RESEARCH ARTICLE SUMMARY

PATTERN FORMATION

The periodic coloration in birds forms through a prepattern of somite origin

Nicolas Haupaix, Camille Curantz, Richard Bailleul, Samantha Beck, Annie Robic, Marie Manceau*

INTRODUCTION: In animals, coat color is often arranged in periodic motifs that vary widely, from striped to spotted patterns. These intricate designs have long fascinated developmental biologists and mathematicians alike. What are the mechanisms underlying the formation of periodic patterns and shaping their diversity? Spatial organization in the developing skin involves prepatterns that precede the color pattern. Self-organizing events have long been thought to act upstream of prepatterns (e.g., through molecular diffusion or pigment cell interaction). Changes in both of these molecular and cellular events may contribute to periodic pattern variation. However, periodic patterns are highly reproducible within species and display specific orientation and periodicity, which suggests that they also rely on preexisting spatial reference.

RATIONALE: Documenting phenotype diversity constitutes a promising framework for the prediction of such spatial landmarks, comparable to mathematical modeling strategies. We surveyed variation in the transient periodic pattern visible in juvenile birds of the galliform group, in which longitudinal stripes are organized in a black-yellow-black sequence in the dorsal region.

RESULTS: By comparing the striped pattern for 10 galliform bird species, we showed that the width of each stripe varied and that their number increased with dorsum size. In contrast, their absolute positions were comparable. We analyzed pigment appearance in the embryonic skin of five representative species and showed that the periodic striped pattern results from the timely production of yellow coloration at specific locations. This yellow-production pulse was not triggered by a certain stage of feather growth or by dynamics of feather follicle production across the dorsum. However, it was linked to the early expression of *agouti*. This well-known pigmentation gene displayed a composite expression pattern in longitudinal bands whose width and position correlated with that of yellow stripes in each species. To test *agouti*'s role, we used a functional approach by exploiting mutant strains of quails: The increase (in the Fawn strain) or decrease (in the recessive black strain) of *agouti* expression levels respectively led to wider or narrower yellow stripes. Comparing pigment distribution across feathers between these gain- or loss-of-function mutants and wild-type quails showed that *agouti* controls stripe width by adjusting the duration of the yellow-production pulse in a dose-dependent manner. Both the position of *agouti*-expressing bands and that of yellow stripes did not change in mutant quails.

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To identify the origin of signals controlling localized *agouti* expression and setting the position of yellow stripes, we performed heterospecific grafting experiments: Embryonic tissues from donor quails were transplanted into pheasant hosts. We found that after transplanting somites (from which dermal cells originate), chimeras locally displayed quail-like expression of *agouti* in the developing skin. Long-term experiments showed that hosts displayed a striped color pattern typical of the donor at the level of the graft. Such changes were not observed when the neural tube (from which pigment-producing cells originate) was grafted. These results showed that the somitic mesoderm autonomously instructs *agouti* expression and consequently the position of yellow stripes.

CONCLUSION: We conclude from this work that the galliform striped pattern is achieved in a two-step mechanism. The somite provides positional information to the developing dermis; this controls the position of *agouti* expression in a prepattern that foreshadows yellow stripes. Their width is then refined by *agouti*, which locally controls yellow production in a dose-dependent manner. This sequential organization of space, combining early landmarks and local mechanisms, may govern the formation (and thus constrain the evolution) of many periodic patterns. ■

The striped pattern of a Japanese quail embryo. Galliform birds display a longitudinal pattern of colored stripes already visible a few days before hatching (here in a Japanese quail, *Coturnix japonica*). Stripes form through differential deposition of black and yellow pigments along growing feathers in the dorsum. Our work shows that this pattern is controlled by a prepattern instructed by the somitic mesoderm.

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RESEARCH ARTICLE

PATTERN FORMATION

The periodic coloration in birds forms through a prepattern of somite origin

Nicolas Haupaix[1], Camille Curantz[1], Richard Bailleul[1], Samantha Beck[1], Annie Robic[2], Marie Manceau[1]*

The periodic stripes and spots that often adorn animals' coats have been largely viewed as self-organizing patterns, forming through dynamics such as Turing's reaction-diffusion within the developing skin. Whether preexisting positional information also contributes to the periodicity and orientation of these patterns has, however, remained unclear. We used natural variation in colored stripes of juvenile galliform birds to show that stripes form in a two-step process. Autonomous signaling from the somite sets stripe position by forming a composite prepattern marked by the expression profile of *agouti*. Subsequently, *agouti* regulates stripe width through dose-dependent control of local pigment production. These results reveal that early developmental landmarks can shape periodic patterns upstream of late local dynamics, and thus constrain their evolution.

Many vertebrates display intricate color patterns characterized by a periodic arrangement of pigments in stripes or spots (*1*). This spatial organization was recently shown to involve genes whose embryonic expression profile forms a prepattern preceding the adult pattern [e.g., the developmental expression of the pigmentation genes *edn3b* and *alx3* foreshadows striped patterns in the fur of cats and African striped mice, respectively (*2*, *3*)]. A long-standing challenge has been to uncover the embryonic pattern-forming events acting upstream of prepatterns to create discrete compartments in the developing skin. Computer simulations of stochastic dynamics such as Turing's reaction-diffusion, which involves the interaction of at least one self-activating factor and its inhibitor diffusing at long range, reproduce periodic motifs that resemble those observed in the wild; this finding suggests that color self-organizes in the skin tissue [see (*4*) for a review]. This hypothesis is supported by a handful of empirical studies. In zebrafish, longitudinal stripes form through interaction [depolarization-repulsion (*5*)] of pigment cells sequentially aggregating locally, forming interstripes, then expanding and compacting within stripe regions (*6*, *7*). In striped cats, the aminopeptidase taqpep creates periodicity by establishing an *edn3b*-expressing prepattern (*2*). Changes in cell behaviors or in the biological parameters of molecular players (e.g., clearance rate, which reflects the rate of molecule elimination; diffusivity) may contribute to natural variation in periodic patterns, consistent with theoretical predictions: Modifying the corresponding parameters of simulations gives rise to a vast array of patterns (*4*). Most periodic color patterns, however, display specific orientation or periodicity and are highly reproducible within species, which suggests that their formation does not entirely rely on stochastic events. Here, we investigated whether early developmental landmarks provide preexisting spatial reference to periodic patterns.

