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The evolution of alternative parasitic life histories in large blue butterflies

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Large blue (*Maculinea*) butterflies are highly endangered throughout the Palaearctic region, and have been the focus of intense conservation research[1–3]. In addition, their extraordinary parasitic lifestyles make them ideal for studies of life history evolution. Early instars consume flower buds of specific host plants, but later instars live in ant nests where they either devour the brood (predators), or are fed mouth-to-mouth by the adult ants (cuckoos). Here we present the phylogeny for the group, which shows that it is a monophyletic clade nested within *Phengaris*, a rare Oriental genus whose species have similar life histories[4,5]. Cuckoo species are likely to have evolved from predatory ancestors. As early as five million years ago, two *Maculinea* clades diverged, leading to the different parasitic strategies seen in the genus today. Contrary to current belief, the two recognized cuckoo species show little genetic divergence and are probably a single ecologically differentiated species[6–10]. On the other hand, some of the predatory morphospecies exhibit considerable genetic divergence and may contain cryptic species. These findings have important implications for conservation and reintroduction efforts.

Maculinea species have become the flagship butterflies for conservation in the UK and Europe[2,11]. The severe decline of *Maculinea* populations during the twentieth century has been well documented, and all species have been included in the red data lists of most European countries[3]. Many management and reintroduction projects have been attempted, with variable results[1]. The extinctions of the large blue (*Maculinea arion*) in the UK, the Netherlands and Belgium, the scarce large blue (*Maculinea teleius*) in the last two countries and the dusky large blue (*Maculinea nausithous*) in the Netherlands[2,12], have spurred increased conservation efforts, with large blue butterfly populations sometimes being used as bioindicators of habitat quality[13].

Maculinea species are also the best-known examples of parasitic butterflies. Initially they feed on the flowers of specific Lamiaceae, Gentianaceae or Rosaceae host plants. When they reach the fourth instar, they drop to the ground and are picked up by *Myrmica*[14] (or in a few cases *Aphaenogaster*[15]) ants and carried into the nest where they feed as parasites. Most currently recognized species, including the widely distributed species *M. arion*, *M. teleius* and *M. nausithous* and the east Asian *Maculinea arionides* prey on ant brood[15–18]. In contrast, *Maculinea alcon* and *Maculinea rebeli* are 'cuckoos', whose larvae are fed primarily on regurgitations from ant workers, trophic eggs and prey items[19,20]. These species have more elaborate adaptations of behavioural and chemical mimicry, and have thus been proposed to be derived relative to species that are strictly predatory[20]. Several additional taxa occurring in the eastern Palaearctic have been proposed as species, including *Maculinea kurentzovi* and *Maculinea cyanecula*, but their status is still unclear and their life histories have not been described (see Supplementary Information)[18].

More than 99% of the estimated 18,000 species of butterflies are herbivorous, but aphytophagy (carnivory and parasitism) has been fully documented in only about 80 species[21]. These are found primarily in the family Lycaenidae, to which *Maculinea* belongs, and are likely to be the result of the close relationship that the caterpillars of this family have with ants. Up to 75% of the approximately 5,000 species of Lycaenidae (*sensu stricto*) associate to some degree with ants, and whereas most of these relationships appear to be mutualistic, as many as 200 (4%) are known or suspected to be parasitic on ants[22].

Maculinea belongs to the *Glaucopsyche* section of the Polyommatini, and *Sinia*, *Iolana*, *Caerulea* and *Phengaris* have been considered its closest relatives[23]. In particular, the Oriental genus *Phengaris* has been proposed as the most likely sister group of *Maculinea* due to its similar morphology and the occurrence in the genus of both predatory and cuckoo parasitism on *Myrmica* ants[4,5,24,25]. The present study reconstructs the evolution of ant parasitism, host plant association and speciation in *Maculinea*, and investigates whether the presently recognized species are likely to represent evolutionarily significant units for conservation.

Our molecular phylogeny includes 32 *Maculinea* specimens representing 31 geographically distinct populations of seven species

covering the entire Palaearctic from Denmark and Spain in the west to Japan and southeastern Russia in the east. We obtained 15 outgroup taxa, representing nine genera within the *Glaucopsyche* section[23]. Maximum parsimony, maximum likelihood and bayesian methods (for each gene region and for all genes combined) were used to analyse information from 3,109 characters from mitochondrial *Cytochrome Oxidase I* and *II* (*COI* and *COII*) and nuclear *Elongation Factor1-alpha* (*EF1-α*) genes. Phylogenetic events were dated by applying published estimates of substitution rates for *COI* and *COII* to a phylogram recovered by maximum likelihood (see Methods).

