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19. 18HA0 at a concentration of 10 to 15 mg/ml was used to grow crystals in sitting drops with a precipitant solution of 1.68 M sodium dihydrogen phosphate, 0.32 M dipotassium hydrogen phosphate, and 0.1 M phosphate-citrate, with pH 5.5 (18).

20. Swine H9 HA [Protein Data Bank (PDB) identification (ID) code: 1jsh] was used as the initial MR model. The final R factor R_{cryst} and free R factor R_{free} values (table S1 legend) are 27.0 and 29.6%, respectively, with only three residues (0.7%) per monomer (Ile^{B10}, Met^{B17}, and Asn^{B60}) in the disallowed regions of the Ramachandran plot. Only Met^{B17} has good density and is positioned at the tip of a loop, proximal to the membrane-fusion loop. The crystal asymmetric unit contains one homotrimer (502 HA0-encoded residues per monomer) with an estimated solvent content of 66%, based on a Matthews' coefficient (V_m), of 3.57 Å³/dalton. For comparison with previous structures, the HA0 sequence is numbered in the same way as the H3 subtype (14) and labeled HA1 11 to 329 and HA2 1 to 175, even though the covalent bond still links HA1 to HA2. Thus, the H1 1918 HA0 structure begins at residue 11 because of an insertion of 10 residues in H3. Insertions in H1 relative to H3 are labeled by the preceding residue with a letter (e.g., Asn^{19A}). The three HA0 chains in the trimer are labeled A, C, and E (HA1) and B, D, and F (HA2). The electron density maps revealed only two disordered regions around residues 78 to 81 of chains HA1 and 10 to 14 of HA2 that, although in disparate regions of the monomer, are in close proximity to other symmetry-related molecules in the crystal. Although B values were high in this region, main-chain atoms could be interpreted from the electron density in both regions, except at Gly¹² and Gly¹³ in the HA2 chain. Occupancy and B values were set to zero for side-chain atoms that were uninterpretable from the electron density.

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22. Angles of rotation reported here for the H5 and H9 subtypes are less than those in (27). Our method of analysis, as described in the legend for Fig. 1, was different but reports a similar trend and reveals a rotation for the 18HA0 as seen for the avian subtype.

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26. The pH ranges from almost neutral on exiting the endoplasmic reticulum to pH 5.9 within the trans-Golgi network and as low as pH 5.4 in the secretory vesicles (27, 28).

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40. The widths of the binding pockets were calculated by

measuring the distance from Gly^{A134} Cα to Gln^{A222} Cα for 18HA0 (13.9 Å), Gly^{A134} Cα to Gln^{A226} Cα for human H3 (15.2 Å), Gly^{A130} Cα to Gln^{A222} Cα for avian H5 (14.0 Å), and Gly^{A128} Cα to Gln^{A216} Cα for swine H9 (15.2 Å).

41. Only one other sequence in the current influenza database of human H1, H2, H3, H5, and H9 subtypes possesses the same patch (H1; A/Alma-Ata/1417/84 virus) (42).

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50. Residues 311 to 324 and 350 to 357 from the human H3 subtype (PDB ID code: 1ha0) and A308 to A321 and B21 to B28 from the avian H5 subtype (PDB ID number: 1jsh) were aligned with residues A311 to A324 and B21 to B28 of 18HA0.

51. Residues A95 to A99, A128 to A161, A179 to A200, and A220 to A230 from 18HA0; A95 to A99, A128 to A161, A179 to A200, and A220 to A230 from the human H3 subtype (PDB ID code: 2hmg); A87 to A92, A124 to A156, A175 to A196, and A216 to A226 from the avian H5 subtype (PDB ID code: 1jsh); A87 to A92, A122 to A150, A169 to A190, and A210 to A220 from the swine H9 subtype (PDB ID code: 1jsh)

were aligned and surfaces were generated with MSMS (46) through the program VMD (47).

