# Stress lines

Environmental factors play a key role in the expression of phenotypic traits and life-history decisions, specifically when they act during early development. In birds, brood size is such an important environmental factor affecting development. Experimental manipulation of brood sizes can result in reduced offspring condition, indicating that conditions during development in enlarged broods have consequences within the affected generation.

But it is unclear whether stress during early development can have fitness consequences extending into the offspring of the next generation. To study such trans-generational fitness effects, a team of researchers from the University of Bielefeld, Germany, and the Museum of Natural Sciences in Madrid, report a breeding experiment with zebra finches (*Taeniopygia guttata*) in which mothers had been raised in different experimental brood sizes (Proceedings of the Royal Society, series B, published online).

The researchers, led by Marc Naguib, found that adult females were smaller as experimental brood sizes in which their mother had been raised increased. Hatching and fledging success of daughters decreased with increasing maternal brood size. These results illustrate that early developmental stress can have long-lasting effects on reproductive success of future generations.

"Such trans-generational effects can be life-history responses adapted to environmental conditions experienced in early life," the authors report.



**Line out:** New research suggests that stress faced by female zebra finches may influence the breeding success of their daughters. (Photo: Adam Jones/Science Photo Library.)

# Insect flight

Michael Dickinson

From Leonardo da Vinci to the Wright brothers, flight has inspired engineers more than any other form of animal behavior. Like any aircraft, an animal capable of active flight must possess three critical features: a light but powerful engine; wings capable of generating sufficient aerodynamic forces; and a control system to keep it from tumbling to the ground. The special properties of the muscles, wings, and brains that satisfy these requirements have made flying animals useful models in muscle biophysics, fluid mechanics, and neurobiology.

The purpose of this primer is to provide an overview of key principles in these three salient areas of flight biology, and is motivated by recent technical advances that are beginning to unravel many long-standing problems. This progress in our understanding of flight biology illustrates the utility of integrative methods, because many key insights have emerged, not simply from a focused analysis of individual elements, but also through more comprehensive approaches that link problems across disciplines. Although flight research embraces a wide variety of different organisms, from dragonflies to flying dragons, I will focus on insects in general, and flies in particular, because they have proven particularly amenable to these interdisciplinary approaches.

**The engine**
Miniaturization is the dominant theme in insect evolution, especially within the species-rich orders that include beetles, wasps and flies. This diversification was possible only because tiny insects evolved a remarkable muscle that is capable of generating high power at high frequency. Understanding the necessity of this peculiar motor starts with a consideration of scaling and aerodynamics. Whereas the lift generated by a

flapping wing scales to the fourth power of body length, an animal's weight scales to the third power. For this reason, small insects must flap their tiny wings faster to create sufficient force to offset gravity. This need for enhanced flapping frequency is even greater than predicted by scaling laws because air viscosity causes a gradual drop in the aerodynamic performance of small wings. Accordingly, the wingbeat frequency of hovering animals ranges from roughly 30 Hz in large hawk moths and hummingbirds to over 1000 Hz in tiny midges. However, the power output of conventional skeletal muscle deteriorates at frequencies well below those used by small, and even moderate-sized, insects. How, then, do these creatures manage to get off the ground?

Conventional skeletal muscle has little difficulty turning on; it is turning off that presents a problem. Contraction is regulated by $Ca^{2+}$, which when released from the sarcoplasmic reticulum (SR) in response to a motorneuron spike, binds to a troponin subunit on the thin filament, which in turn moves an associated protein, tropomyosin, to uncover the binding site where myosin can bind to actin. This entire process is fast because the electrochemical force driving $Ca^{2+}$ into the cytoplasm is enormous. In contrast, deactivation is a slow process because it requires the active pumping of $Ca^{2+}$ into the SR against its electrochemical gradient. Muscles powering an oscillating appendage, however, must deactivate quickly so that they are ready for the next cycle and do not resist the action of their antagonists during the reciprocal stroke. Very fast oscillatory muscles, such as those on rattlesnake rattles, have enormous amounts of SR, but the hypertrophy of SR comes at the expense of contractile filaments and mitochondria, creating a fundamental trade-off between deactivation speed and power. Many flying animals, including all bats and birds and some insects, use conventional twitch muscle to fly, but the cellular biophysics of contraction limits their flapping frequency and therefore their size.

