LETTERS

Speciation by hybridization in *Heliconius* butterflies

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Speciation is generally regarded to result from the splitting of a single lineage. An alternative is hybrid speciation, considered to be extremely rare, in which two distinct lineages contribute genes to a daughter species. Here we show that a hybrid trait in an animal species can directly cause reproductive isolation. The butterfly species *Heliconius heurippa* is known to have an intermediate morphology and a hybrid genome[1], and we have recreated its intermediate wing colour and pattern through laboratory crosses between *H. melpomene*, *H. cydno* and their F_1 hybrids. We then used mate preference experiments to show that the phenotype of *H. heurippa* reproductively isolates it from both parental species. There is strong assortative mating between all three species, and in *H. heurippa* the wing pattern and colour elements derived from *H. melpomene* and *H. cydno* are both critical for mate recognition by males.

Homoploid hybrid speciation—hybridization without change in chromosome number—is considered very rare[2–4]. This has been explained by the theoretical prediction that reproductive isolation between hybrids and their parents is difficult to achieve[3,5,6]. However, if a hybrid phenotype directly causes reproductive isolation from parental taxa, this difficulty can be overcome. Such a role for a hybrid phenotype has been convincingly demonstrated only in *Helianthus* sunflowers[7]. In animals, the evidence for homoploid hybrid speciation is less convincing. Putative hybrid species are known with mixed genomes[8–11], but in these examples shared genetic variation could also be a result of introgression subsequent to a bifurcating speciation event.

Heliconius cydno and *H. melpomene* are two closely related species that overlap extensively in lower Mesoamerica and the Andes[12]. Speciation in these butterflies has not involved any change in chromosome number[13] but is instead associated with shifts in colour patterns that generate both assortative mating and postzygotic isolation due to predator-mediated selection[14–17]. *Heliconius cydno* is black with white and yellow marks, whereas *H. melpomene* is black with red, yellow and orange marks. Both species exhibit strong positive assortative mating based on their wing colour patterns and also differ in habitat use[18] and host plant preference[19], but interspecific hybrids do occur at low frequency in the wild[15]. *Heliconius heurippa* has an intermediate wing pattern, which has led to the suggestion that this is a hybrid species[1,20]. Its hindwing is indistinguishable from that of sympatric *H. m. melpomene*, whereas the yellow band on its forewing is similar to that of parapatric *H. cydno cordula*. Ecologically, *H. heurippa* is most similar to *H. cydno*, which it replaces geographically in the eastern Andes of Colombia.

Here we first establish that *H. heurippa* is currently genetically isolated from its putative parents and provide evidence that its genome is of hybrid origin. A bayesian assignment analysis using 12 microsatellite loci scored in populations from Panama, Colombia and Venezuela divides *H. cydno* ($n = 175$), *H. melpomene* ($n = 167$) and *H. heurippa* ($n = 46$) individuals into three distinct clusters (Fig. 1). Hence, *H. heurippa* is genetically more differentiated than any geographic race sampled of either species. Moreover, analyses of polymorphism at two nuclear genes (*Invected* and *Distal-less*) show no allele sharing between *H. cydno* and *H. melpomene*, whereas the *H. heurippa* genome appears as an admixture, sharing allelic variation with both putative parental species (Supplementary Fig. 2, and C.S., C.D.J. and M.L., unpublished observations).

To test the hypothesis of a hybrid origin for the *H. heurippa* colour pattern, we performed inter-specific crosses between *H. cydno*

