Acknowledgements

This research was supported by the US Geological Survey and a grant from the LexEn programme of the National Science Foundation.

Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to F.H.C (e-mail: chapelle@usgs.gov) or D.R.L. (e-mail: dlovley@microbio.umass.edu). GenBank accession numbers for sequences IUA5, IUA6, IUA162 and IUA69 are AF448449, AF448450, AF448 451 and AF448452, respectively.

Contribution of *Distal-less* to quantitative variation in butterfly eyespots

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The colour patterns decorating butterfly wings provide ideal material to study the reciprocal interactions between evolution and development. They are visually compelling products of selection, often with a clear adaptive value, and are amenable to a detailed developmental characterization[1]. Research on wing-pattern evolution and development has focused on the eyespots of the tropical butterfly *Bicyclus anynana*[2]. There is quantitative variation for several features of eyespot morphology[3–5] but the actual genes contributing to such variation are unknown. On the other hand, studies of gene expression patterns in wing primordia have implicated different developmental pathways in eyespot formation[6–11]. To link these two sets of information we need to identify which genes within the implicated pathways contribute to the quantitative variation accessible to natural selection. Here we begin to bridge this gap by demonstrating linkage between DNA polymorphisms in the candidate gene *Distal-less (Dll)* and eyespot size in *B. anynana*.

The comparison of gene expression patterns across species has been a common approach in evolutionary developmental biology. This approach can identify steps in developmental pathways that have been altered during evolution, but it fails to identify the actual genetic changes that have occurred[12]. Despite the recognized importance of understanding the generation of the variants that can be sorted by natural selection[13], the genes contributing to standing quantitative variation in morphological traits are largely unknown. Recent work has focused on bristle number in *Drosophila melanogaster*[14]. Unfortunately the direct developmental mechanisms through which polymorphisms in candidate genes contribute to variation in bristle number, and the ecological significance of this variation, are difficult to determine. Here we investigate the genetic basis of eyespot size in *B. anynana*, a character of more obvious adaptive significance[2,15] and whose developmental basis can be dissected using manipulative experiments[3].

Studies of gene expression patterns in pre-adult wing primordia have implicated a series of genes in butterfly wing patterning and, in particular, in eyespot formation[6–11]. Genes including *Dll* and genes of the *hedgehog* pathway have circular regions of expression that correspond to the position of eyespot centres[8,9] whose organizing properties have been clearly demonstrated[16,17]. *Dll* is particularly interesting because its expression patterns appear to reveal different stages in eyespot formation and parallel adult variants of eyespot morphology[8]. These results suggest that *Dll* is involved in regulating the formation and diversity of eyespot patterns in butterfly wings[8]. However, although *Dll* expression patterns implicate the Dll protein in eyespot formation, it is unclear how standing variation at this locus contributes to inter-individual variation in eyespot patterns. Here we test whether segregating variation at *Dll* contributes to short-term response to selection on eyespot size.

We used artificial selection to establish lines that differed in the size of the two dorsal forewing eyespots of *B. anynana* (see Methods). After nine generations of selection, the lines show markedly different phenotypes; the 'high' line has large eyespots and the 'low' line, small eyespots (Fig. 1a, b). The rapid and gradual response to selection indicates that there is substantial additive genetic variance for eyespot size. Realized heritabilities of around 0.6–0.7 (Fig. 1a) are comparable to previous estimates for the size of the posterior eyespot[3]. The selected butterflies also show quantitative differences in *Dll* expression: wing discs of the high line have considerably larger areas of *Dll* expression around the location of the centre of the presumptive eyespots. These differences are already apparent in late final instar larvae (Fig. 1c), and more marked in pupal wing discs (Fig. 1d). The observed ontogeny of *Dll* expression is comparable to that described previously[8].

The changes in *Dll* expression might be caused by DNA polymorphisms in *Dll* itself or in upstream regulators of *Dll*. To distinguish between the hypotheses of *cis* versus *trans* polymorphisms affecting *Dll* expression, a series of crosses were made using the high and low selection lines (Fig. 2a). The progeny from a backcross between a hybrid butterfly and each of the parental lines have