At least four times within the evolutionary history of insects,

a new type of 'asynchronous' flight muscle emerged that can generate power at high frequency, thus permitting adaptive radiation into new niches and habitats. In principle, the solution is simple; get rid of the SR and fill the entire muscle volume with the stuff that counts — contractile filaments and mitochondria. In asynchronous flight muscle, actin–myosin binding is regulated mechanically rather than chemically. The term asynchronous comes from the fact that individual contractions are not correlated with pre-synaptic motor neurons spikes as they are in typical skeletal muscle. Rapid stretch, not depolarization-mediated $Ca^{2+}$ release, activates crossbridges to generate force, and rapid shortening de-activates crossbridges to relax the muscle. Because deactivation speed is not limited by diffusion, asynchronous muscle needs little SR. The motor neurons of asynchronous muscles fire continuously, but at a rate that is much lower than the contraction frequency. This low level of excitation is thought to maintain a tonic level of calcium that is sufficient to keep the crossbridges in a stretch-activate-able state. Recent evidence suggests, however, that by varying the spike rate of the asynchronous muscle, motorneurons can raise and lower the tonic calcium level to regulate power output as required for different flight maneuvers.

In addition to its peculiar physiology, asynchronous muscle has an odd anatomical arrangement. The entire exoskeleton of an insect is topologically an uninterrupted hollow sphere. Joints, including those attaching the wings to the body, consist of flexible rubbery sections surrounded by stiffer regions. Most insect muscle inserts directly onto invaginations of the exoskeleton called apodemes, which serve as tendons. In contrast, stretch-activated power muscles are classified as indirect because they insert broadly onto the walls of the thorax, not onto apodemes at the base of the wings. At the base of the wing a complicated hinge serves as a motion-amplifying gearbox to

transform the tiny strains imparted by the flight muscles into the sweeping motion of the wings. The back and forth motion of the wings is created by an orthogonal arrangement of two antagonist groups, dorso-longitudinal muscles (DLMs) and dorso-ventral muscles (DVMs) (Figure 1A). Contraction of the DLMs drives the wing forward and stretches the DVMs; this in turn activates the DVMs to drive the wings backward and stretches the DLMs to continue the self-sustaining cycle.

What is the molecular basis of stretch-activation? This question has general implications in muscle physiology because vertebrate heart muscle exhibits stretch activation, as does all skeletal muscle to a small degree. Stretch activation does not require the cell membrane; it is a feature intrinsic to the protein structure of the sarcomere. Somehow the extension of two adjacent Z-disks increases the net probability that myosin heads undergo a force-generating step. Many current hypotheses emphasize the role of a direct physical link between thin and thick filaments that would serve as a stretch 'sensor' to influence the probability of myosin binding. Suspects include myosin regulatory light chain, which spans the distance from thick and thin filaments adjacent to myosin heads, and projectin and kettin, molecules that tether the ends of the thick filaments to the Z-disk.