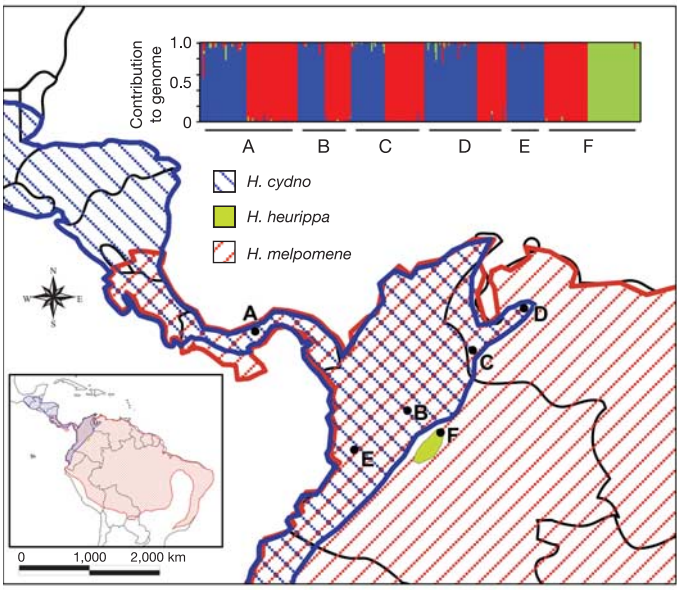

Figure 1 | Geographic distributions and genetic differentiation between *H. cydno*, *H. melpomene* and *H. heurippa*. *H. heurippa* is sympatric with *H. melpomene* and parapatric with *H. cydno* in eastern Colombia. We used the software Structure 2.1 (ref. 29) with the multilocus microsatellite data set to assign individuals to species and detect admixed individuals (namely hybrids). We ran Structure 2.1, varying the burn-in (10^4 to 10^5) and run length (10^5 to 10^6), number of clusters (one to four), ancestry type (with and without admixture) and allele frequency estimation (correlated and independent) to obtain the highest probability model for the data set. The upper inset shows the results obtained with the best model (three clusters, admixture and independent estimations of allele frequencies). The relative contributions of the three clusters to each individual's genome are shown in the following colours: blue, *H. cydno*; red, *H. melpomene*; green, *H. heurippa*. Collection site codes: A, Pipeline Road, Panama; B, Parcela 33, Colombia; C, San Cristóbal, Venezuela; D, La Gira, Venezuela; E, Ocache, Colombia; F, Villavicencio, Colombia. An expanded view of the clusters is shown in Supplementary Fig. 1.

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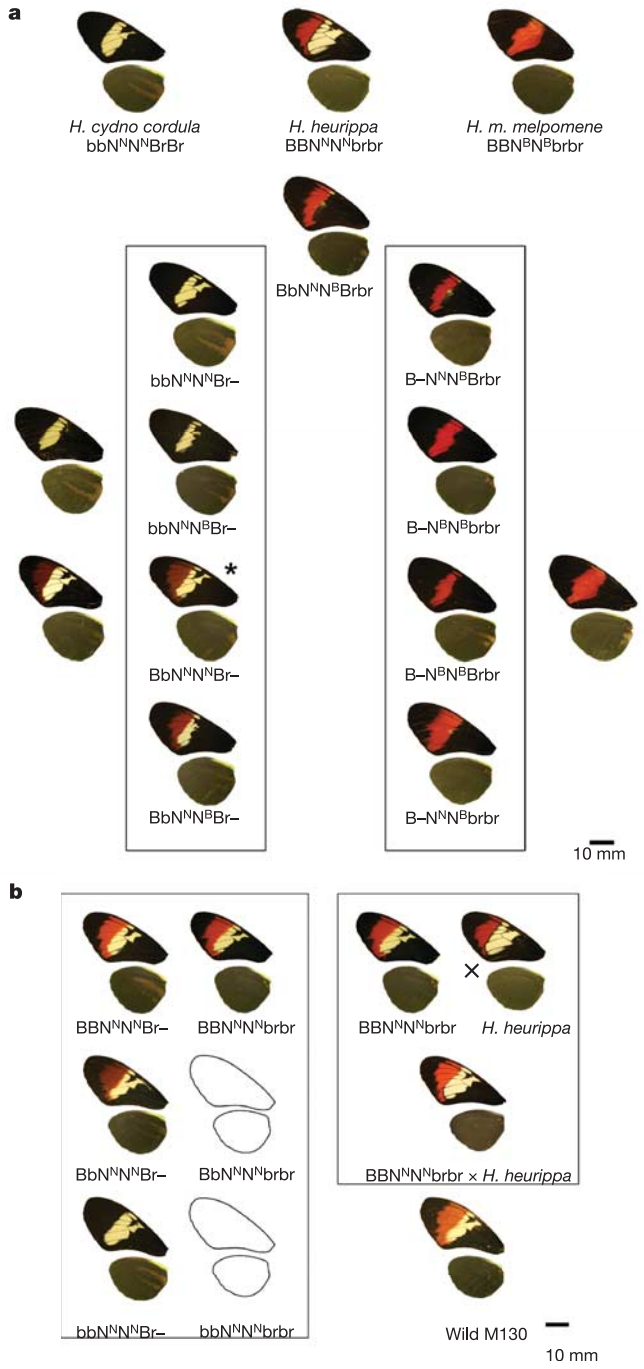