Stripes vary in number and width but have comparable positions relative to the dorsal midline

To predict potential spatial landmarks, we surveyed phenotypic variation in the transient striped pattern visible along the dorsum of juvenile birds of the galliform group (*8*, *9*). In flat-skin specimens for 10 species chosen for their representative varying patterns (table S1), we compared feather types according to pigmentation along barbs (i.e., primary branches). We distinguished two main types:

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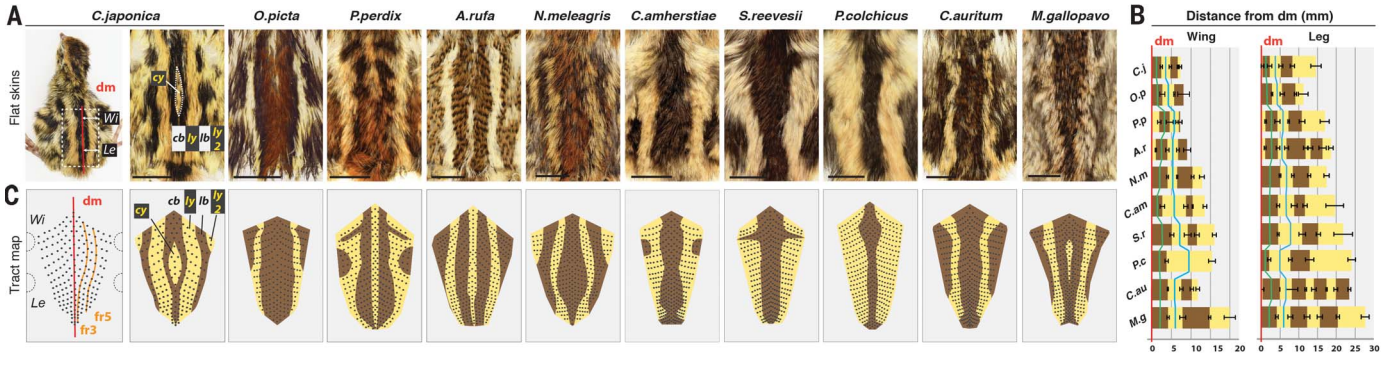

Fig. 1. Stripes vary in width and shape, but not position, in galliforms. (**A**) Flat-skin preparations of dorsal skin regions (white rectangle) in juvenile individuals of 10 galliform bird species (n = 2 to 5 per species, see table S1) show their alternating yellow and black stripes, labeled as *cy* (central yellow), *cb* (central black), *ly* (lateral yellow), *lb* (lateral black), and *ly2* (additional lateral yellow) stripes. This pattern is symmetrical over the dorsal midline (dm, in red) and was thus used as a landmark reference. Scale bars, 1 cm. (**B**) Measures of absolute distances from the dorsal midline to the boundary between each stripe at wing and leg levels [indicated by *Wi* and *Le* in (A) and (C)] demonstrate interspecies variation in stripe width. We also observed changes in the number of lateral stripes despite differences in dorsum size (species are shown in size order, with smallest at top). Conversely, *cb* and *ly* stripes have comparable positions. Green lines represent *cb* distance from the dorsal midline (2 to 4 mm at wing level, 2 to 3 mm at leg level); blue lines represent *ly* distance from the dorsal midline (3 to 9 mm at wing level, 3 to 6 mm at leg level). C.j, *Coturnix japonica*; O.p, *Oreortyx picta*; P.p, *Perdix perdix*; A.r, *Alectoris rufa*; N.m, *Numida meleagris*; C.am, *Chrysolophus amherstiae*; S.r, *Syrmaticus reevesii*; P.c, *Phasianus colchicus*; C.au, *Chrosoptylon auritum*; M.g, *Meleagris gallopavo*. Error bars indicate SD. (**C**) Feathers of the dorsal tract organize in feather rows (fr; shown in orange in *C. japonica*). Their spatial arrangement varies between species. Plotting feather types at each position of the tract produces precise spatial representations of the color pattern.

black (*b*; entirely eumelanic) and yellow (*y*; with a pheomelanin band and black base and tip) (fig. S1). This allowed us to identify a common stripe sequence, symmetrical over the dorsal midline and extending from the wing to the tail: The most central stripe is black (*cb*)—sometimes containing a few central yellow feathers (*cy*)—and is flanked by two lateral yellow stripes (*ly*). The latter can be ventrally bordered by lateral black stripes (*lb*) and additional lateral yellow stripes (*ly2*) (Fig. 1A). We observed that the number of stripes often increased with dorsum size, hence variation in color pattern does not result solely from scaling. The position of each stripe relative to dorsum size varied between species (fig. S2). In comparison, absolute distances between the dorsal midline and the center of the stripes closest to the median (i.e., *cb* and *ly*) were comparable; this suggests that these stripes are positioned early, prior to dorsal skin expansion (Fig. 1B). The width of each stripe also varied between species. To detail this variation, we compared stripe boundaries relative to feather tracts, which are feather-covered skin areas separated by near-glabrous regions. Within tracts, feathers form longitudinal rows whose number, spacing, and length along the anteroposterior axis is typical to each species (*10*, *11*) (fig. S3). We plotted feather types at each position of the dorsal tract and found that color boundaries are highly reproducible within species' tracts (e.g., in *Coturnix japonica*, the *cb-ly* boundary is characterized by the consistent production of *b/y* split feather types). However, these color boundaries vary between species relative to feather row number (i.e., stripe width) and along the anteroposterior axis (i.e., stripe shape; Fig. 1C). This suggests that variation in stripe width or shape results from differences in local mechanisms occurring during feather tract formation.

