizing parts of the extinct species' genome, using gene editing technologies (notably CRISPR-Cas9) to insert this into the target genome. It is then proposed that nuclear transfer from cells with the new DNA will create living organisms with characteristics coded by the new alleles. Using this approach it has been proposed, for example, that all of the differences that an extinct woolly mammoth genome has relative to the living Asiatic elephant genome could thus be edited into an Asian elephant genome to 'resurrect' a woolly mammoth genome (Lynch et al., 2015). However, significant technical challenges remain: (i) DNA is a fragile molecule and there are limits to how far back in time it will be possible to go since degradation of ancient DNA creates gaps and uncertainties. The more gaps, the greater the proportion of the reconstructed genome that must be derived from a near relative; (ii) Genome engineering requires a suitable near relative to provide the appropriate gene sequences to fill gaps around the scaffold of the reconstructed genome and, in mammals, to act as gestational surrogates; (iii) The resulting hybrids will not be genetically identical to the extinct form, and the expression of hybrid traits might be unpredictable, not least due to epigenetic effects.

The technical challenges of fully reconstructing the genomes of extinct species are thus immense (Shapiro, 2015 in: Adams, 2016). Though whole-genome sequencing technologies have become more accessible allowing for the generation of genomic datasets for multiple individuals in species of conservation concern, more research would be needed to advance the ability to decipher the genomic architecture of complex traits important for species persistence (Johnson et al., 2016). Additional advances would then be required in ART techniques, both generally and for application to specific species, to allow the proposed nuclear transfer into egg cells and subsequent successful embryo development to occur. It will likely take much research to overcome the issues of embryo non-viability that are generally occurred when ART approaches involving genetic manipulation are applied to living species (c.f. white rhino).