Maculinea is recovered as a monophyletic group, with members of *Phengaris* as its closest relatives (Fig. 1). Although the criteria used to define a genus are largely subjective, the apparent paraphyly of *Phengaris* and the proximity of all three *Phengaris* specimens to *Maculinea* raises the possibility that the two genera should be synonymized under *Phengaris* Doherty, 1891, with the junior name *Maculinea* van Eecke, 1915 rendered invalid. The relationships among the remaining outgroup taxa are not well supported. Approximately five million years ago, *Maculinea* taxa separated into two main clades that correspond with the cuckoo and the predatory lifestyles (Fig. 2). The predatory clade is further divided into two
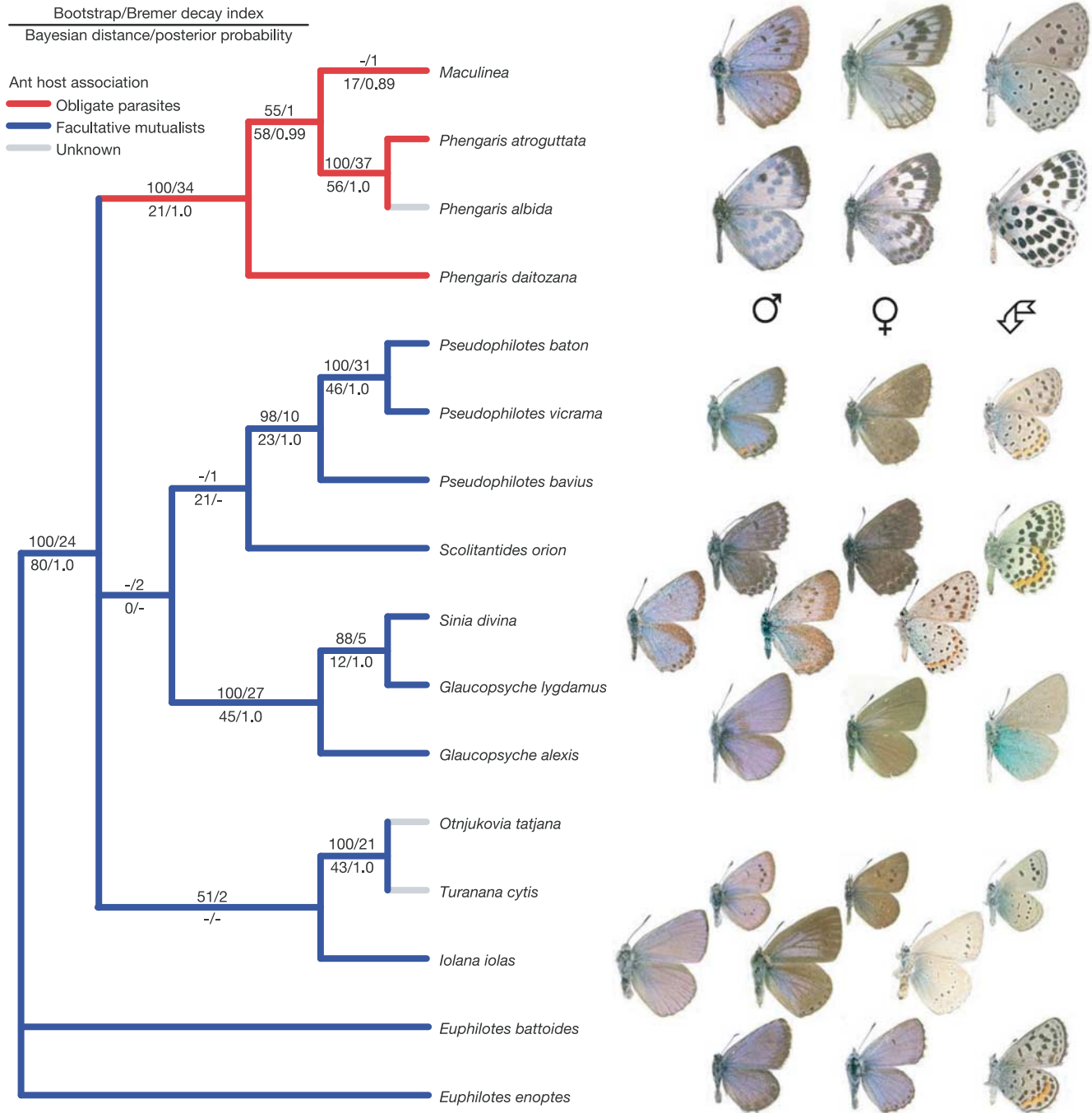

