Reversing *Wolbachia*-based population replacement

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Genetic manipulation that reduces the competence of a vector population to transmit pathogens would provide a useful tool to complement current control strategies, which are based primarily on the reduction/exclusion of vector populations and the prophylactic/therapeutic treatment of the vertebrate host population. Genetic drive is an important component of vector population replacement strategies, facilitating the replacement of natural populations with a genetically modified population. Genetic drive is reviewed here, emphasizing strategies that would employ infections of intracellular *Wolbachia* bacteria as a vehicle for population replacement. Also discussed are strategies for the retarding, arresting or reversing of *Wolbachia*-based population replacement. These strategies are based upon altering the conditions required for transgene invasion and are a prudent safeguard, should unexpected detrimental effects become associated with transgene spread.

The resurgence of vector-borne diseases has led some to express pessimism at the prospect of control by existing means [1,2]. Reasons for the intractability of arthropod-borne diseases to conventional control approaches include: (1) a decline in the public health infrastructure; (2) the emergence of pesticide and drug resistance in arthropod vectors and parasites; and (3) the legislated reduction of available pesticides owing to environmental and public health concerns. Thus, there is a need for development of novel strategies to complement current control measures.

One control strategy that is receiving attention is based upon the replacement of arthropod disease vectors with genetically modified populations that are refractory to pathogen transmission [3]. Recent advances, including the identification of agents that block pathogen transmission and appropriate promoters, together with the development of transgenic techniques for important vectors [2,4–8], have moved the prospect of vector replacement strategies into the realm of 'near-term feasibility'. However, as with any new technology, concerns have been raised regarding the potential risks associated with the release of transgenic vectors, including concerns about the gene-drive strategy that will be used to spread the desired transgene into the field population [9–14]. In particular, prior research with gene-drive techniques has failed to emphasize strategies for retarding or reversing population replacement, should unexpected detrimental effects become associated with transgene spread, giving rise to concerns of a 'Pandora's box' scenario.

Genetic drive

Gene-drive systems are an important component of vector population replacement strategies and provide mechanisms for the autonomous spread of desired transgenes into the targeted population. Although replacement strategies based upon inundative releases of transgenic insects may be used in early technique-proving tests, it is likely that full-scale implementation of population replacement strategies will require gene-drive strategies. Compared with strategies that rely upon inundative releases and mendelian inheritance, strategies that employ gene-drive would require relatively small 'seedings' of transgenic individuals into a field population. Perhaps more important than increased cost efficacy, gene-drive strategies can facilitate population replacement with transgenic individuals that have a lower fitness relative to the natural population [15–17].

Primary candidates for gene-drive strategies are autonomous transposons and paratransgenesis. Transposon strategies are based upon the replicative movement of transposable elements between chromosomes at the time of mating. Thus, a majority of the offspring resulting from matings between an individual with and an individual without the transposon will harbor the transposon, increasing the rate of transgene spread relative to mendelian inheritance. Appropriate examples are provided by prior modeling and population cage experiments [18–22], as well as the global spread of the *P* element throughout fruit flies within the past century [23].

Gene-drive strategies based upon paratransgenesis would employ viruses or bacterial symbionts as expression vehicles [24–29]. Candidates for paratransgenic gene-drive include endosymbiotic *Wolbachia* bacteria that induce cytoplasmic incompatibility (CI). In insect populations that harbor differing *Wolbachia* infection types, CI results in reduced egg hatch from females that lack an infection present in their mates (Box 1) [30]. Thus, infected females are at a reproductive advantage relative to females that lack one or more of the infections. The reproductive advantage afforded by CI to infected females promotes the spread of the maternally inherited *Wolbachia* infection. This *Wolbachia*-induced 'cytoplasmic drive' has been observed in both laboratory and field studies [30–32]. Importantly, model predictions and observations of laboratory and field populations illustrate the ability of

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Box 1. A model for *Wolbachia*-induced cytoplasmic incompatibility

Wolbachia-induced cytoplasmic incompatibility (CI) can be described using a 'poison' and 'antidote' model. The *Wolbachia*-induced 'poisoning' occurs in males, resulting in embryonic developmental arrest when *Wolbachia*-infected males mate with uninfected females. The 'antidote' is provided by the maternally inherited *Wolbachia* infections, such that CI does not occur in broods of similarly infected mates. Thus, in populations that include infected and uninfected individuals (see Fig. I), a pattern of unidirectional CI can result. Infected females can successfully mate with either male type and are at a reproductive advantage relative to uninfected females, resulting in the spread of the *Wolbachia* infection.