Figure 2 | Reconstruction of the *H. heurippa* wing pattern. All fore- and hind-wings shown in dorsal and ventral views, respectively. **a**, First row, *H. cydno cordula*, *H. heurippa* and *H. m. melpomene*; second row, *H. cydno cordula* × *H. m. melpomene* F_1 hybrid. Backcrosses to *H. cydno cordula* and *H. m. melpomene* are shown in the left and right boxes, respectively. Other individuals are wild hybrids from San Cristóbal, Venezuela, shown next to their putative genotypes. Three major loci regulate the patterns: complete (BB, *H. melpomene*), intermediate (Bb, heterozygotes) or no (bb, *H. cydno*) expression of a red band on the dorsal forewing; complete (N^NN^N, *H. cydno*), intermediate (N^NN^B, heterozygotes) or no (N^BN^B, *H. melpomene*) expression of a yellow band on the dorsal forewing; and complete (BrBr, *H. cydno*), intermediate (Brbr, heterozygotes) or no (brbr, *H. melpomene*) expression of a brown pincer-shaped mark on the ventral hindwing. Further crosses were performed using individuals with the phenotype marked with an asterisk (see **b**). **b**, Left box: offspring from crosses between individuals marked with an asterisk in **a**. The B and Br loci are linked, explaining the absence of the two recombinant genotypes BbN^NN^Nbrbr and bbN^NN^Nbrbr. Right box: offspring of a cross between a laboratory hybrid with genotype BBN^NN^Nbrbr and *H. heurippa*, showing that the pattern breeds true. The other individual (M130) is a wild hybrid from San Cristóbal, Venezuela, with a phenotype very similar to *H. heurippa*.

cordula and *H. m. melpomene* to reconstruct the steps of introgressive hybridization that could have given rise to *H. heurippa*. The colour pattern differences between *H. m. melpomene* and *H. cydno cordula* are determined largely by three co-dominant loci controlling the red and yellow bands on the forewing and the brown pincer-shaped mark on the ventral hindwing (see Fig. 2a)[21,22]. Most *H. cydno* × *H. melpomene* F_1 hybrids seem intermediate to both parents (Fig. 2a), with both a yellow (*cydno*) and a red (*melpomene*) band in the median section of the forewing, whereas the ventral side of the hindwing shows a reduced brown mark intermediate between the parental species.

Female F_1 hybrids resulting from crosses between *H. melpomene* and *H. cydno* are sterile in accordance with Haldane's rule[1,23], and thus only male F_1 hybrids backcrossed to either *H. cydno cordula* or *H. m. melpomene* females resulted in offspring. Backcrosses to *H. melpomene* produced offspring very similar to pure *H. m. melpomene*, and further backcross generations never produced individuals with forewing phenotypes similar to *H. heurippa* (Fig. 2a). However, after only two generations a phenotype virtually identical to *H. heurippa* (Supplementary Fig. 3) was produced by backcrossing an F_1 male to an *H. cydno cordula* female and then mating selected offspring of this cross (Fig. 2b). In offspring of crosses between these *H. heurippa*-like individuals the pattern breeds true, showing that they are homozygous for the red forewing band (BB) and the absence of brown hindwing marks (brbr) characteristic of *H. melpomene*, and similarly homozygous for the yellow forewing band (N^NN^N) derived from *H. cydno*. The pattern of these *H. heurippa*-like individuals also breeds true when crossed to wild *H. heurippa* (Fig. 2b), implying that pattern genes segregating in our crosses are homologous with those in wild *H. heurippa*.