Figure 1 Phylogeny of the obligately parasitic genera *Phengaris* and *Maculinea* in relation to outgroup taxa that have facultative, mutualistic relationships with ants. The strict consensus maximum parsimony (MP) tree of 47 *Maculinea* and outgroup taxa was inferred from 3,109 base pairs (bp) of the genes *COI*, *COII* and *EF1-α*. The *Maculinea* part of the tree (32 specimens) is collapsed, and is shown in detail in Fig. 2. Photographs show representative members of each genus except *Otnjukovia*. The strict consensus tree was constructed from 1,277 MP trees (tree length (TL) = 1,284; consistency index (CI) = 0.628; and retention index (RI) = 0.804). Bayesian inference recovered a similar topology.

groups: *M. arion*, *M. arionides* and *M. cyanecula* forming one, and *M. teleius* and *M. nausithous* the other.

Representatives of predatory species, especially *M. nausithous*, from different populations show considerable genetic divergence and may represent cryptic species (Supplementary Table 7). The cuckoo clade of *M. alcon* + *M. rebeli*, on the other hand, shows a distinct lack of structure, with a long, strongly supported basal branch and extremely limited differentiation among terminal branches (Fig. 2 and Supplementary Fig. 6). The minor extent of these differences, especially relative to the differences between populations of the predatory clades, suggests that *M. rebeli*'s status as a separate species is questionable. All extant local differentiation of *M. alcon* and *M. rebeli* between host plants and host ants[6–8] may in fact derive from a single recent ancestor that arose considerably

less than one million years ago. However, the local ecological adaptations of some of the representatives of the *M. alcon* complex[6,8,19] support the argument that they are evolutionarily significant units and merit specific conservation measures.

Ancestral state reconstruction using maximum parsimony indicates that the ancestors of both the *Maculinea* clade and the *Phengaris* + *Maculinea* clade were butterflies from the eastern Palaearctic or Oriental region whose caterpillars mined the inflorescences of Gentianaceae or Lamiaceae during their initial instars and later parasitized *Myrmica* ants either as predators, or as mixed predators/cuckoos. Parsimony reconstruction does not distinguish between these alternative ancestral states, although both require fewer steps than a putative cuckoo ancestor without a predatory capacity. However, a maximum likelihood reconstruction taking
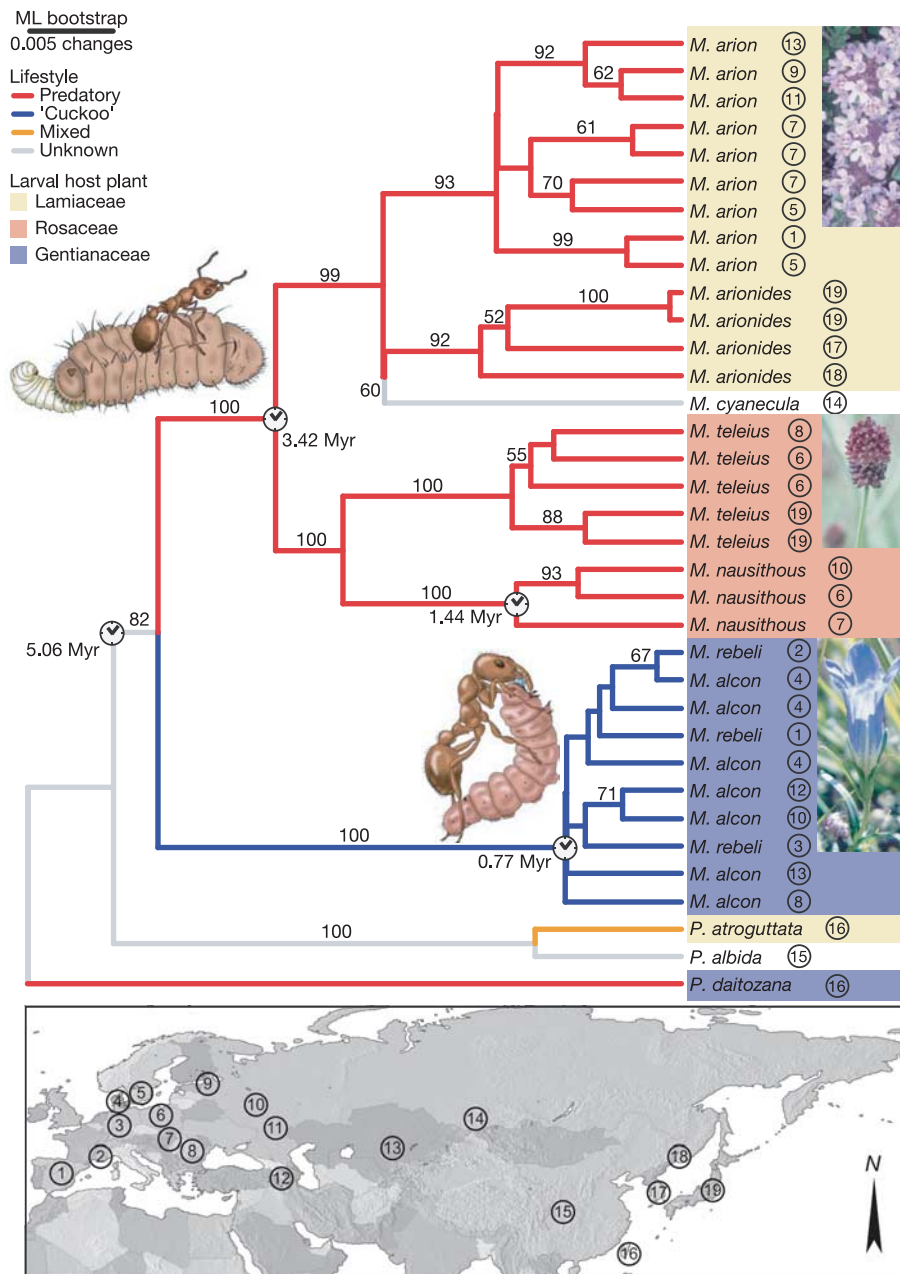