Interspecies differences in *agouti* expression correlate with stripe pattern variation

We took advantage of both the absence of variation in stripe position and the presence of differences in stripe width or shape to link the juvenile color pattern to (i) pigment production and (ii) tract formation during embryogenesis (in contrast to phenotypic surveys classically limited to observations of adult patterns). We used five species representative of variation in the width or shape of stripes, the presence or absence of *cy/lb* stripes, and the organization of the dorsal tract (namely *C. japonica*, *Alectoris rufa*, *Perdix perdix*, *Phasianus colchicus*, and *Syrmaticus reevesii*). We first compared the appearance of pigments in these species. We found that pigment production starts a few days before hatching (when all feather follicles of the dorsal tract are visible; fig. S4) and occurs in a medial-to-lateral wave: Eumelanin pigments are first produced in the most central feathers, forming one (*P. colchicus*, *S. reevesii*) or two (*C. japonica*, *A. rufa*, *P. perdix*) longitudinal black bands, and then progressively appearing laterally. This forms the *cb* stripe. Eumelanin production is transiently

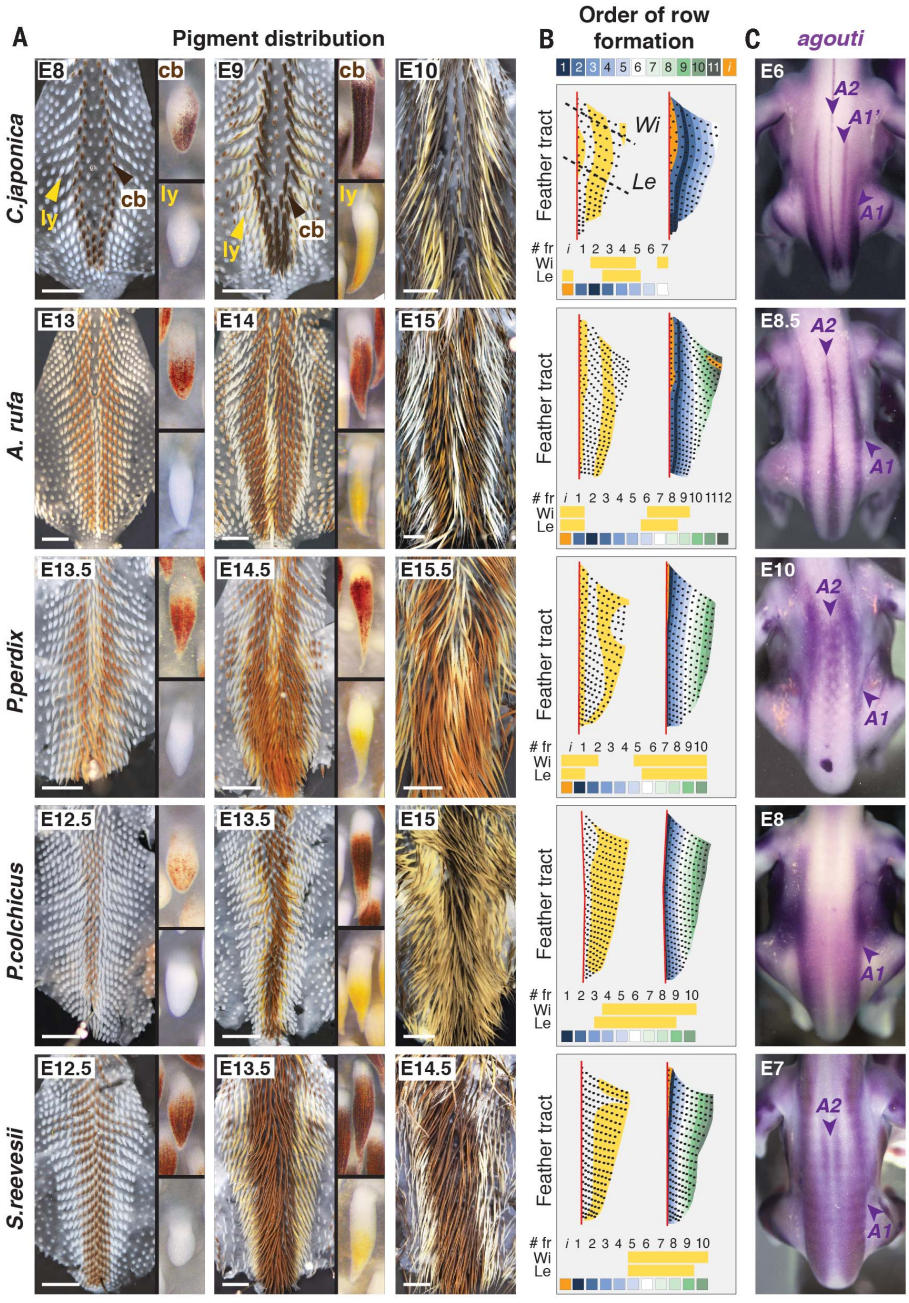

Fig. 2. Stripes form through yellow pulses independent of tract differentiation. (**A**) Flat-skin preparations of dorsal embryonic skins in five species (left) and high-magnification views of developing feathers in *cb* (upper right) and *ly* (bottom right) stripes allow visualization of the sequential production of pigments. Scale bars, 0.25 cm. (**B**) Color pattern diagrams (left) and color-coded representations of each feather (#fr) at wing (Wi) and leg (Le) levels show the positions of *cy* and *ly* stripes (which sometimes differ between Wi and Le; *C. japonica* has an additional *ly2* stripe). A blue-to-green color code on diagrams (right) and for each feather (squares) shows the order of formation of feather rows (1 to 11, depending on species). Intercalate feathers (*i*, in orange) may form after tract formation. We observed no correlation between the order of formation of feather rows and their position in the tract (i.e., #fr), nor with the position of *cy* and *ly* stripes. (**C**) In situ hybridizations for *agouti* (in purple) reveal the two most lateral expressing bands present in all species (*A1*), two intermediary bands visible only in *C. japonica* (*A1'*), and one *A2* band visible in *C. japonica*, *A. rufa*, *P. perdix*, and to a lesser extent, *S. reevesii*. (*A1'*)*A1* and *A2* (purple arrowheads) foreshadow *ly* and *cy* stripes [compare with (B)]. E, embryonic day of development.

replaced in lateral feather rows by pheomelanin, forming the banded pattern in feathers of *ly* stripes. The most lateral feathers produce only eumelanin (forming *lb* stripes), and the most central feathers transiently produce pheomelanin (forming the *cy* stripe; Fig. 2A). Thus, the striped pattern results from localized "pulses" of yellow pigment production (*cy* and *ly* stripes) in an otherwise black-producing tissue.