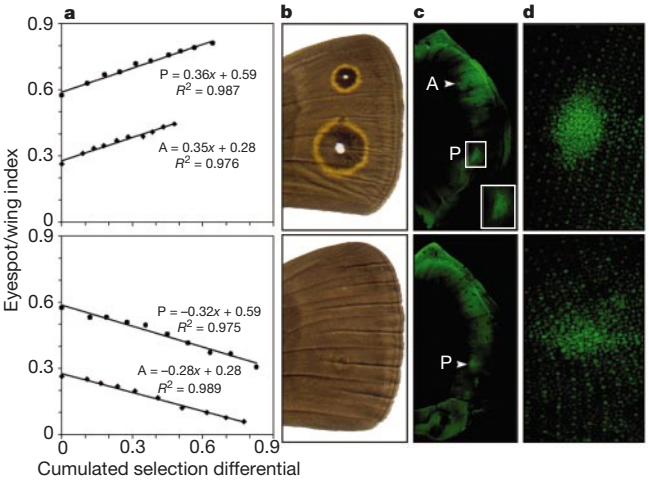

Figure 1 *Bicyclus anynana* selection lines divergent for the size of the dorsal forewing eyespots. **a**, Response to artificial selection for large (high line, top) and small (low line, bottom) eyespots. For each generation, the selection differential is the difference between the mean trait value for all butterflies and that of the selected group[29]. Realized heritabilities are twice (selection has targeted females only)[29] the absolute value of the slopes estimated from least-squares regressions for both eyespots (anterior (A) indicated by diamonds, posterior (P) by circles). **b**–**d**, Eyespot size phenotypes and *Dll* expression in butterflies from the high and low selection lines. Butterflies from the high line have large adult eyespots (**b**, top). They also show enlarged domains of *Dll* expression in the centre of the putative eyespots both in late larval wing discs (**c**, top; inset with higher magnification of central area of posterior (P) eyespot; arrow points at centre of anterior (A) eyespot) and in pupal wings (anterior eyespot centre shown in **d**, top). Butterflies from the low line have small, often absent, adult eyespots (**b**, bottom) and small areas of *Dll* expression in wing primordia (**c**, **d**, bottom). In the larval disc in **c** (bottom) there is no visible *Dll* expression associated with the anterior eyespot; arrow points to the posterior eyespot centre. In the pupal disc there are small domains of *Dll* expression as shown here for the anterior eyespot (**d**, bottom).

either both *Dll* alleles from the same origin or one allele from each parental line. To distinguish *Dll* alleles from high and low origin we cloned a *B. anynana Dll* homologue (GenBank accession number AF404825), which showed 99% amino acid identity with the published *Precis coenia* Dll[18]. We sequenced 1,250 base pairs (bp) of this gene in individuals from the selection lines (see Methods), and identified 27 segregating sites (corresponding to levels of DNA polymorphism comparable to *Drosophila*[19]). We used two of these polymorphisms to distinguish high (H) and low (L) *Dll* alleles and to genotype individuals from the crosses (see Methods). The high backcross produced progeny of genotype HH or HL, and the low backcross produced HL or LL (Fig. 2a). Eyespot sizes of butterflies from distinct genotypic classes were compared to test for an association between *Dll* genotype and that phenotype (see Methods).

We observe that high and low *Dll* alleles segregate with eyespot size in the backcrosses (Fig. 2b). Significant differences were found between the size of the posterior eyespot of HH versus HL backcross females and for both the anterior and posterior eyespots in HL versus LL males (Table 1). Our results show that there are differences in the estimated allelic effects between males and females and between the two target eyespots. Work in *Drosophila* and other organisms has frequently detected this type of sex-specific effect and correlated character-specific effects[14,20,21]. As the identified *Dll*-linked factor explains only part of the difference between selection lines (Table 1), other genes must have been involved in the response to selection (for ventral eyespot size at least five loci were implicated[22]). These genes may include upstream *trans*-regulators of *Dll*.

Segregation of a molecular marker with eyespot size is evidence of linkage but does not necessarily implicate the marker site as directly contributing to the trait. The phenotypic differences between backcross genotypic marker classes depend on the effect of the quantitative trait locus (QTL) linked to the marker site and on the recombination fraction between the two[23]. Although an interval mapping approach is necessary to distinguish between the effect of a QTL and its location[24], we can calculate the log odds (LOD) score (see Methods) for a putative QTL some recombination fraction away from the markers in *Dll*. For both the posterior and anterior eyespots in males from the low backcross, the LOD score was maximized at *Dll* (Table 1), although a 1 LOD support interval indicates that the QTL could be as far as 40 cM from *Dll*. The effect detected for the posterior eyespot in females from the high backcross might be due to the same QTL that affects males (its LOD score did not peak at *Dll* but the value at *Dll* was not significantly lower than its maximum value; Table 1) or to a second QTL linked to, but not at *Dll*. The analyses in Table 1 indicate that a QTL at *Dll* is the most parsimonious explanation for the observed data, although a linked QTL cannot be ruled out. As *B. anynana* is thought to have approximately 26 linkage groups (26–29 is the haploid chromosome number in other *Bicyclus* species[25]), linkage to *Dll* rules out a large number of other candidate genes. Mapping of the QTL to one of 26–29 linkage groups localizes it to about 4% of the genome. To improve this resolution and locate the causative sites within *Dll*, future work will use population-based association studies[26,27].