Furthermore, in a wild population of sympatric *H. m. melpomene* and *H. cydno cordula* in San Cristóbal, Venezuela, we observed natural hybrids at an unusually high frequency (8%), including some individuals very similar to our laboratory-produced *H. heurippa*-like butterflies (Fig. 2b). Microsatellite data show that these individuals have genotypes indistinguishable from that of *H. cydno* and must therefore be at least fifth-generation backcrosses (Supplementary Fig. 4). This shows that multiple generations of backcrossing can occur in the wild and that female hybrid sterility is not a complete barrier to introgressive hybridization. The fact that the *H. heurippa* pattern can be generated by laboratory crosses between *H. melpomene* and *H. cydno*, and is also observed in wild hybrids between the two species, establishes a probable natural route for the hybrid origin of *H. heurippa*.

The next step in species formation is reproductive isolation. We therefore tested the degree to which *H. heurippa* is isolated from *H. melpomene* and *H. cydno* by assortative mating. No-choice mating experiments showed a reduced probability of mating in all interspecific comparisons, with *H. heurippa* females particularly unlikely to mate with either *H. cydno* or *H. melpomene* (Table 1). When a male of each species was presented with a single female, *H. heurippa* males were tenfold more likely to court their own females than the other species (Supplementary Fig. 5). In mating experiments with choice, there was similarly strong assortative mating, although occasional matings between *H. cydno* and *H. heurippa* were observed (Table 2). Isolation due to assortative mating, on average more than 90% between *H. heurippa* and *H. melpomene* and more than 75% between *H. heurippa* and *H. cydno*, is therefore considerably greater than that caused by hybrid sterility (about 25% isolation between *H. heurippa* and *H. melpomene*, and zero between *H. heurippa* and *H. cydno*)[1] or predator selection against hybrids (about 50%)[24]. Therefore, strong assortative mating, in combination with geographic isolation

Table 1 | Relative mating probabilities in no-choice experiments

Male	Female		
	H. melpomene	*H. cydno*	*H. heurippa*
H. melpomene	1 (17)	0.178 (0.084–0.309, 45)	0.073 (0.023–0.163, 55)
H. cydno	0.120 (0.048–0.231, 50)	1 (27)	0.022 (0.001–0.096, 45)
H. heurippa	0.100 (0.031–0.022, 40)	0.440 (0.255–0.637, 44)	1 (22)

For each female type, probabilities were estimated relative to that of intra-specific mating, which was set to 1. Numbers in parenthesis show the 95% maximum-likelihood support limits and the number of females used.

from *H. cydno* and postzygotic isolation from *H. melpomene* has contributed to the speciation of *H. heurippa*.

We next investigated the role of colour pattern in mate choice. Experiments with dissected wings showed that both elements of the forewing colour pattern of *H. heurippa* were necessary for the stimulation of courtship (Fig. 3). *H. heurippa* males were less than half as likely to approach and court the *H. m. melpomene* or the *H. cydno cordula* pattern than their own (Fig. 3). When either the red or yellow bands were experimentally removed from the *H. heurippa* pattern, this led to a similar reduction in its attractiveness, demonstrating that both hybrid elements are necessary for mate recognition by male *H. heurippa* (Fig. 3).

Similar results were obtained when these experiments were replicated with printed-paper models (Fig. 3), showing that the colour pattern itself was the cue rather than pheromones associated with the dissected wings. Additional experiments showed that males of both *H. m. melpomene* and *H. cydno cordula* showed a greatly reduced probability of approaching and courting the *H. heurippa* pattern than their own (Supplementary Figs 6 and 7). Given the incomplete postzygotic reproductive isolation between all three species[1], this pattern-based assortative mating must have a continuing role in generating reproductive isolation between *H. heurippa* and its relatives.

Novel patterns in *Heliconius* probably become established through a combination of genetic drift and subsequent fixation of the novel pattern driven by frequency-dependent selection[25]. Such an event could have established the hybrid *H. heurippa* pattern as a geographic isolate of *H. cydno*. Subsequently, the pattern was sufficiently distinct from both *H. melpomene* and *H. cydno* that mate-finding behaviour also diverged in parapatry, generating assortative mating between all three species (Supplementary Fig. 8). This two-stage process indicates a possible route by which the theoretical difficulty of a rapid establishment of reproductive isolation between the hybrid and the parental taxa could have been overcome[5,6]. Furthermore, because we are proposing divergence in mate behaviour in a geographically isolated population, reinforcement or some other form of sympatric divergence is not required for speciation to occur.