To test whether yellow pulses occur at certain stages of tract differentiation, we reconstructed the sequence resulting in the spatial arrangement of follicles across the dorsal tract in all species and compared it with the position of the yellow-producing domains. Developing follicles stained with the β-*catenin* marker (*12*) first correspond to one (*P. colchicus*, *S. reevesii*) or two (*C. japonica*, *A. rufa*, *P. perdix*) longitudinal bands; rows of follicles are then added in a wave traveling ventrally [consistent with previous observations in the chick (*13*)], and lastly in the most central area, completing the dorsal tract (fig. S5). Tracing back *cy* and *ly* feather rows to their order of formation showed that this order differs between species (Fig. 2B). Thus, the position of *cy* and *ly* stripes is not set by a mechanism taking place at given stages of tract formation.

We next tested whether yellow pulses are triggered by feather growth: We compared, in the *ly* stripe, (i) stages at which feathers switch to pheomelanin or back to eumelanin production, and (ii) the size and proximodistal position of the pheomelanin band in fully grown *y* feathers (as a readout for the onset and duration of the pulse). We found no correlation between these and the timing of yellow production (fig. S6). Thus, yellow pulses are not temporally linked to stages of feather growth. Because yellow-producing domains have shared positions and varying widths that do not correlate with dynamics of tract formation, we predicted that they rely on prepatterns established before skin differentiation. The signaling peptide Agouti controls the production of pheomelanin pigments in a timely fashion in the hair (*14*) and marks prepatterns of light color domains in the embryonic skin (*3*, *15*) and along individual feathers (*16*). We found that prior to follicle formation in the five bird species, two *agouti*-expressing bands (*A1*) form in the dermis on both sides of the neural tube. In *C. japonica*, *A. rufa*, *P. perdix*, and weakly in *S. reevesii*, a thinner central band of *agouti* (*A2*) also appears a few hours after *A1*, and in *C. japonica* only, additional thin bands (*A1′*) intercalate between the *A1* and *A2* bands, a few hours after *A2* appearance (Fig. 2C and fig. S7). Thus, *agouti* expression is composite in space and time. In all species, the pattern of *agouti*-expressing bands resembled that of yellow stripes.

Agouti foreshadows yellow stripes and controls their width in a dose-dependent manner

To confirm this spatial correlation, we linked the position of each band to presumptive feather rows using double in situ hybridization for *agouti* and β-*catenin* in *C. japonica*. We found that the *A1′* *agouti* band is expressed in the presumptive domain of the second feather row, consisting of