The same 'poison–antidote' model applies when differing *Wolbachia* infection types occur within the host population, resulting in a pattern of bidirectional cytoplasmic incompatibility in matings between individuals that harbor different infection types (Fig. Ib).

Multiple *Wolbachia* infection types that occur within the same host individual (superinfection) can lead to patterns of additive unidirectional incompatibility (Fig. Ic). Thus, in mixed populations, superinfected females are at a reproductive advantage because they can mate successfully with all male infection types. Single-infected females can mate successfully with single-infected or uninfected males, and are at an advantage relative to uninfected females that can only mate successfully with uninfected males.

Examples of unidirectional, additive unidirectional and bidirectional CI have been described in numerous insects, including important vector species [a–d]. The CI pattern [a] of four *Culex pipiens* strains (BARRIOL, SELAX, SPHAE and ESPRO) are illustrated in Fig. Id.

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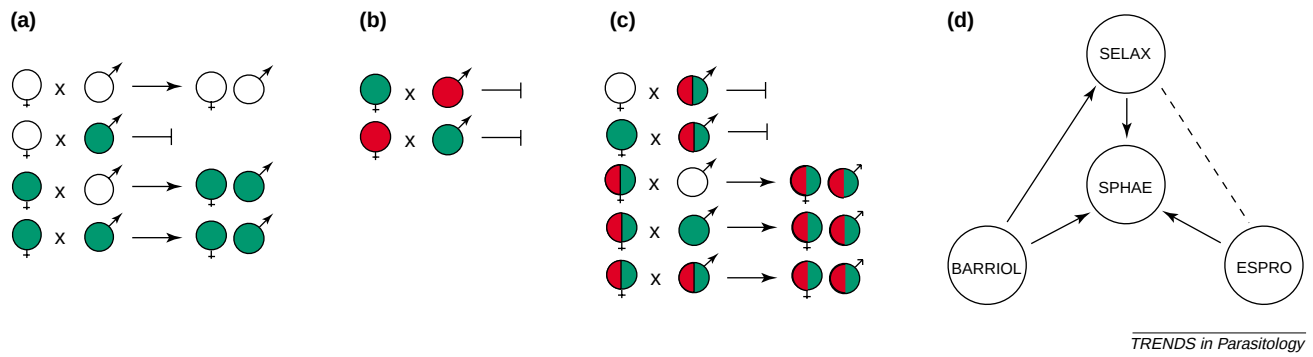

Fig. I. Examples of cytoplasmic incompatibility. (a) Unidirectional cytoplasmic incompatibility (CI) can occur in host populations that include *Wolbachia*-infected (green) and uninfected (white) host individuals. (b) Examples of bidirectional CI can result in matings between individuals with differing infection types (red versus green). (c) *Wolbachia* superinfection (shaded red and green) can result in patterns of additive unidirectional incompatibility. Owing to maternal transmission of *Wolbachia* infections, the infection type in offspring is expected to be similar to that of the mother. (d) Examples of unidirectional (arrows) and bidirectional (broken line) incompatibility types are provided by the different strains of *Culex pipiens*, SELAX, SHAE, ESPRO and BARRIOL (see Ref. [a]). The direction of the arrows indicates the expected direction of population replacement in populations that harbor both cytotypes.

Wolbachia to spread into host populations despite fitness costs [25,31,33]. Thus, transgenes linked to a *Wolbachia* infection would be expected to spread into a targeted population following the seeding of the targeted population with females infected with the transgenic *Wolbachia* (Fig. 1).