Dll codes for an evolutionarily conserved transcription factor characteristically expressed in insect appendages[18]. In butterflies it has been redeployed in a spatial coordinate system operating within specific wing regions and leading to eyespot formation[6–8]. Our results suggest that this same gene contributes to inter-individual variation in eyespot size in *B. anynana*. The co-segregation of DNA polymorphisms in *Dll* with eyespot variation, coupled with quantitative changes in *Dll* expression between phenotypically divergent lines, suggests that not only the *Dll* pathway but molecular variants at *Dll* itself contribute to within-species variation in eyespot patterns and are thus likely to contribute to the evolutionary diversification of butterfly wing patterns. Other candidate genes possibly involved in generating variation in eyespot patterns include *decapentaplegic*[6], *engrailed*[9,11], *spalt*[11] and *Ultrabithorax*[10]. Ultimately, we can ask how such genes interact with each other, the environment, and the pigment biochemical pathways to provide the evolutionary potential for the spectacular diversity seen in butterfly wing patterns. Our results show that a combination of approaches from evolutionary and developmental biology can contribute more to understanding how evolutionarily relevant variation is generated than either approach used alone. □

Table 1 Significant differences in eyespot size between *Dll* genotypes

Parameter	Female posterior (HH × HL)	Male anterior (HL × LL)	Male posterior (HL × LL)
Size difference*			
Trait values (*T*)	0.025	0.046	0.117
T/base s.d.	0.42	0.80	2.17
T/(high – low)	0.05	0.14	0.22
Probability of no eyespot†			
given HH	0	NA	NA
given HL	0	0.11	0.14
given LL	NA	0.24	0.48
P-value‡			
Mann–Whitney *U*	0.0132	0.0103	0.0004
Permutation test	0.001	0.007	0.004
Maximum LOD score			
Value	0.81	1.24	2.67
Position§ (cM)	20‖	0	0

NA, not applicable.
* Phenotypic differences between genotypic classes (HH, HL and LL) for the posterior eyespot of females from the high line backcross and for both eyespots of males from the low line. The differences between average phenotypes of alternative genotypes are given in raw trait units (*T*), units scaled to base population standard deviations (s.d.), and expressed as a proportion of the total difference between high and low selected lines.
† Probability of the eyespot being absent conditional on genotype.
‡ *P*-values for the one-way Mann–Whitney *U*-test and permutation tests indicate that all differences are statistically significant.
§ Distance from *Dll*.
‖ LOD score at *Dll* is 0.73.

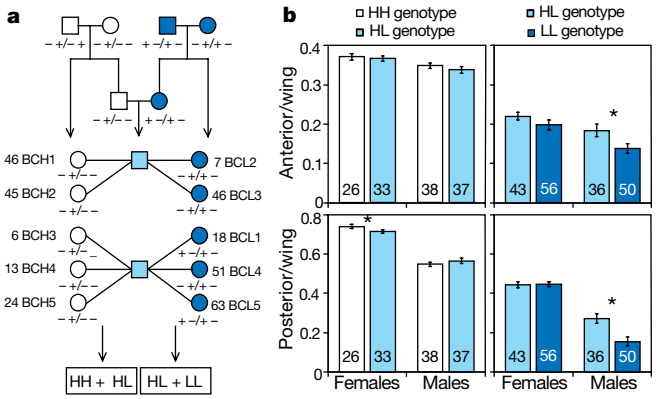

Figure 2 Test for an association between alleles at the *Dll* locus and dorsal eyespot size in *B. anynana*. **a**, Crosses between phenotypically divergent lines produced after nine generations of artificial selection. Individuals from the high and low lines and heterozygotes are represented by white, dark-blue and light-blue symbols, respectively. To minimize intra-line genetic variation we used single mating pairs (females shown as circles and males as squares). The backcrosses between two hybrid males (*Lepidoptera* females appear not to have recombination[30]) and five females from each divergent line produced a total of five backcross families in each direction (BCH1–5 and BCL1–5 for high and low, respectively). The numbers before each BC family indicate total number of informative progeny. H and L represent high and low *Dll* alleles, respectively. *Dll* locus genotypes based on the two sequence polymorphisms are given for all single pairs used (see Methods; _ is not known). **b**, Mean eyespot/wing size index (± standard error) for males and females of each of the two genotypic classes (different colours) for each backcross direction (high line on the left and low line on the right). Asterisks indicate statistically significant differences at the 5% level (Table 1). Numbers inside columns represent sample sizes.