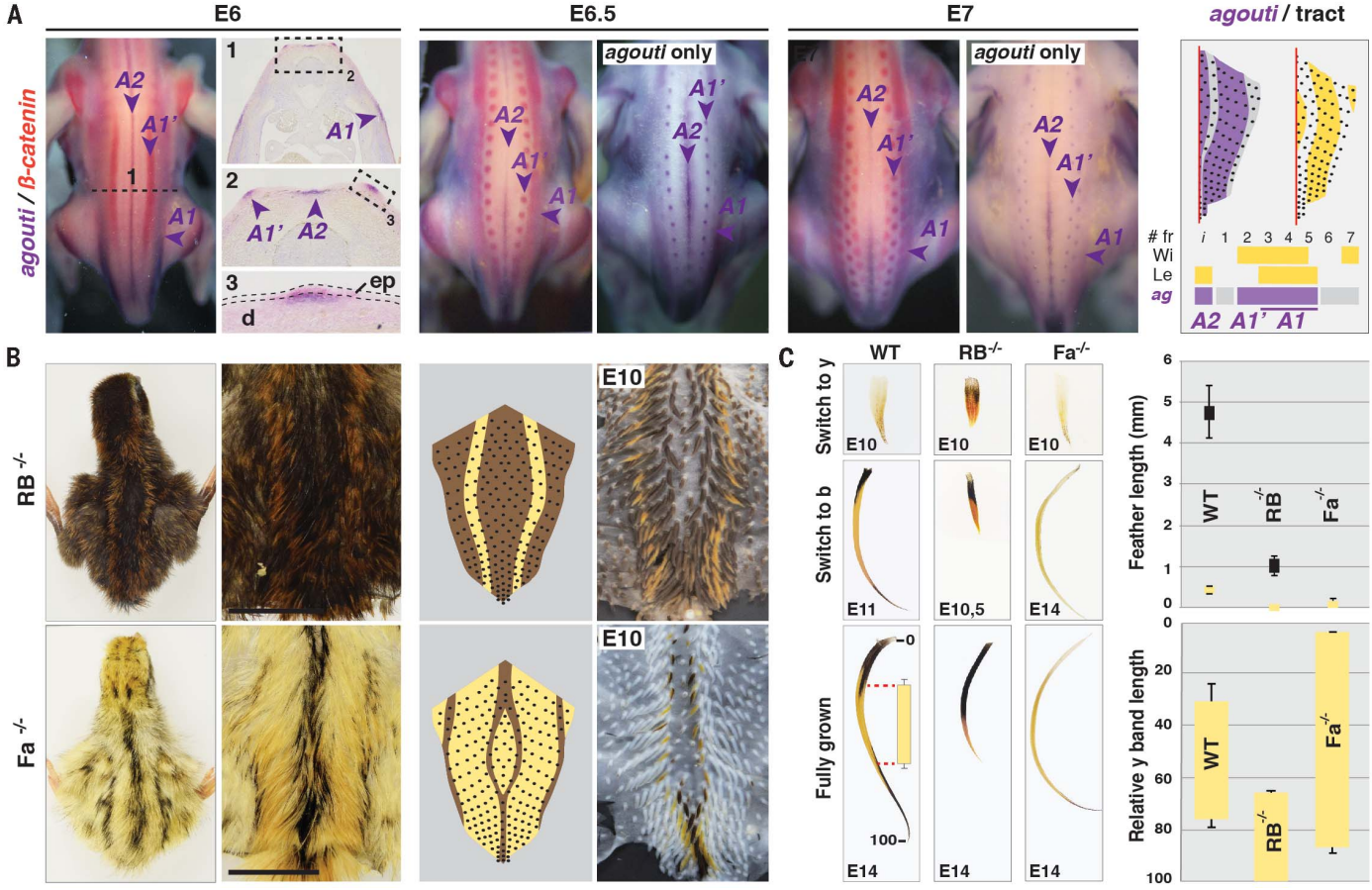

Fig. 3. *agouti* marks a striped prepattern and controls stripe width. (**A**) Double in situ stains for *agouti* (purple) and β-*catenin* (red) at E6, E6.5, and E7 show (in whole-mount embryos and sections) that *A1′* corresponds to fr#2, *A1* corresponds to fr#3 to 5, and A2 corresponds to fr#*i* (putative domains correspond to interlimb level on an E16 dorsal tract map). ep, epidermis; d, dermis. (**B**) Juvenile flat skins, pattern maps, and embryonic flat skins of mutant strains of *C. japonica* show a reduction ($RB^{-/-}$; upper panels, *n* = 5) or extension ($Fa^{-/-}$; lower panels, *n* = 5) of *cy* and *ly* stripe width. (**C**) Observations and quantifications of *y* feather length (as detailed in fig. S6; upper graph) when they first switch to yellow production (yellow dot plots) or back to black production (black dot plots) show that the yellow pulse occurs earlier in $RB^{-/-}$ than in wild-type individuals. Measures of the size of the yellow band relative to the length of fully grown feathers (fig. S6 and see scheme in *C. japonica*; lower graph) show that the yellow pulse occurs for a longer duration in $Fa^{-/-}$ than in wild-type individuals. Error bars indicate SD.

the first *b/y* feather of the *ly* stripe, whereas *A1* corresponds to the third through fifth rows, forming the rest of the *ly* stripe, and *A2* covers the central rows that form the *cy* stripe. In this species, *agouti* is later restricted to feather follicles of these stripes (Fig. 3A). Thus, the composite expression of the (*A1′*)*A1* and *A2 agouti* bands reveals a prepattern that foreshadows the position of *ly* and *cy* yellow stripes, respectively. To understand how *agouti* expression affects stripe patterning, we used a functional quantitative approach and assessed the striped phenotype in mutant *C. japonica* strains. In recessive-black quails ($RB^{-/-}$), *agouti*'s spatial pattern is maintained with no change in the position or width/shape of the *A1*, *A1′*, and *A2* bands (fig. S8), but a frameshift mutation in *agouti*'s coding sequence results in a marked decrease in transcript expression levels (*17*). We found that these birds display overall shorter feathers organized in thinner *ly* stripes (i.e., only feather rows 3 and 4 produce yellow, versus rows 2 and 5 in wild-type individuals), and no visible *cy* stripe (Fig. 3B). These changes in *ly* stripe width result from a decrease in the duration of the yellow pulse: Relative to wild-type quails, feathers of $RB^{-/-}$ quails switch back to eumelanin production earlier (consequently producing fewer *y* feathers containing smaller yellow bands; Fig. 3C). Conversely, quails of the Fawn mutant strain ($Fa^{-/-}$) are homozygous for a duplication at *agouti* responsible for an increase in its expression levels (*17*, *18*). In $Fa^{-/-}$ birds, the spatial pattern of *agouti* remains unchanged (fig. S8) but the *ly* (rows 1 or 2 through row 6) and *cy* stripes are wider, with *y* feathers displaying longer yellow bands because of a delayed switch to eumelanin production (i.e., increase in the duration of the yellow pulse; Fig. 3, B and C). These loss- or gain-of-function experiments show a role for *agouti*: At given positions (i.e., expressing bands), this gene regulates stripe width through a dose-dependent control of the duration of the yellow pulse. Agouti is a peptide diffusing in a paracrine manner; its localized expression may thus create a signaling gradient to which dermal cells respond according to their position: Certain thresholds of *agouti* levels trigger yellow production to locally fine-tune the border of yellow stripes and modulate the length of the yellow band (consistent with the latter being increased in feathers at the center of *ly* stripes relative to those at the edge; fig. S1). This raises the appealing possibility that striped pattern evolution is governed by differential regulation of *agouti* expression levels.