Figure 2 Phylogeny of *Maculinea* taxa with *Phengaris* as outgroup. The predatory, cuckoo and mixed strategies of exploiting host ants are indicated by branch colour, and initial larval host plant by background colour. Numbers in circles refer to map locations where specimens with corresponding numbers were collected. Estimated ages of key nodes are indicated (Myr, million years ago), based on a convergent rate of nucleotide substitution for *COI* in insects[30]. This smoothed maximum likelihood phylogram of 35 taxa was inferred from 3,109 bp of the genes *COI*, *COII* and *EF1-α* under the GTR + I + Γ nucleotide substitution model (−lnL = 4,655.43), with bootstrap values based on 500 pseudoreplicates.

into account branch lengths favours by 2 to 1 the predatory ancestral condition. This is consistent with most biological arguments for the evolution of feeding strategies in this group[19,20] (Supplementary Figs 8 and 9, Supplementary Table 8).

The life history of the most basal ant-parasitic species, *Phengaris daitozana*[4,5,24], might represent that of a hypothetical predatory ancestor. Closely related *Glaucopsyche* larvae sometimes over-winter as pupae in ant nests, presumably because of the stability and security afforded by the nest[26,27]. The life history shown by *P. daitozana* may have evolved simply through a shift from pupal to larval diapause[4,5,24], necessitating additional feeding in the spring before pupation. *P. daitozana* completes its nutrition at this point by feeding on ant brood. Larvae of the more derived *Phengaris atroguttata*, however, after a similar initial bud-mining phase on the flowers of Lamiaceae, are actively carried into the nest by *Myrmica* workers, where they feed both as cuckoos and as predators of the ant brood[4,5,24]. Because our study includes all species of *Maculinea* and *Phengaris* with known life histories, and our conclusions are not affected by the paraphyly or monophyly of *Phengaris*, new biological observations will be necessary to reach a better understanding of the evolution of parasitic strategies in this group.

Phylogenetic conservatism in host ant use is exhibited at the subfamily level: *Maculinea* + *Phengaris* form a clade whose species parasitize the ant subfamily Myrmicinae, almost exclusively the genus *Myrmica*. Similarly, all of the described species in the related polyommatine genus *Lepidochrysops*, whose independently evolved phyto-parasitic lifestyle appears to have been associated with significant diversification (about 120 species), parasitize ants in the subfamily Formicinae, primarily the genus *Camponotus*[22,26]. Other groups of parasitic Lycaenidae show similar degrees of specialization, with four species of Liphyrini all attacking weaver ants, *Oecophylla* (Formicinae), and an estimated 27 species of *Thestor* and five species of *Trimenia* associating with pugnacious ants, *Anoplolepis* (Formicinae)[22]. A recent phylogeny of the Australian butterfly genus *Acrodipsas* shows a shift in which the most basal species feed on ants in the subfamily Dolichoderinae, whereas all known derived species parasitize ants in the Myrmicinae[28].