Potential advantages of *Wolbachia*-based gene-drive strategies include the general applicability of this strategy, made possible by the broad host range of *Wolbachia* infections [34,35], and the ability to generate new CI types artificially by transfection [36–41]. Furthermore, naturally occurring and artificially generated *Wolbachia* superinfections (i.e. individuals co-infected with two or more *Wolbachia* types) can have an additive effect on CI, such that superinfected females are at a reproductive advantage relative to females that lack one or more of the *Wolbachia* types (Box 1) [37,42,43]. Thus, *Wolbachia* superinfections can allow repeated population replacements within a

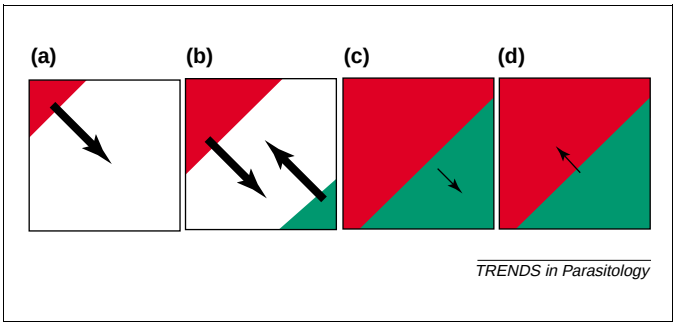

Fig. 1. Strategies for transgene drive and transgene drive reversal using *Wolbachia* infections. (a) *Wolbachia*-infected transgenic mosquitoes (red) are seeded into a vector population (white). The reproductive advantage afforded to released females by unidirectional incompatibility results in the autonomous spread of the *Wolbachia* infection and desired transgene (arrow), replacing the natural population. (b) If undesired effects result from transgene spread, an additional *Wolbachia* infection (green) that is unidirectionally or bidirectionally incompatible with the 'red' *Wolbachia* infection can be released, resulting in a (c) slowing or (d) reversal of the transgene spread. Larger arrow sizes indicate increased rates of *Wolbachia* or transgene spread.

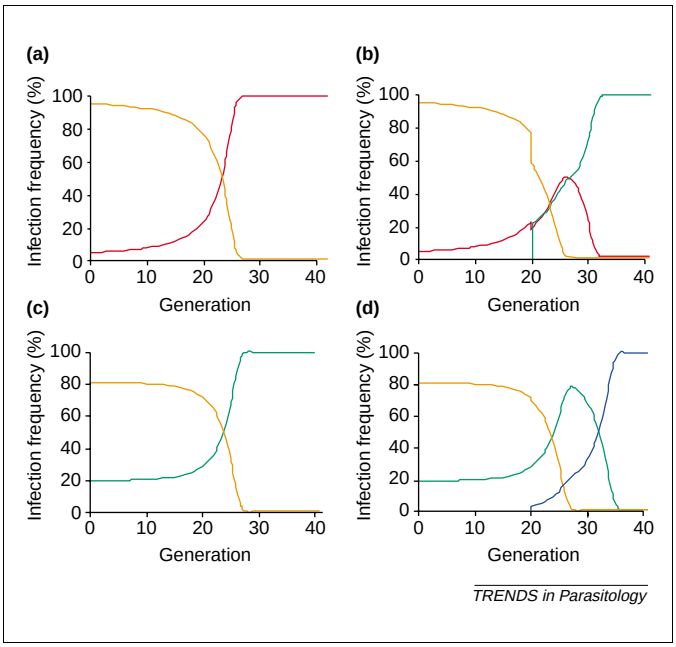

Fig. 2. Model simulations based on *Culex pipiens* strains ESPRO, SPHAE, SELAX and BARRIOL predict strategies for population replacement and replacement reversal. (a) Predicted population replacement resulting from a release 'seeding' of a transgenic ESPRO *Cx. pipiens* strain (red line) into a SPHAE population (orange line). The transgenic ESPRO infection spreads following a single release at generation one, replacing the SPHAE cytotype within 30 generations. (b) If negative effects were to become associated with transgene spread, the transgenic ESPRO infection can be eliminated by a single release (at generation 20) of the bidirectionally incompatible SELAX infection (green line). Simulations provide additional examples (c and d) of strategies that employ unidirectionally incompatible *Wolbachia* infections in *Cx. pipiens*. Predicted population replacement (c) resulting from a release 'seeding' of a transgenic SELAX *Wolbachia* infection into a population with the SPHAE cytotype. The transgenic SELAX infection spreads following a single release at generation one, replacing the SPHAE cytotype within 30 generations. (d) If negative effects were to become associated with transgene spread, the transgenic SELAX infection can be eliminated by the subsequent release (at generation 20) of the unidirectionally incompatible BARRIOL infection (blue line). The illustrated simulations are generated using a previously described model [33]. The cytoplasmic incompatibility levels are as described for the SPHAE, ESPRO and SELAX cytotypes in *Cx. pipiens* [49]. Simulations assume a 0.1% maternal transmission failure rate and a 2% *Wolbachia* reduction of host fecundity [33]. Simulations assume: (a) a single release of ESPRO individuals (5% of total population) at generation one; (b) an additional release of the SELAX cytotype (20% infection frequency) at generation 20; (c) a single SELAX release (20%) at generation one; and (d) an additional release of the BARRIOL cytotype (3%) at generation 20.