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Conserved Genetic Basis of a Quantitative Plumage Trait Involved in Mate Choice

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A key question in evolutionary genetics is whether shared genetic mechanisms underlie the independent evolution of similar phenotypes across phylogenetically divergent lineages. Here we show that in two classic examples of melanistic plumage polymorphisms in birds, lesser snow geese (*Anser c. caerulescens*) and arctic skuas (*Stercorarius parasiticus*), melanism is perfectly associated with variation in the *melanocortin-1 receptor (MC1R)* gene. In both species, the degree of melanism correlates with the number of copies of variant *MC1R* alleles. Phylogenetic reconstructions of variant *MC1R* alleles in geese and skuas show that melanism is a derived trait that evolved in the Pleistocene.

The genetic basis of independent origins of the same phenotype is important to models of phenotypic evolution. There are few data, especially for vertebrates, because the loci underlying phenotypic evolution in natural populations are rarely known. The lesser

snow goose (*Anser c. caerulescens*) and arctic skua (or parasitic jaeger, *Stercorarius parasiticus*) have prominent melanistic plumage polymorphisms (Fig. 1) showing clinal variation in the frequency of melanistic morph phenotypes across their arctic breeding ranges (1, 2). In both species, there is quantitative variation in the degree of melanism among adult individuals with the melanistic phenotype ("blue" snow geese and "intermediate" and "dark" skuas) and discrete separation between these and the nonmelanistic phenotypes ("white" geese and "pale" skuas).

These polymorphisms influence mate choice. In snow geese, mate color preference follows parental color, leading to assortative

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mating. It is believed to result from juveniles learning their parents' color at an early age and using this information in mate choice decisions (3). In arctic skuas, both female preference for dark males and positive assortative mating have been documented (4, 5). Pale arctic skuas of both sexes begin breeding at a younger age than dark birds, whereas dark male skuas, including first-time breeders and experienced breeders mating with a new female, breed earlier than pale birds in their first year of breeding but not subsequently (2, 4). Life history differences among white and blue snow geese have yet to be demonstrated (6). We studied a candidate

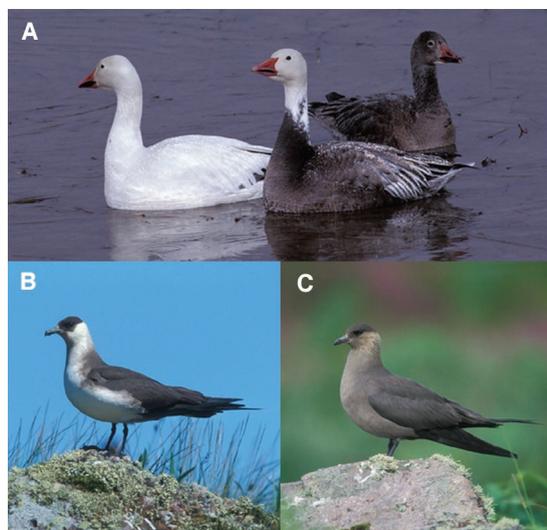
gene underlying these plumage differences in geese and skuas, the *melanocortin-1 receptor* (*MC1R*) gene, which is expressed in melanocytes in developing feather buds and is a key regulator of melanogenesis in vertebrates, including birds (7–11).

The frequencies of color morphs of lesser snow geese show an approximate east-west cline across their arctic breeding range from eastern Canada to eastern Siberia, with blue individuals common in the east and comparatively rare in the west. We sampled white and blue geese from three polymorphic eastern populations (Akimiski Island, Baffin Is-