Our study provides the first experimental demonstration of a hybrid trait generating reproductive isolation between animal species, and the first example of a hybrid trait causing pre-mating isolation through assortative mating. None of the theoretical treatments of homoploid hybrid speciation have considered the effects of assortative mating[5,6]. If variation for mate preference were incorporated, the theoretical conditions favouring hybrid speciation might not be as stringent as has been supposed. Finally, two other species, *H. pachinus*[20] and *H. timareta*[26], have also been proposed as having *H. cydno*/*H. melpomene* hybrid patterns, indicating that this process might have occurred more than once. However, whether these cases represent a particularity of *Heliconius* or a common natural process that has been undetected in other animal groups studied less intensively remains a matter of further study. Suggestively, other proposed cases of homoploid hybrid speciation in animals occur in well-studied groups such as African cichlids[8–10] and *Rhagoletis* flies[11].

Table 2 | Number of matings in tetrad mate-choice experiments

Female	Male	
	H. melpomene	*H. heurippa*
H. melpomene	15	0
H. heurippa	0	12
	H. cydno	*H. heurippa*
H. cydno	5	3
H. heurippa	0	5
	H. melpomene	*H. cydno*
H. melpomene	10.5	0
H. cydno	0	5.5

Mating results of 0.5 are due to simultaneous mating of both pairs during the experiment.

METHODS

Crosses. Crosses were performed in La Vega, Colombia, between January 2000 and December 2002 with the use of *H. m. melpomene* (Virgen de Chirajara, 4.213° N, 73.795° W) and *H. cydno cordula* (Barro Negro, 6.016° N, 72.091° W), both from the Colombian Eastern Cordillera. We isolated virgin females with older males to produce inter-specific F_1 offspring and backcrosses. After mating, females were kept individually in $2 \times 3 \times 2\ m^3$ insectaries and supplied with pollen and nectar from *Psiguria* and *Lantana* flowers, and *Passiflora* vines for oviposition[22]. Eggs and the larval and adult stages were reared as described

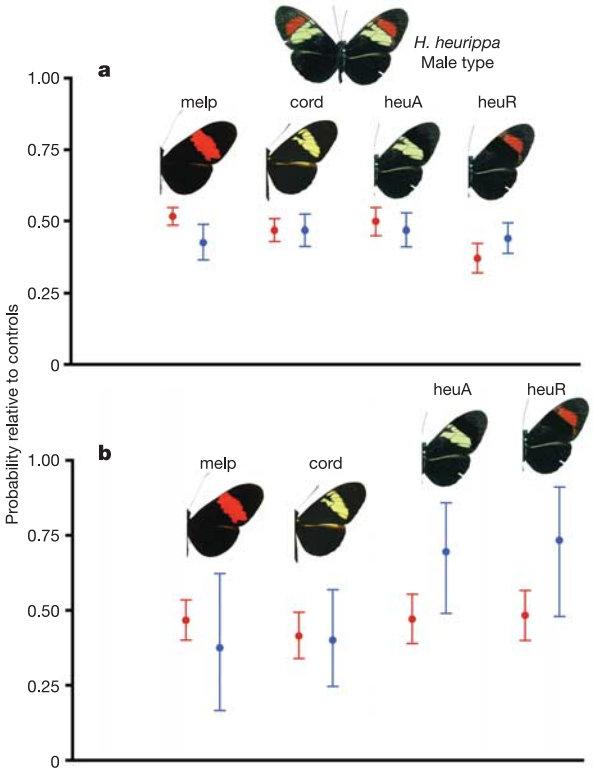

Figure 3 | Relative probabilities of *H. heurippa* males approaching and courting colour pattern models. Values are estimated relative to the probability of approach (**a**) and courtship (**b**) of the *H. heurippa* control pattern shown at the top (probability equal to 1). Model patterns are *H. m. melpomene* (melp), *H. cydno cordula* (cord), *H. heurippa* modified to remove the red band (heuA) and *H. heurippa* modified to remove the yellow band (heuR). Red, real wings; blue, paper wings. Error bars show maximum-likelihood support limits (see Supplementary Information for an explanation).

previously[23]. Colour pattern segregation and designation were studied with the use of nomenclature described previously[22].