Methods

Artificial selection

Nine generations of artificial selection on eyespot size targeted the ratios between eyespot diameter and a linear measurement of wing size. To select on the two dorsal forewing eyespots simultaneously (anterior, A, and posterior, P), the sum of the rank order of A/wing and P/wing was used (rA + rP). From an outbred laboratory stock[3], high and low lines were derived by selecting for larger and smaller eyespots, respectively. To establish these lines, 1,058 females were measured and allowed to mate with males at random. The 45 females with most extreme rA + rP values in each direction were used as parents for the next generation. In each of the following generations, 150–200 females per line were scored and the 30–40 most extreme selected.

Bicyclus anynana Dll

A *B. anynana Dll* homologue was obtained from an embryonic complementary DNA library using a probe from the *P. coenia Dll* gene (GenBank accession number AF404110). DNA sequence polymorphisms that distinguish high and low line *Dll* alleles were obtained by sequencing 1,250 bp of the 3′ untranslated region (UTR) of *B. anynana Dll* (GenBank AF404825; bases 2,079–3,328) in three low and two high butterflies. For sequencing we used the same *B. anynana*-specific primers that were used to amplify the 3′ UTR *Dll* fragment: 5′-ACTTATAAAACACACAAAATGGCGTA-3′ and 5′-TTAAAATTATGTATC ACAACAGACTGG-3′.

Genotyping assays

To type a C/T polymorphism (GenBank AF404825; base 2,194) located within the recognition sequence for the endonuclease *Bsp*HI, 20–100 ng genomic DNA were used in a 25-μl polymerase chain reaction (PCR) with 2.5 U Taq polymerase in its recommended buffer (Pharmacia), 0.5 mM of each primer (5′-ACTTATAAAACACACAAAATGGCGTA-3′ and 5′-ATGCTGTAATAATAATCGCATA-3′) and 0.1 mM of each dNTP. Cycling conditions were 1 min at 96 °C followed by 35 cycles of 45 s at 94 °C, 45 s at 57 °C and 1 min at 72 °C. Amplicons were ethanol-precipitated, resuspended in a 20-μl restriction digestion mix containing 5 U *Bsp*HI in its recommended buffer (New England Biolabs), incubated overnight at 37 °C, and scored on agarose gels. Individuals homozygous for the presence or absence of the cut site are scored +/+ and −/−, respectively; heterozygotes are scored +/−.

To type a 10-bp insertion-deletion polymorphism (GenBank AF404825; position 2,263), we designed two primers in the forward direction: 5′-GGTCACGGTATAAAA CGTGAACG-3′ (insertion-specific) and 5′-GCCACGGTATAAAACGTGAGT-3′ (deletion-specific). These were used in separate PCRs with the same reverse primer (5′-GGGTTGAACACAATTGCTGCT-3′). PCRs were carried out as above except for the annealing temperature (here 63 °C) and scored on agarose gels. Homozygotes for the insertion or the deletion (genotypes +/+ and −/−, respectively) have a band only for one of the primer combinations, whereas heterozygotes (+/−) have bands for both. Genotypes for both sites are given in the order of the polymorphisms along the cDNA sequence (*Bsp*HI, indel) with homologous chromosomes separated by a solidus (/).

These assays were used to genotype the butterflies from our crosses. The *Bsp*HI C/T polymorphism was fully informative in all backcross high (BCH) families. To genotype the low direction recombinants we used both assays. The *Bsp*HI polymorphism was fully informative in the backcross low families BCL1 and BCL5. For the BCL4 family, the two polymorphisms were used to provide an informative haplotype. For the families BCL2 and BCL3 only half of the progeny had informative genotypes for both polymorphisms; a total of 28 progeny of genotype −+/+− were not included in the segregation analysis.