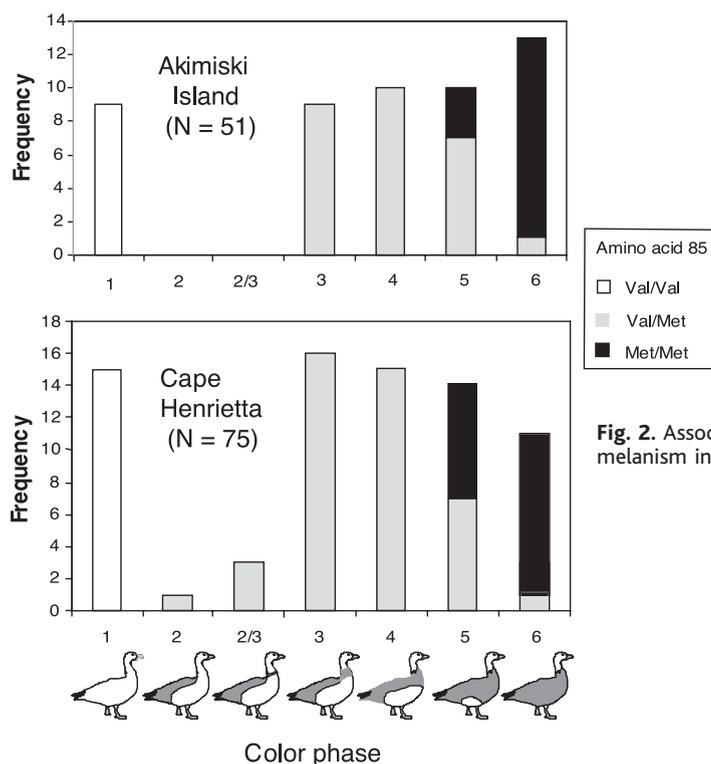
land, and Cape Henrietta) and white geese from two western populations (Queen Maude Gulf and Wrangel Island). A nonsynonymous point substitution (Val⁸⁵→Met⁸⁵) in the *MC1R* gene was perfectly associated with the blue phenotype throughout the entire range of lesser snow geese across North America (12). All blue geese sampled ($N = 91$) were heterozygous or homozygous for the Met⁸⁵ allele, whereas all white geese ($N = 116$), were homozygous for the Val⁸⁵ allele (Fisher's exact test, $P < 0.001$). Furthermore, there was a strong correlation between the number of Met⁸⁵ alleles and the degree of melanism (Fig. 2) in the two polymorphic populations examined. The degree of melanism in blue lesser snow geese was scored from phase 1 (palest) to phase 6 (darkest) (13). All phase 2, 3, and 4 individuals were heterozygotes, whereas phase 5 and 6 birds comprised increasing proportions of Met⁸⁵ homozygotes.

Blue and white populations probably only came into contact about a century ago (14), but population histories cannot account for the association of *MC1R* with phenotype, because if white and blue birds are considered as separate populations (that is, phase 1 birds versus phase 2 to 6 birds), differentiation at the *MC1R* locus among morphs at Baffin Island [fixation index (F_{st}) = 0.78, $P < 0.01$] and Akimiski Island (F_{st} = 0.56, $P < 0.01$) is far greater than that at other nuclear loci (microsatellite loci: Baffin Island, average over 10 loci, F_{st} = 0.02, $P <$

Fig. 1. Color morph phenotypes in lesser snow geese and arctic skuas. (A) Left, white (phase 1) lesser snow goose; right, blue (phase 6) lesser snow goose; behind is an immature bird. (B) Pale arctic skua. (C) Intermediate arctic skua. [(A), copyright A. Morris/VIREO; (B) and (C), copyright M. Lane/Alamy]



A Lesser snow goose



B Arctic skua

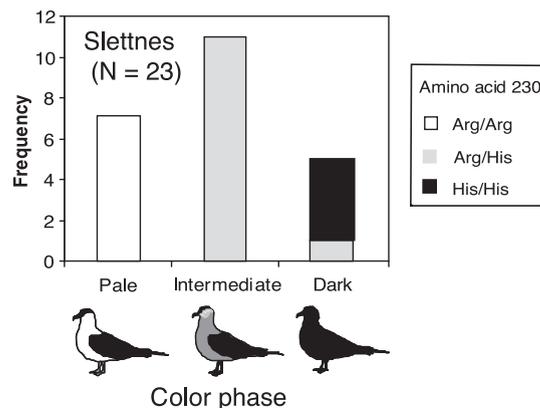


Fig. 2. Association between copy number of variant *MC1R* alleles and degree of melanism in lesser snow geese (A) and arctic skuas (B).