vector population, should it become necessary because of transgene inactivation, disassociation of the transgene from *Wolbachia*, or the development of resistance [29].

There is an additional advantage of *Wolbachia*-based gene-drive strategies. Model simulations suggest strategies for retarding or reversing *Wolbachia*-based population replacement (Fig. 2). This capability would provide a prudent safeguard in the event that unexpected, undesired results become associated with transgene spread.

Reversal by bidirectional incompatibility

Using previously developed models, we have examined two strategies for retarding, arresting and reversing the spread of a *Wolbachia* transgene driver. The first strategy would employ bidirectionally incompatible *Wolbachia* infections (Box 1). Our predictions are based upon observations of both natural and artificially generated examples of bidirectional CI [30,44–47] and a previously defined model [33].

Model simulations demonstrate that the release of individuals infected with a *Wolbachia* type that is bidirectionally incompatible with the advancing *Wolbachia* transgene driver could be used to slow, halt or reverse the spread of a transgenic *Wolbachia* infection (Fig. 1). As previously described [33,48], there is no stable equilibrium within insect populations that harbor two or more bidirectionally incompatible *Wolbachia* types. In a population harboring bidirectionally incompatible *Wolbachia* infections, a 'battle' between the incompatible cytotypes will continue until all but one infection has been eliminated, and the insect population is uniformly infected with compatible *Wolbachia* types. Thus, the spread of a transgenic *Wolbachia* infection can be slowed, stopped or reversed by the release of an additional nontransgenic *Wolbachia* type that is bidirectionally incompatible with the transgenic infection (Fig. 2b).

The model [33] demonstrates that key parameters determining whether the transgenic *Wolbachia* infection will continue to spread are the CI levels induced by both the transgenic and nontransgenic infections, as well as by the *Wolbachia* maternal transmission rates and the *Wolbachia* effects on host fitness. If these parameters are identical between the *Wolbachia* types, then the infection that occurs at the highest frequency within the population is expected to 'win' the *Wolbachia* battle [33]. However, *Wolbachia* infections often vary in parameters that affect infection dynamics [30]. Specifically, the infection that induces a higher level of CI ('strong' infection) will displace a 'weaker' infection type that induces a lower CI level [33]. Similarly, the infection exhibiting a higher maternal transmission rate will be at an advantage. Infections that reduce host fitness will be at a disadvantage relative to infections that induce little or no cost to host fitness or that are mutualistic [32]. Figure 3 illustrates the effect of varying infection dynamics on the predicted 'winner' of a *Wolbachia* battle.

An understanding of the parameters that determine *Wolbachia* infection dynamics will aid in the appropriate design of applied population replacement and replacement-reversal strategies. For example, negative effects associated with transgene spread might not be observed immediately following the implementation of the population replacement strategy. Therefore, the transgenic *Wolbachia* infection will have multiple generations in which to invade the host population before implementation of a replacement-reversal strategy, and the transgenic *Wolbachia* infection will have a frequency advantage over subsequently released infections. In the example illustrated in Fig. 2a, the transgenic infection frequency increases fivefold in the 20 generations following the release. Therefore, to remove the transgenic infection using a nontransgenic *Wolbachia* infection, releases of the nontransgenic infection initiated at generation 20 would need to be significantly larger ($\geq$20% of the population; Fig. 2b) than the initial transgenic release (5%). By reserving a 'stronger' *Wolbachia* infection for a replacement-reversal strategy (Fig. 3), lower infection frequencies are sufficient to remove a 'weaker' transgenic infection, should it prove necessary.