Genotype–phenotype association

Given that the phenotypes of the progeny from the BCL families did not follow a normal distribution, we have used a nonparametric statistical test (one-way Mann-Whitney *U*-test) to compare eyespot/wing size between different genotypic classes. The reason for applying one-tailed tests is the *a priori* expectation that the high and low alleles should, respectively, increase and decrease eyespot size. A permutation approach[28] was used to test the significance of the observed Mann–Whitney *U*-test results. Within each sex and backcross combination genotypes were randomly permuted 1,000 times with respect to paired eyespot size measures and the Mann–Whitney *U*-test statistic estimated. Distributions for the nth largest test statistic were then calculated and compared to the corresponding observed nth largest test statistic in the non-permuted data set. This approach allows *P*-values to be assigned to the most significant (second most significant, and so on) eyespot/sex/backcross combination, without correcting for potential correlations over characters, sexes, or backcross direction.

QTL position

To identify the most likely location of the QTL contributing to eyespot variation relative to the marker in *Dll* the following log likelihood equation was maximized for values of r between 0.0 and 0.5:

$$\log\text{likelihood} = \sum_i \begin{bmatrix} \text{if } (y_i = 0)\{\log[\theta_1(1-r) + \theta_2 r] + \log[\theta_1 r + \theta_2(1-r)]\} \\ \text{else } \{\log[(1-\theta_1)(1-r)z(y,\mu,\sigma) + (1-\theta_2)rz(y,\mu+\beta,\sigma)] \\ \quad + \log[(1-\theta_1)rz(y_i,\mu,\sigma) + (1-\theta_2)(1-r)z(y_i,\mu+\beta,\sigma)]\} \end{bmatrix}$$

where y_i is the phenotype of the ith butterfly, θ_1 is the probability (eyespot is absent; genotype is LL), θ_2 is the probability (eyespot is absent; genotype is HL), β is the expected (eyespot size; eyespot is present and genotype is HL) minus the expected (eyespot size; eyespot is present and genotype is LL), $z(y_i, a, \sigma)$ is the normal probability distribution function with mean a and standard deviation σ evaluated at y_i, and r is the recombination fraction between the *Dll* marker and the putative QTL. Base ten LOD[23] scores were calculated for different values of r by maximizing the above function for θ_1, θ_2, μ, β, σ minus the same function maximized for the same parameters at $r = 0.5$ (unlinked QTL).

Dll expression patterns

Wing discs from late final instar larvae and 20–24 h pupa were stained with cross-reactive Dll antibody (1:100) as in ref. 8. As a secondary antibody we used donkey anti-rabbit Texas red (1:200; Jackson Laboratories). Images were collected on an MRC 1024 ES laser confocal microscope using a ×10 objective for the entire larval wings and ×20 for the eyespot centres.

Received 16 August; accepted 8 November 2001.

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Acknowledgements

We thank K. Koops for rearing the butterflies, and B. Zwaan for help with the crosses; C. Brunetti, J. Selegue and S. Carroll for the *B. anynana* cDNA library, the *P. coenia Dll* probe and the Dll antibody; N. Glansdorp for help with wing extractions, and G. Lammers

for help with the confocal microscopy; K. Broman for deriving the likelihood function; F. Marec for information on butterfly chromosomes; M. Brittijn for help with the figures; and A. Monteiro, H. Teotónio and B. Zwaan for comments on the manuscript. This work was supported by the Portuguese Foundation for Science and Technology and the Luso-American Foundation under the Gulbenkian Program (P.B.), the Human Frontier Science Program Organization (P.M.B.) and the US National Institute of Health (A.D.L.).

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Visual categorization shapes feature selectivity in the primate temporal cortex

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The way that we perceive and interact with objects depends on our previous experience with them. For example, a bird expert is more likely to recognize a bird as a sparrow, a sandpiper or a cockatiel than a non-expert[1]. Neurons in the inferior temporal cortex have been shown to be important in the representation of visual objects; however, it is unknown which object features are represented and how these representations are affected by categorization training. Here we show that feature selectivity in the macaque inferior temporal cortex is shaped by categorization of objects on the basis of their visual features. Specifically, we recorded from single neurons while monkeys performed a categorization task with two sets of parametric stimuli. Each stimulus set consisted of four varying features, but only two of the four were important for the categorization task (diagnostic features). We found enhanced neuronal representation of the diagnostic features relative to the non-diagnostic ones. These findings demonstrate that stimulus features important for categorization are instantiated in the activity of single units (neurons) in the primate inferior temporal cortex.