0.05; Akimiski Island, average over 9 loci, $F_{st} = 0.01$, NS) or in mitochondrial DNA (mtDNA) [Akimiski Island, fixation index (Φ_{st}) = 0.00 (15)].

These data show that the *MC1R* locus is the major determinant of melanism in lesser snow geese, and the Met⁸⁵ allele is equivalent to the melanic allele hypothesized from modeling of transmission genetics in wild populations (13, 16). The Val⁸⁵→Met⁸⁵ substitution may be directly responsible, because it is in the outer part of the second transmembrane domain of *MC1R*, which is known to control *MC1R* activity and amount of melanin (7, 8, 17), but the involvement of closely linked sites outside the region sequenced cannot be ruled out. There is no evidence that variation at other segregating *MC1R* sites sequenced contributes to the plumage phenotype. Variation among individuals of phases 2 to 6 that are Val/Met⁸⁵ heterozygotes is not associated with variation at any of the eight other variable *MC1R* sites (18).

In the greater snow goose (*Anser c. atlanticus*) and Ross' goose (*Anser rossii*), which are close relatives of the lesser snow goose, blue morph individuals occur only occasionally. White morphs of these taxa were homozygous for the Val⁸⁵ allele, whereas the single blue Ross' goose examined was a Val⁸⁵/Met⁸⁵ het-

erozygote. Most *MC1R* haplotypes in Ross' goose were shared with snow geese (Fig. 3). Reconstruction of the evolutionary history of the Met⁸⁵ allele in a haplotype network (Fig. 3) shows that it is derived from the Val⁸⁵ allele and that it occurs in a single haplotype group (haplotype group 13). The derived position and low number of Met⁸⁵ haplotypes (2) as compared to Val⁸⁵ haplotypes (26) suggest a relatively recent origin of Met⁸⁵ haplotypes, and hence melanism. Coalescent simulations (19) estimate the age of the Val⁸⁵→Met⁸⁵ mutation to be 380,000 ± 188,000 (SD) years. These results are consistent with suggestions that melanism arose and became fixed in an isolated eastern population of lesser snow geese in the Pleistocene (14). Another proposed Pleistocene vicariance event that occurred in the common ancestor of snow and Ross' geese, based on the presence of two divergent mtDNA clades in both taxa (20, 21), must have pre-dated the Val⁸⁵→Met⁸⁵ mutation, because the two mtDNA clades are present in similar frequency in both blue and white geese.

Color morph frequencies in the arctic skua show a latitudinal cline, with the proportion of pale birds increasing northward in the circumpolar breeding range (2). A separate nonsynonymous point substitution in the *MC1R* gene (Arg²³⁰→His²³⁰) is as-

sociated with melanic plumage in adult arctic skuas. All melanic birds sampled ($N = 16$) in the polymorphic population at Slettnes, Norway, were heterozygous or homozygous for the His²³⁰ allele. Pale birds ($N = 12$) from the same polymorphic population in Slettnes and a monomorphic population at Komi, northeast Russia, were homozygous for the Arg²³⁰ allele (Fisher's exact test, $P < 0.01$). The degree of melanism correlates with copy number of the Arg²³⁰ allele (Fig. 2), with all intermediate birds being heterozygous at site 230, whereas a high proportion (83%) of dark birds were His²³⁰ homozygotes. Genetic differentiation among pale and intermediate to dark skuas is high at *MC1R* ($F_{st} = 0.56$, $P < 0.02$) and absent in mtDNA [$\Phi_{st} = 0.01$, NS (22)]. Together these data strongly suggest that *MC1R* is the major locus determining plumage coloration in arctic skuas that had been predicted from genetic modeling (4). The Arg²³⁰→His²³⁰ mutation may be causative, because a histidine at the homologous site in the third intracellular loop of *MC1R* is also associated with melanism in rock pocket mice (*Chaetodipus intermedius*) (23, 24).