Detailed population-level information about host ant use is only available for *Maculinea* species in Europe[7,10,14,20]. Here, the host ant species used by *Maculinea* have been shown to be of prime importance for conservation[1,2,11] and reintroduction programmes[1,2]. Between them, the five European *Maculinea* species use host ants from all of the species-groups of free-living *Myrmica* found on the continent[29]. However, any single population of *Maculinea* normally depends on only one or two host ant species[6–8,14,20], and can show considerable local adaptation to its hosts[6–8]. Although they comprise the most genetically homogeneous clade in the phylogeny, *M. alcon* + *M. rebeli* use a total of seven *Myrmica* species as major hosts within Europe (Supplementary Table 10). The relatively broad range in host ant species together with the observation that individual populations are typically highly specific with respect to ant association suggest that cuckoo taxa may be undergoing rapid ecological divergence[6]. In contrast, the most genetically divergent species, the predatory *M. nausithous*, uses only two host ant species across its entire recorded range (Supplementary Table 10).

The present results overturn widely held inferences about *Maculinea* evolution and conservation. Future conservation and reintroduction programmes of predatory *Maculinea* species will need to take the possibility of cryptic species into account, and would thus benefit from genetic screening of alternative source populations. In contrast, the recognition and conservation of evolutionarily significant units for cuckoo species will need to be based on persistent ecological and behavioural adaptations of local populations. Finally, the now extremely sparse and threatened populations of Oriental *Phengaris* species should receive high conservation priority to enable further study of the evolutionary origins of the unusual parasitic life histories in this clade. □

Methods

Methods are described in a more detailed manner, and are fully referenced, in the Supplementary Information.

Specimen sequences

Total genomic DNA was extracted from 47 specimens of *Maculinea* and outgroup taxa. Two mitochondrial genes, *COI* and *COII*, and a nuclear gene, *EF1-α* were amplified by polymerase chain reaction (PCR). The direct sequencing of double-stranded PCR products yielded fragments of equal lengths, and alignments were unambiguous for both mitochondrial and nuclear gene fragments.

Phylogenetic analysis

Phylogenetic analyses were conducted using maximum parsimony (MP; PAUP* 4.0b10), maximum likelihood (ML; PHYML) and bayesian inference (BI; MrBayes 2.01). Monophyly of *Maculinea* and *Phengaris* + *Maculinea* was inferred using the total data set of 47 specimens, whereas relationships within *Maculinea* were inferred using a subset consisting of 32 specimens of *Maculinea* and three of *Phengaris* as outgroups to minimize potential for long-branch attraction. Both separate and combined phylogenetic analyses of mitochondrial and nuclear genes were performed. Hierarchical likelihood ratio tests (hLRTs) were used to determine the best-fitted substitution model for each data set in ML and BI analyses. To ensure that BI was not trapped in local optima, each BI analysis was run three times, and average log-likelihood (lnL) values at stationarity were calculated and compared for convergence. Nonparametric bootstrap values were used to estimate the support of tree branches recovered by MP and ML.

Dating main phylogenetic events

An ML phylogram recovered from the *COI* + *COII* data set of 32 *Maculinea* and three *Phengaris* specimens in PHYML was used to date main phylogenetic events. The likelihood ratio test (LRT) found a significant deviation from substitution rate consistency ($P < 0.0001$) across different branches on the ML topology. A nonparametric rate-smoothing (NPRS) algorithm was therefore used to homogenize evolutionary rates across the topology. The topology was then calibrated by applying a published estimate of substitution rate[30] in *COI* to the mean uncorrected pairwise distance for the single calibration node.

Ancestral character state reconstruction

Ancestral character state analyses were performed using Mesquite v. 1.01. Both MP and ML character optimizations were applied to the ML phylogram for 47 taxa inferred from the combined analysis of *COI* + *COII* and *EF1-α* genes under the GTR + I + Γ model of DNA substitution (Supplementary Fig. 6) trimmed to include only one sample for each of the *Phengaris* and *Maculinea* species. For MP analyses, host plant families were coded as a multistate unordered character. Two hypotheses for the parasitic strategy were considered: coding the *Phengaris* and *Maculinea* cuckoo strategies as equivalent, or as one-step different. Two coding options were used to cover all possibilities and explore the implications of both models. ML optimizations were done using the Markov k-state one-parameter model.