No-choice experiments. Experiments without choice measure the reluctance of males and females to mate inter-specifically; such experiments simulate a natural situation in which males encounter females singly. In the insectaries mentioned above, a virgin female (one to three days old) of each species was presented to ten mature males (more than ten days old) for two days. Males were used only once. Matings were recorded every 30 min from 06:00 until 15:00. Experiments were performed between all combinations of the three species, including control experiments between conspecifics. To detect any unobserved mating, all the females were checked after the experiments for the presence of a spermatophore in their reproductive tract.

Tetrads. Experiments with choice were performed to estimate mating probability in a situation in which males encounter females from different species simultaneously. Pairwise experiments were performed between each combination of the three species, in which a single recently emerged virgin female and a mature male (more than ten days old) of each species were placed in a $2 \times 3 \times 2\,m^3$ insectary over the course of a single day. Thus, each experiment involved four butterflies, for example a female and male *H. heurippa* and a female and male *H. cydno* for the comparison between those two species. The first mating only was recorded for each experiment, and individuals were not reused. In cases in which both pairs mated simultaneously they were scored as each having half a mating. At least 15 experiments were performed for each pairwise comparison. Mating probabilities were estimated by likelihood (Supplementary Methods).

Colour pattern models. In *Heliconius*, males use colour patterns to locate females and are choosy in mating, presumably because of the large and costly spermatophore transferred to females[27]. We investigated male preferences for different colour pattern models. Between 10 and 20 males in a $2 \times 3 \times 2\,m^3$ insectary were presented with either dissected natural wings or a printed colour pattern model, fixed to a length of flexible clear nylon. Models were manipulated to simulate *Heliconius* flight in the centre of a spherical area (60 cm in diameter) demarcated by references in the insectary roof. Randomly ordered pairs of 5-min experiments were performed: first, a control flight with a model of the male's own colour pattern, and second, an experimental flight with a different colour pattern. Entry to the sphere was recorded as 'approach' and sustained fluttering directed at the model as 'courtship'. At least 25 replicates were performed for each comparison. In addition, models were made in which the *H. heurippa* pattern was modified to show either the yellow band without red (Heu-A) or the red band without yellow (Heu-R). For the dissected wing models, permanent black marker pen (Pilot ultrafine point no xylene SCA-UF) was used to cover the corresponding band. Paper models were made from digital photographs of wings taken with a Sony Cyber-shot dsc-s85 camera that were printed with a high-performance inkjet printer (Hewlett Packard Deskjet 3820) on special photo-quality calcium paper. Only paper models with a reflectance spectra similar to real wings were used. The data were used to estimate the probabilities Q_{ij} that males of type j approached or courted models of type i relative to that of their own type j (which was set to one), using likelihood. Confidence intervals for parameters were obtained as the values that decreased the difference between two $\log_e$ likehoods by two units (Supplementary Methods).

Microsatellites. Twelve microsatellite loci were genotyped (Hel02, Hel04, Hel05, Hm01, Hm02, Hm03, Hm04, Hm05, Hm06, Hm13, Hm19 and Hm22)[28] for 60 individuals of *Heliconius heurippa* and at least 24 individuals from each of five populations of both *H. melpomene* and *H. cydno*. Collection sites were as follows: *H. c. chioneus* and *H. m. rosina* from Pipeline Road, Panama (9.122° N, 79.715° W); *H. c. cordula*, *H. m. melpomene* from San Cristóbal, Venezuela (7.767° N, 72.225° W); *H. c. chioneus* and *H. m. melpomene* from Parcela 33, Colombia (5.066° N, 74.561° W); *H. heurippa* and *H. m. melpomene* from near Villavicencio, Colombia (4.151° N, 73.635° W); *H. c. weymeri* and *H. c. cydnides* from Ocache, Colombia (3.703° N, 76.493° W); *H. c. barinasensis* and *H. m. melpomene* from La Gira, Venezuela (9.334° N, 70.730° W). The genetic structure of populations was analysed with bayesian assignment tests as implemented in Structure 2.1 (ref. 29).

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information The sequences have been deposited in GenBank under accession numbers DQ445384–DQ445414 (*Distal-less*) and DQ445416–DQ445457 (*Invected*). Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to M.L. (mlinares@uniandes.edu.co) or J.M. (mavarezj@si.edu).