A different nonsynonymous substitution at the same amino acid site (Arg²³⁰→Cys²³⁰) was found in the relatively dark great skua (*Catharacta skua*), a species representing the second lineage within the skua family (Stercorariidae). Thus, melanism may have evolved independently in the great skua lineage since it shared a common ancestor with arctic and long-tailed skuas (*Stercorarius longicaudus*). This hypothesis is supported by phylogenetic studies indicating that the common ancestor of today's skua lineages may have been *Stercorarius*-like, with barred juvenile and white-breasted alternate adult plumage similar to that of gulls (25). The His²³⁰ allele in arctic skuas is present in a derived position on a haplotype network (Fig. 3), suggesting that ancestral arctic skuas were pale. Coalescent simulations estimate an upper bound for the age of the Arg²³⁰→His²³⁰ mutation in arctic skuas at 340,000 ± 248,000 (SD) years, indicating a Pleistocene origin for the polymorphism.

The precise effects of *MC1R* variation on plumage coloration and patterning are surprisingly diverse. In snow geese, the dosage of variant *MC1R* alleles affects patterning: Color phases differ in the amount of the body covered in melanic feathers. In contrast, melanic arctic skua phases show graded differences in the amount of melanin in individual feathers over the neck, breast, and belly. In bananaquits (*Coccyzus flaveola*), the Glu⁹²→Lys⁹² variant *MC1R* allele acts as a melanin switch, so that all individuals with one or two copies of this allele have completely melanized feathers throughout their plumage (10). *MC1R* variation is also associated with patterning effects in domestic

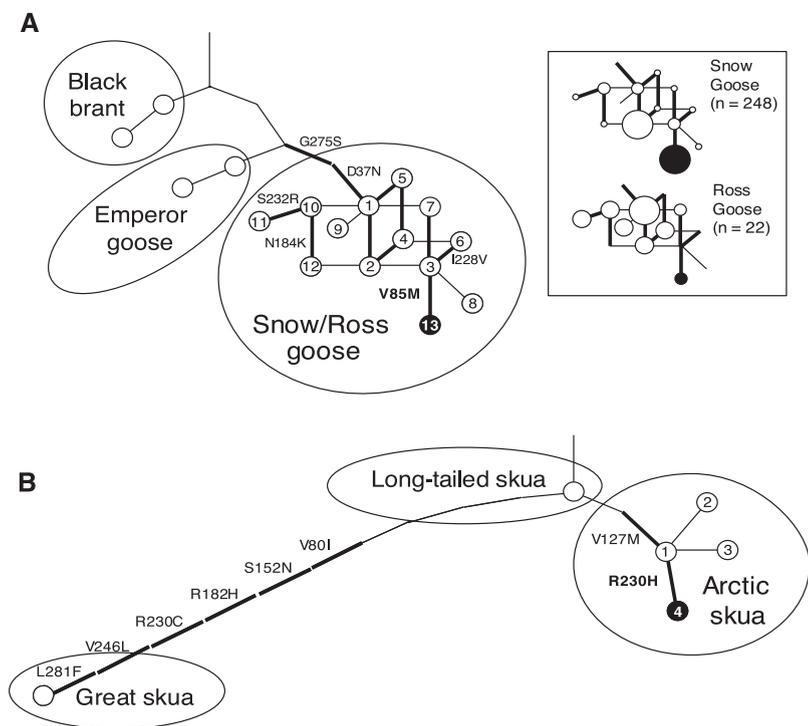


Fig. 3. *MC1R* haplotype networks in (A) geese and (B) skuas, obtained using TCS software (30, 31). Thick lines represent nonsynonymous substitutions; thin lines are synonymous substitutions. Networks are rooted using chicken *MC1R*. Numbers indicate haplotype groups (snow and Ross' geese) or haplotypes (arctic skuas). Haplotype groups in geese are haplotypes that were defined ignoring variation at two hypervariable synonymous sites in *MC1R* (nucleotide sites 378 and 408) [see methods (12) for details]. The inset in (A) shows haplotypes sampled in snow geese and Ross' geese, with the area of the circles approximately proportional to haplotype frequency. Black and white circles correspond to melanic and nonmelanic haplotypes, respectively.