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An obligate brood parasite trapped in the intraspecific arms race of its hosts

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Reciprocal selection pressures often lead to close and adaptive matching of traits in coevolved species. A failure of one species to match the evolutionary trajectories of another is often attributed to evolutionary lags[1,2] or to differing selection pressures across a geographic mosaic[3,4]. Here we show that mismatches in adaptation of interacting species—an obligate brood parasitic duck and each of its two main hosts—are best explained by the evolutionary dynamics within the host species. Rejection of the brood parasite's eggs was common by both hosts, despite a lack of detectable cost of parasitism to the hosts. Egg rejection markedly reduced parasite fitness, but egg mimicry experiments revealed no phenotypic natural selection for more mimetic parasitic eggs. These paradoxical results were resolved by the discovery of intraspecific brood parasitism and conspecific egg rejection within the hosts themselves. The apparent arms race between species seems instead to be an incidental by-product of within-species conflict, with little recourse for evolutionary response by the parasite.

Avian obligate brood parasites depend entirely on other species to raise their offspring, often inflicting severe fitness costs on hosts. Brood parasitism provides a model system for investigating the dynamics of antagonistic coevolution, because of the reciprocally hostile relationship between parasite and host[1,2,5–8]. In some parasitic taxa, extreme fitness costs of parasitism to hosts have favoured the evolution of egg discrimination and rejection by hosts, which in turn has led to the evolution of egg mimicry and host specialization in the parasite[5–8]. The black-headed duck (*Heteronetta atricapilla*) of southern South America is unique in comparison with all other species of obligate brood parasites in that its highly precocial chicks leave the host nest within a day of hatching (Fig. 1g) and require no post-hatching parental care[9,10]. This parasite should impose few fitness costs on its hosts and, accordingly, the ecological and evolutionary dynamics of host–parasite interactions should differ markedly from those of all other brood parasites.

We conducted a large-scale observational and experimental study of host–parasite interactions in black-headed ducks during four breeding seasons on seven wetlands in the pampas of Argentina. Brood parasitism was common (29.3% of 1,927 potential host nests of 11 species parasitized). Several attributes of the brood parasitism were counter to those expected for a precocial brood parasite. First, the parasites used very few host species (Fig. 1a), and parasitized these hosts at a high frequency (Fig. 1b). Despite the diversity of species used at least occasionally as hosts in our study (11 species), 80% of the 974 duck eggs we found occurred in nests of just two species of coots (Fig. 1a, e, f), with almost half occurring in a single host, the red-gartered coot (*Fulica armillata*). Because hatching success of the duck eggs is highest with this host (Fig. 1c), an estimated 58% of all ducklings hatch from nests of this one species and 83% from both coot species combined (see Methods). Dependence on such a narrow range of hosts was unexpected because the ability to use a wide diversity of hosts has been proposed as a key factor in the evolution of obligate brood parasitism in *Heteronetta*[2,10]. Second, the parasitic eggs had low hatching success in both main hosts (Fig. 1c), despite similar incubation periods of host and parasite. Third, both main hosts showed high levels of egg rejection (Fig. 1d). A strong negative correlation between the frequency of egg rejection and the hatching success of duck eggs for each host on each wetland (Spearman rank correlation $r_s = -0.99$, $n = 8$, $P < 0.01$) indicates that egg rejection markedly decreases the reproductive success of black-headed ducks and is a main source of egg mortality.

Egg rejection has arisen independently in a wide variety of birds to counter the costs of interspecific brood parasitism[1,2,5–8]; its occurrence here therefore implies some cost of parasitism to hosts. Such costs would have to be borne during incubation because the ducklings leave the nest within a day of hatching. Using both naturally and experimentally parasitized nests, we assessed costs known to be suffered by hosts of brood parasitism, including smaller host clutch size, longer incubation period, increased egg loss[11] and increased nest predation risk from the non-cryptic duck eggs[12] (Fig. 1h). We detected no costs of parasitism for red-gartered coots, whereas parasitized red-fronted coot (*F. rufifrons*) nests suffered higher egg loss rates than unparasitized nests (Table 1). Whereas many costs of parasitism are reduced by, and thus select for, egg rejection, 'unrecoverable' costs—such as incidental egg