The somitic mesoderm controls localized *agouti* expression and yellow stripe position

Contrary to stripe width, the position of stripes is conserved between species; this suggests that early developmental landmarks establish *agouti*'s composite prepattern. We studied the earliest visible expression of *agouti* in the embryonic skin in all five species and found it comparably located in the dermis above the neural tube (*A2*; fig. S9) and the dorsomedial part of differentiating somites (*A1*; Fig. 4A). Previous quail-chick grafting experiments showed that both the neural tube and somites contribute to the skin lineage. Feather follicle cells originate from the somite dermomyotome, and their spatial distribution is mesoderm-dependent (*13*, *19*). Conversely, pigment-producing cells (melanocytes) derive from the neural crest (*20*, *21*), but despite evidence

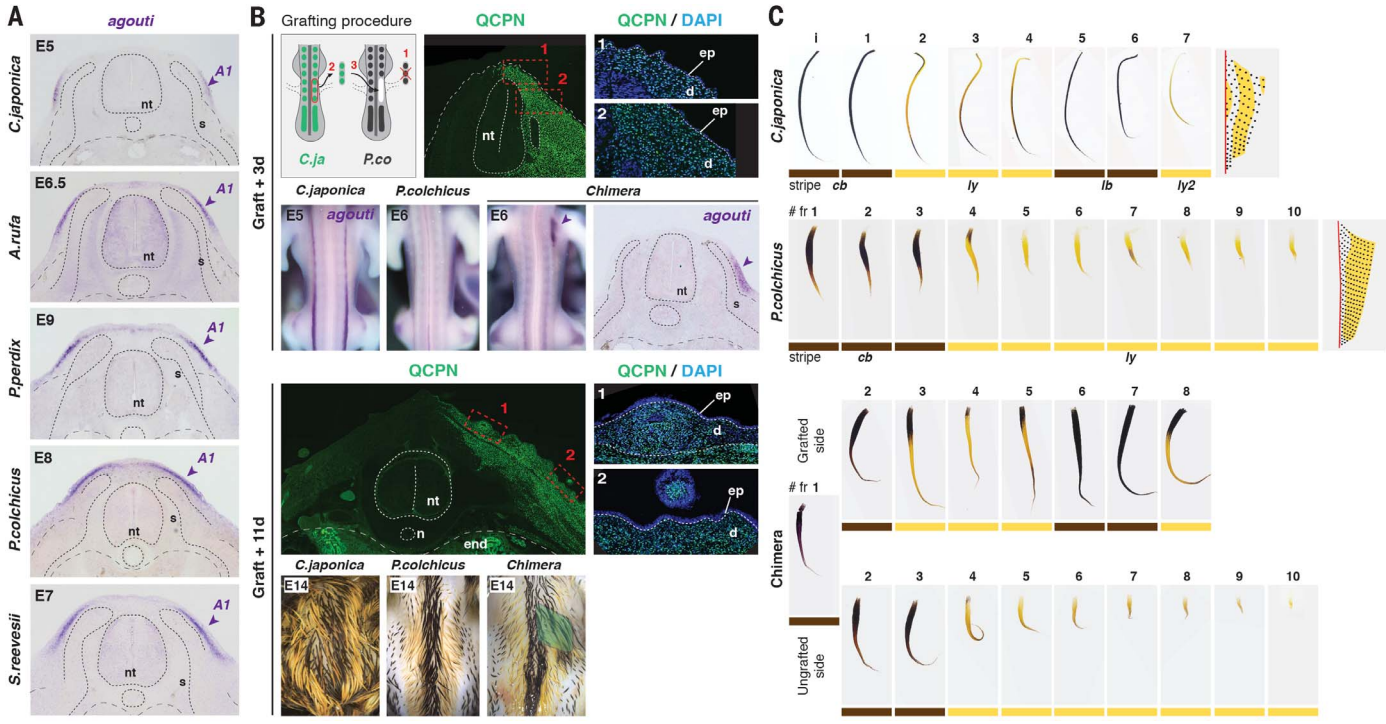

Fig. 4. Stripe color pattern has a somitic origin. (**A**) Sections of in situ hybridization for *agouti* (in purple) show that in all species, *A1* bands (purple arrowheads) locate in the embryonic dermal area above the developing somite. nt, neural tube; s, somite. (**B**) *C. japonica*'s three last formed somites at HH13 (i.e., immediately posterior to the wing bud) were grafted in place of those of *P. colchicus*; 3 days after grafting, immunohistochemistry for QCPN (in green) revealed that *C. japonica*'s cells invaded the host tissues (n = 8). High-magnification views with DAPI stains (in blue) show that $QCPN^+$ cells locate in the developing dermis but not in the epidermis in regions corresponding to *A1/A1′* (red square 2) and *A2* (red square 1). At that stage, grafted regions in chimeras strongly express *agouti* in position of the *A1* band (purple arrowhead, n = 7), similar to *C. japonica* but contrary to *P. colchicus* embryos. Eleven days after grafting, $QCPN^+$ cells also locate in the dermis, including the papillae of feather follicles, but not in the epidermis. Relative to *C. japonica* and *P. colchicus* controls (left panels, n = 3 per species), feathers of the chimera at the same stage (right panels, n = 4) resemble those of the host except in the grafted area (in green). (**C**) The chimera's grafted side (third series from top) has the same stripe and feather pattern (see #fr) as *C. japonica* (first series and color pattern diagram); conversely, its ungrafted side (fourth series) has the same stripe and feather pattern as *P. colchicus* (second series and color pattern diagram). In chimeras, the first feather (#fr1) is common to the grafted and ungrafted sides. n, notochord; end, endoderm.