chickens (8, 26), but not with small-scale feather tip melanization across species of *Phylloscopus* warblers (27). In addition, snow geese, arctic skuas, and bananaquits show that *MC1R* frequently controls variation between eumelanin production and the absence of melanin, whereas in chickens and most mammals it controls relative amounts of eumelanin and red/yellow pheomelanin (8, 9, 26).

Variation in plumage color in geese and skuas provides a rare example where the major molecular genetic determinant of a quantitative trait has been identified in wild populations. The association of *MC1R* variation with naturally occurring melanism in three divergent avian lineages (Anseriforms, snow geese; Charadriiforms, arctic skuas; and Passeriforms, bananaquits) reveals a conserved mechanism of plumage color evolution through many tens of millions of years of avian history (28). The repeated involvement of *MC1R* is surprising, because over 100 loci are known to affect pigmentation in vertebrates (29). This presumably reflects some combination of a high mutability to functionally novel *MC1R* alleles, a relative absence of deleterious pleiotropic effects of these alleles, and the visibility of dominant or codominant melanic *MC1R* alleles to natural selection. Our results provide strong support for the notion that, at least in the case of melanism in birds, evolution is driven by mutation rather than selection on existing standing genetic variation.

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Supporting Online Material

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Genetic Basis of Natural Variation in *D. melanogaster* Antibacterial Immunity

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Many genes involved in *Drosophila melanogaster* innate immune processes have been identified, but whether naturally occurring polymorphism in these genes leads to variation in immune competence among wild flies has not been tested. We report here substantial variability among wild-derived *D. melanogaster* in the ability to suppress infection by a Gram-negative entomopathogen, *Serratia marcescens*. Variability in immune competence was significantly associated with nucleotide polymorphism in 16 innate immunity genes, corresponding primarily to pathogen recognition and intracellular signaling loci, and substantial epistasis was detected between intracellular signaling and antimicrobial peptide genes. Variation in these genes, therefore, seems to drive variability in immunocompetence among wild *Drosophila*.

Efficacy of immune response is a critical determinant of fitness, and higher eukaryotes have accordingly evolved sophisticated mechanisms for suppressing bacterial infection. For invertebrates, this is mediated by generalized, or innate, immune pathways, which include phagocytosis by scavenging macrophages and the extracellular circulation of short antibiotic peptides (1, 2). Artificially generated mutations that abolish the function of key genes have resulted in severe immune deficiencies [(1) and citations therein]. In addition, studies of population-level variation have suggested that *Drosophila* immunity genes evolve under positive natural selection (3–5). However, phenotypic effects of

naturally occurring polymorphism in innate immune genes have not previously been studied in invertebrates. We sought to measure variability in immunocompetence among *Drosophila melanogaster* in wild populations with the aim of attributing that variation to gene candidates involved in immune function. We initially focused on loci encoded on the *D. melanogaster* second chromosome, examining 21 protein-coding genes of diverse function (Table 1). Seven candidate genes are hypothesized to be involved in microbial recognition: four class C scavenger receptors [*SR-CI*, *SR-CII*, *SR-CIII*, and *SR-CIV* (6)] and a three-gene cluster of putative peptidoglycan recognition proteins [*PGRP-SCIA*, *PGRP-SCIB*, and *PGRP-SC2* (7)]. Candidates involved in signal transduction are three Toll-like receptors (*Tehao*, *18-Wheeler*, and *Toll-4*), the rel transcription factor *Dif*, rel inhibitor *cactus*, and intracellular signaling genes *imd* and *IK2* (2). The remaining seven genes encode the secreted antibacterial peptides Defensin, Metchnikowin, Attacins A, B and C, and Dipterocins A and B (8–12).

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