Perception is arguably the end product of a categorization process[2]. A number of studies have tried to elucidate the ways that features are extracted and represented by non-human primates in the context of a categorization task[3–10]. These studies have shown that rhesus monkeys are capable of representing natural objects at basic (for example trees versus non-trees)[5,6,8], superordinate (animals or food)[2,3,7] and subordinate (individual wire objects, faces or fish)[9,10] levels. However, it still remains to be shown which object features are represented and how these representations are affected by the categorization training. The inferior temporal cortex area has a critical role in visual object recognition and responds to complex stimuli[9,11] as well as to greatly simplified versions of these stimuli[12]. Moreover, activity in the human temporal cortex is thought to be sensitive to the categorization level of the stimuli and to depend on the expertise of the observer[13,14] . The aim of our study was to test whether inferior temporal cortex neurons respond selectively to object features that constitute the relevant dimensions for visual object categorization.

We trained two monkeys in a categorization task and used parameterized line drawings of faces and fish as stimuli (Fig. 1). Each schematic face consisted of an outline and four varying features: eye height (EH), eye separation (ES), nose length (NL) and mouth height (MH)[15]. The schematic fish also had four features: the shape of the dorsal fin (DF), tail (T), ventral fins (VF) and mouth (M). The four-dimensional vectors of parameters

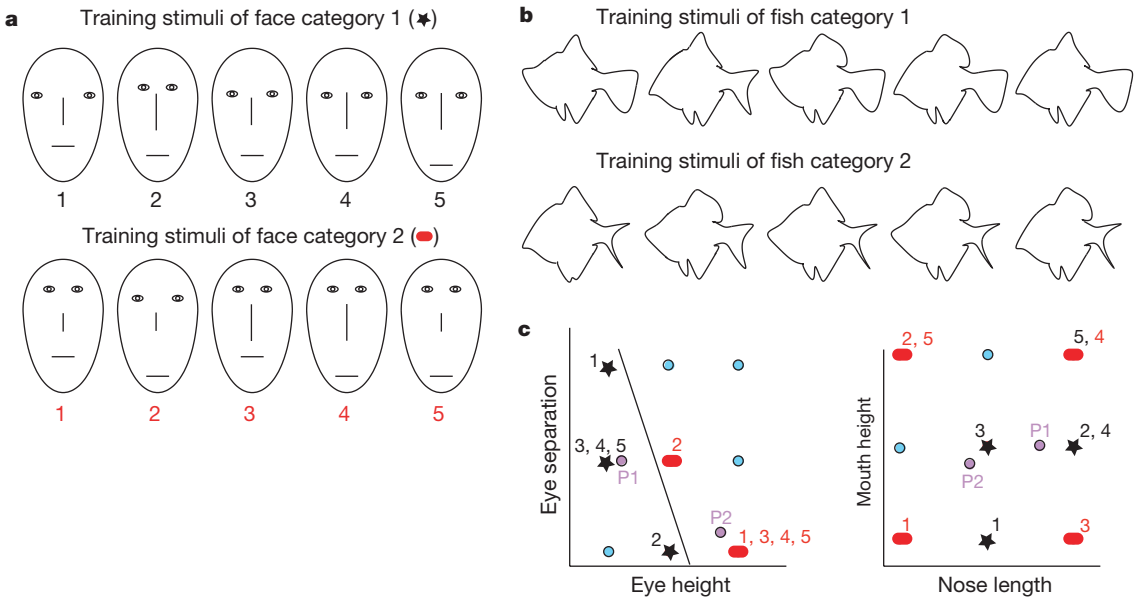

Figure 1 Stimuli and categories. **a**, The first stimulus set consisted of line drawings of faces with four varying features: eye height, eye separation, nose length and mouth height. **b**, The second stimulus set consisted of fish outlines with four varying features: the shape of the dorsal fin, tail, ventral fins and mouth. In both stimulus sets, each feature could take one of three discrete values. The categories were separable along two of the four stimulus features, but information about only one of the diagnostic features was insufficient for optimal performance. The monkeys were presented with one stimulus at a time. **c**, Two-dimensional representation of the stimulus space. Black stars represent the stimuli of the first category and red ovals represent the stimuli of the second category. Each number indicates the position of one corresponding stimulus from **a**. As the stimuli differ along four dimensions, the two-dimensional representations in this figure result in overlap of stimuli that are distinct. The purple circles (P1 and P2) represent the prototypes. Cyan circles represent test exemplars that did not belong to a fixed category. The two categories were linearly separable along the eye height, eye separation (EH, ES) dimensions (solid line) but not along the nose length, mouth height (NL, MH) dimensions.