that their differentiation responds to cues from the feather papillae (22), the means by which they govern color patterning remained unclear, as grafted strains were homogeneously colored (and quail melanocytes produced black pigments in the host). We thus performed somite and neural tube transplantations from *C. japonica* donors into *P. colchicus* hosts (because both species display a typical striped pattern; Fig. 4B). Three days after transplanting the right half of the *C. japonica* neural tube (prior to major migration of the neural crest) in place of that of *P. colchicus* at the equivalent stage, no expression of *agouti* (normally visible in *C. japonica* but not *P. colchicus* embryos at that stage; fig. S7) was detected in the dermis of chimeras. *C. japonica* cells (stained with the specific QCPN marker) were observed in the developing neural tube, dermis, and epidermis. The same was true 11 days after grafting [i.e., embryonic day 14 (E14), when coloration is visible in lateral stripes]; at that stage, some $QCPN^+$ cells coexpressed the melanocyte marker Trp1 (fig. S10). In these chimeras, the grafted and ungrafted parts of the skin displayed similar color (and feather) patterns (fig. S11). This demonstrates that the neural tube, despite generating melanocytes producing black pigments by default (20, 21), does not spatially control the expression of *agouti* expression or the activity of melanocytes (and thus the position of yellow-producing domains). Conversely, 3 days after replacing the undifferentiated somites of *P. colchicus* located below wing level by those of *C. japonica*, $QCPN^+$ cells invaded the whole dermal compartment and we observed strong expression of *agouti* in the grafted dermis in the position of the *A1* band (Fig. 4B). Eleven days after transplantation, $QCPN^+$ cells did not express Trp1 (fig. S12). Growing feathers of the grafted side were longer than those of the ungrafted side, spatially organized according to *C. japonica*'s dorsal tract [fig. S11 and consistent with previous work (13)], and displayed the color pattern seen below wing level in *C. japonica* (note that it differs from leg level; see Fig. 2B). Specifically, *lb* feathers were present in the lateral part of the tract and yellow pigments were produced in feather rows 2 to 4, thereby reducing the width of the *ly* stripe in the grafted side relative to the ungrafted side (Fig. 4C). Thus, the localized expression of *agouti* and production of yellow pigments (and therefore, the striped coloration pattern) are autonomously instructed by the somitic mesoderm.

Discussion

Results from this study show that the striped pattern is achieved in a two-step mechanism. The somite procures positional information, creating specific compartments in the dermis at precise locations (controlling the position of *agouti*-expressing bands and consequently yellow stripes). This prepattern is then refined according to expression levels of *agouti* (temporally controlling pigment-type production and consequently the width of each stripe). These results raise the possibility that most natural patterns, including periodic designs, rely on (and are constrained by) a stepwise organization of space that combines late, local events producing periodicity and early positional sources ensuring reproducibility, which is the key to fitness and proper directionality (in this case, longitudinal stripes depend on information from axial structures). Our work thus opens new avenues following from current theoretical models: Simulations of Turing's reaction-diffusion (and other self-organizing systems) can be framed using initial conditions corresponding to developmental landmarks. Extending empirical work on other natural patterns will better define such initial conditions and thus shed light on developmental constraints to color pattern evolution.

Materials and methods

Specimen sampling

Previously euthanized specimens of 1- to 3-day-old hatchlings (for 10 species of galliform birds; table S1) and fertilized eggs (for *C. japonica*, *A. rufa*, *P. perdix*, *P. colchicus*, and *S. reevesii*) were obtained from authorized local breeders (Ferme de Chanteloup, Caringa, Mr. Bouly de Lesdain, Les Bois de Vaux) and stored at −20°C prior to processing. After egg incubation in Brinsea ovaeasy 190 incubators, embryos at various stages of development were dissected in PBS and fixed in 4% formaldehyde.

Stripe pattern analyses

Flat skin specimens were prepared from frozen carcasses through an incision of the skin along the ventral midline and cautious separation of the scalp from body muscles. Dissected skins were stretched flat, left to dry for 1 week, and imaged. Measures of distances between stripe boundaries were performed from the dorsal midline to the wings and legs (Fig. 1A) using a Vernier caliper (*C. japonica* and *A. rufa*, $n = 5$; *Oreortyx picta* and *Chrosoptylon auritum*, $n = 2$; all other species, $n = 3$). Within the measured area, all species displayed *cb*, *ly*, *lb* (*Chrysolophus amherstiae*, and *S. reevesii* only at leg level), and *ly2* (except *S. reevesii*) stripes. Only *C. japonica*, *A. rufa*, *P. perdix*, and *Meleagris gallopavo* had *cy* stripes. Spatial reference maps of dorsal tracts were obtained by (i) plucking out all feathers (using forceps), which revealed their respective positions along longitudinal rows (i.e., feather rows) from the neck to the tail, and (ii) recording color at each position (coded yellow when feathers displayed pheomelanin and black when they only contained eumelanin).

Pigments, tracts, and feather spacing analyses

Embryonic flat-skin specimens were obtained by performing longitudinal openings from neck to tail in embryos of *C. japonica*, *A. rufa*, *P. perdix*, *P. colchicus*, and *S. reevesii* and separating the developing skin tissue from the body (using microscissors and forceps). Dissected skins were fixed in 4% formaldehyde and stored in 80% glycerol. Developing feathers were plucked, mounted on glass slides in fluoromount (Southern Biotech), imaged, and measured using Fiji software (23).