A specific example is provided by *Wolbachia* infections

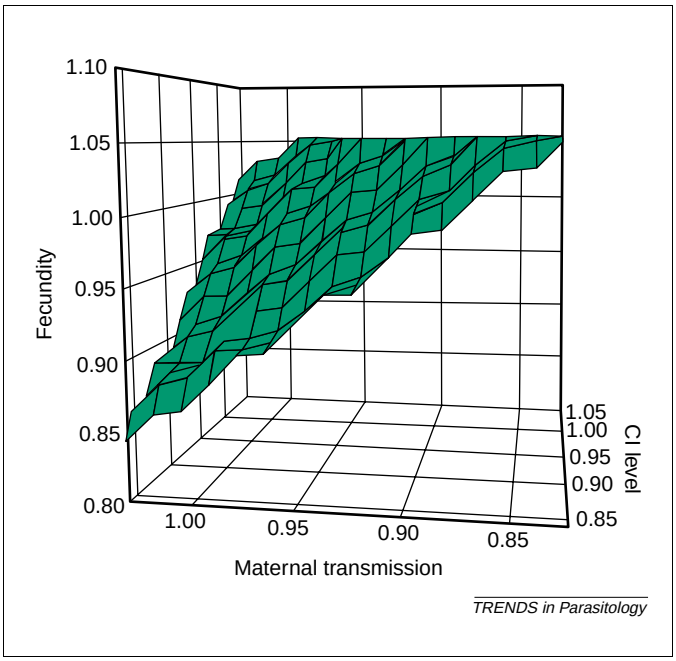

Fig. 3. The predicted 'winner' in a battle between two bidirectionally incompatible *Wolbachia* infections (A and B) within a host population. The A infection is predicted to win at points above the surface plot (shaded green). At points below the surface plot, the B infection is predicted to win. To generate the surface plot, the B infection parameters were held constant at 95% cytoplasmic incompatibility (CI) level, 99.9% maternal transmission and 2% fecundity cost associated with the *Wolbachia* infection. The A infection parameters of CI level, maternal transmission rate and *Wolbachia* effect on host fecundity are varied (the surface plot illustrates 1052 points generated by varying each parameter independently at steps of 0.02). For each of the 1052 examined sets of conditions, the model simulations were continued until only the A or B cytotype remained in the population.

in *Culex pipiens*. The ESPRO and SELAX cytotypes (Box 1) are unidirectionally incompatible with the SPHAE cytotype [49]. Thus, a transgenic ESPRO infection could be employed to replace the SPHAE population (Fig. 2a). If negative effects were to become associated with transgene spread, then releases of a nontransgenic SELAX infection could be used to remove the transgenic infection from the population (Fig. 2b). In the example illustrated in Fig. 2b, the ESPRO infection must exceed an infection frequency of 54% to replace a population with the SELAX cytotype. By contrast, the ESPRO infection need only exceed a 1% infection frequency to invade a population with the SPHAE cytotype. Thus, the introduction of the SELAX cytotype at generation 20 (Fig. 2b) alters the conditions required for ESPRO cytotype to invade the host population.

The examples illustrated in Fig. 2 assume a panmictic population [33]. In spatially complex populations, models and field observations demonstrate the spread of *Wolbachia* infections as a 'traveling wave' of infection [25,31,50]. Thus, a transgenic ESPRO infection that is spreading into a SPHAE population as part of a gene-drive strategy would be expected to be at or near fixation in areas approaching the site(s) of the release 'seedings' and at lower infection frequencies within the hybrid zone that forms along the expanding invasion front. By replacing the SPHAE population with the SELAX cytotype, the hybrid zone of incompatibility along the expanding front would change from unidirectionally incompatible matings to bidirectionally incompatible matings (Box 1). As described above, the infection frequency within the hybrid zone that is required for ESPRO invasion would therefore increase from 1% to 54%. Hence, depending upon infection frequency and the 'strength' of the competing cytotypes, the perimeter of the ESPRO infection would continue to expand (but at a slower rate; Fig. 1c) or would contract (Fig. 1d; replacement reversal).