The "switch to *y*" measure corresponds to the distance (in mm) from the tip of the feather to the distal limit of the yellow band (*C. japonica*, E10, $n = 12$; *A. rufa*, E13.5, $n = 12$; *P. perdix*, E13.5, $n = 12$; *P. colchicus*, E14, $n = 21$; *S. reevesii*, E14, $n = 23$; $RB^{-/-}$, E10, $n = 22$; $Fa^{-/-}$, E10, $n = 18$). The "switch to *b*" measure corresponds to the distance (in mm) from the tip of the feather to the distal limit of the black base (*C. japonica*, E11, $n = 32$; *A. rufa*, E15, $n = 32$; *P. perdix*, E15, $n = 7$; *P. colchicus*, E15, $n = 38$; *S. reevesii*, E14.5, $n = 24$; $RB^{-/-}$, E10.5, $n = 14$; $Fa^{-/-}$, $n = 0$, in this strain no eumelanin was seen before hatching). The "relative *y* band length" measure (in %) corresponds to the length of the yellow band normalized to the length of the whole, fully grown *y* feather from its base (0) to its tip (100); see schemes in Fig. 3 and fig. S6 (*C. japonica*, E13, $n = 24$; *A. rufa*, E22, $n = 8$; *P. perdix*, E22, $n = 6$; *P. colchicus*, E22, $n = 19$; *S. reevesii*, E22, $n = 8$; $RB^{-/-}$, E14, $n = 6$; $Fa^{-/-}$, E14, $n = 6$).

Distance ratios were calculated between #fr1-2, fr2-3… to fr5-6 for three series along the feather row (see scheme in fig. S11).

Expression analyses

In situ hybridization experiments were performed as described (24) using antisense riboprobes synthesized from vectors containing a 269-bp fragment of *C. japonica*, *A. rufa*, or *S. reevesii*'s coding sequences for *agouti*, or an 881-bp fragment of *C. japonica*'s coding sequence for β-*catenin*. For double in situ hybridizations, riboprobes were labeled with digoxigenin or fluorescein and sequentially revealed with anti-digoxigenin-AP or anti-fluorescein-AP antibodies (both 1:2000, Roche) and NBT/BCIP (Promega) or fast-red (Abcam) substrates.

Primers: *agouti*-F: TGCTCTGCTACAGTTTGCTCAG; *agouti*-R: TGGTTTGCAGGTTTTGAA); β-*catenin*-F: AGCTGACTTGATGGAGTTGGA; β-*catenin*-R: TCGTGATGGCCAAGAATTTC).

Heterospecific grafting

Quail-pheasant grafting procedures were adapted from previous quail-chick grafting experiments (25): At HH13 in *C. japonica*, the three last formed somites (just posterior to the wing bud), or the right half of the neural tube alongside these somites, were ablated using glass microneedles and cleaned of potential additional neighboring tissue in PBS. Transplants were immediately transferred into HH13 *P. colchicus* hosts in which equivalent tissues had been previously removed (see schemes in Fig. 4 and fig. S10). Grafted eggs were kept humid by adding ampicillin-containing PBS solution on the yolk surface, closed with tape, and reincubated (37°C, 50% humidity) for 3 or 11 days (i.e., a stage corresponding to E14 in *P. colchicus*). Chimeras were dissected (feathers were rapidly removed for analysis) and fixed in 4% formaldehyde. The extent of the graft was determined by screening chimeras for the presence of (i) pigments in the dermis and epidermis, which are normally absent in *P. colchicus* but visible in *C. japonica* individuals at that stage (fig. S11), and (ii) $QCPN^+$ cells on transverse sections.

Immunohistochemistry

Control and grafted embryonic specimens were embedded in gelatin/sucrose, sectioned using a CM 3050S cryostat (Leica), mounted in fluoromount, and immunostained using primary antibodies directed against QCPN (DSHB; 1:10) and Trp1 (Bruce Morgan laboratory; 1:20), and Alexa-conjugated secondary antibodies (Molecular Probes; 1:500). Cell nuclei were revealed using 4′,6-diamidino-2-phenylindole (DAPI; Southern Biotech).

Imaging

Whole-mount flat skins and stained/grafted embryos were imaged using an AF-S Micro NIKKOR 60-mm f/2.8G ED macro-lens equipped with a D5300 camera (Nikon) and a MZ FLIII stereomicroscope (Leica) equipped with a DFC 450C camera (Leica). Tissue sections were imaged using a BX53 fluorescence microscope (Olympus; for in situ hybridization or fluorescent stains) or a CSU-W1 spinning-disk confocal microscope (Zeiss) equipped with a CMOS flash 4 camera (Hamamatsu; for Trp1/QCPN fluorescent stains).

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SUPPLEMENTARY MATERIALS

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Figs. S1 to S12
Table S1
References (*23*–*25*)

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Science

The periodic coloration in birds forms through a prepattern of somite origin

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How birds change their stripes

From stripes to spots, animals often exhibit periodic coloration. Discrete embryonic domains (prepatterns) precede the periodic feather patterns observed in birds. After documenting natural variation in the striped pattern of galliform birds, Haupaix *et al.* performed long-term skin grafts to transfer the pattern of one species to another (see the Perspective by Prud'homme and Gompel). This approach revealed that periodic stripe formation obeys developmental landmarks upstream of local refining mechanisms. The somitic mesoderm first instructs stripe position through the early expression of the pigmentation gene *agouti*, which then controls stripe width by modulating pigment production in a dose-dependent manner. Thus, during feather patterning, a two-step process is at play.

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