As previously described [33], host population reproductive rates and survivorship dynamics are not expected to affect significantly the outcome of either replacement or replacement-reversal strategies. However, host migration rates will have an important effect on strategies. With higher host migration rates, the *Culex* population acts increasingly as a panmictic population, such that the 'winning' cytotype is determined by the infection frequency of the total population [49]. However, with decreasing migration rates, 'winning' cytotypes are determined by increasingly localized infection frequencies, such that there is an increasing probability that stable patches of differing infection types will form, separated by a stable, hybrid zone of incompatibility. In the latter scenario, the transgene spread could be arrested, resulting in isolated patches of the transgenic cytotype and facilitating the subsequent removal of individuals harboring the transgene by additional bidirectionally incompatible releases or conventional insecticidal control measures. Interestingly, the stable maintenance of adjacent CI populations that are separated by a hybrid incompatibility zone is similar to previous descriptions of naturally infected *Culex* populations [30].

Reversal by unidirectional incompatibility

As described above, *Wolbachia* superinfections can have an additive effect on CI, such that the superinfection is at a reproductive advantage relative to single infections (Box 1). Thus, an uninfected insect population can be invaded sequentially by *Wolbachia*, initially by a single-infected cytotype and subsequently by a superinfected cytotype. Naturally occurring examples of superinfections have been described in Hymenoptera [51,52], Lepidoptera [53–55], Coleoptera [56] and Diptera [42,43,45,57–59]. Furthermore, both double- and triple-infections have been generated by artificial transfection using microinjection to transfer embryonic cytoplasm [37,43]. The elaborate pattern of crossing types observed in the *Cx. pipiens* species complex has been hypothesized to reflect single- and superinfections of *Wolbachia*, resulting in uni- and bidirectionally incompatible crosses [30].

Superinfections provide an additional strategy for reversing *Wolbachia* gene-drive and displacing an undesired transgenic *Wolbachia* infection. Specifically, a nontransgenic superinfection that is unidirectionally incompatible with a transgenic infection could be used to reverse and remove a *Wolbachia*-linked transgene from a population (Fig. 2d). Returning to the example provided by *Culex*, the BARRIOL cytotype is unidirectionally incompatible with both the SPHAE and SELAX cytotypes (Box 1) [49]. Thus, if the SELAX cytotype was used to drive a transgene into a SPHAE population (Fig. 2c), then a nontransgenic BARRIOL infection could be used subsequently to reverse and remove the transgenic SELAX

cytotype (Fig. 2d). The resulting host population would be the BARRIOL cytotype and without the transgene.

Compared with the use of bidirectionally incompatible infections to retard/reverse transgene spread, the use of a unidirectionally incompatible infection could permit smaller releases (i.e. population 'seeding'; Fig. 2d). Thus, if a SELAX-linked transgene had been driven to fixation within a population, then subsequent releases of the BARRIOL cytotype must only exceed a 10% infection frequency threshold to replace the transgenic SELAX infection. By contrast, replacement reversal following fixation of a transgenic ESPRO cytotype would require releases of the bidirectionally incompatible SELAX cytotype such that SELAX frequency exceeds 46%.

The *Culex* examples above are based primarily upon prior characterization of CI levels [49]. Improved estimates of maternal transmission rates and infection effects on *Culex* fecundity will enhance model predictiveness and aid in the design of applied strategies. Model predictiveness will also be improved with additional understanding of migration rates in the targeted host population and an expansion of the current model to incorporate spatial heterogeneity and host migration. The above strategies will be affected by naturally occurring horizontal or paternal transmission of *Wolbachia* infections, which could result in the mixing of infection types and transgenic superinfections. Previous observations of *Wolbachia* suggest that transmission occurs primarily by maternal inheritance, but that horizontal transmission can occur [60]. Thus, additional studies are required to establish the rates of horizontal and paternal transmission in natural populations of targeted species.

Concluding remarks

Prior to field application of strategies, the results predicted by model simulations should be compared with the results obtained in population cage tests. The results will be useful in discussions of the potential benefits and risks of population replacement and replacement-reversal strategies. Although the current model [61,62] has been used previously to describe mosquito populations [63] and transposon dynamics [18], a primary criterion for its selection was its flexibility, permitting the description of a wide range of insect hosts and variable forms of density-dependant mortality [62]. The latter criterion is important because the *Wolbachia*-based strategies have the potential for use in a broad range of medically and economically important insects. As specific pest/vector species are targeted, variation between population cage tests and model predictions will aid in the recognition of important parameters that are not incorporated into our general model.

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