A new hypothesis is based on the discovery that asynchronous flight muscle has two isoforms of troponin C, a normal type (F2), and a peculiar but more abundant type that has lost one of its $Ca^{2+}$ binding sites (F1). By substituting troponin isoforms in skinned fibers, researchers have shown that F1 is necessary for stretch activation, but the F2 is not. One intriguing possibility is that the altered isoform still functions by moving tropomyosin away from target zones, but now responds mechanically to the tension along the thin filament imposed by stretch. Even if true, this mechanism is not mutually exclusive of many other current hypotheses. Further, given the likelihood of multiple
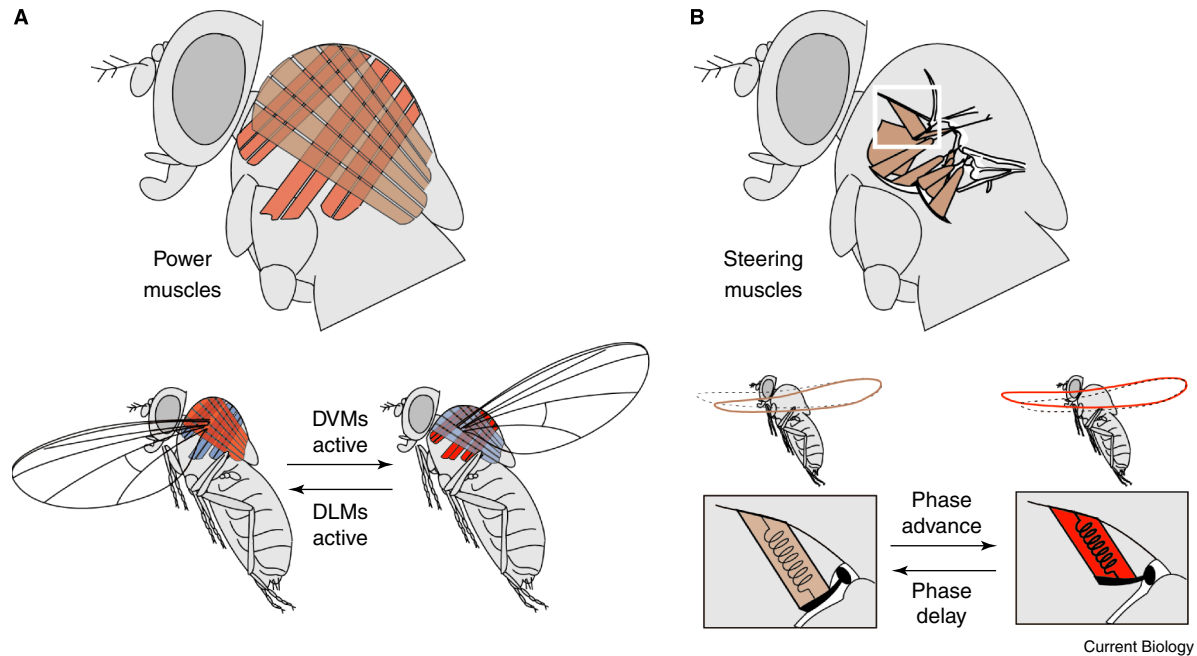
Figure 1. Functional organization of flight muscle in flies.

(A) Large asynchronous flight muscles provide the power required for flight. Antagonist sets of muscles oscillate to drive the wings back and forth via their indirect insertions on the sides of the thorax. (B) Tiny steering muscles alter wing motion through their actions on apodemes at the base of the wing. Some of the muscles serve as controllable springs whose stiffness is regulated by the firing phase of their motor neurons.

evolutionary origins, there may not be a single mechanism either within or across taxa. It will be both intriguing and informative to determine whether insects have made the leap to stretch activation the same way each time.

### Aerodynamics

The myth that engineers cannot explain the aerodynamics of insect flight persists despite an extensive amount of research to the contrary. This is unfortunate, because a cohesive theory of flapping flight has emerged from a collective effort in biology, physics and aeronautics. There are several mutually compatible ways of explaining the lift created by a conventional airfoil. Most simply, as a wing translates it diverts the oncoming air downward, and the resulting change in momentum of this air is equal to upward force acting on the wing. For conventional aircraft, this is most efficient at gentle angles of attack, under which conditions the stream of oncoming air separates at the leading edge of the wing, follows the contour of the wing surface, and rejoins smoothly near the trailing edge. This flow configuration is stable, and is thus well modeled by relatively simple

time-invariant models. Modern airfoils are designed with graceful contours so that the flow of air stays attached to the upper surface of the wing, rather than separating to form a turbulent wake that causes a precipitous drop in lift known as stall.

At first glance, insects appear to do everything wrong. First, although slightly corrugated for rigidity, their wings are flat and lack any streamlined shape. Second, they move their wings through the air at very large angles of attack, well above the threshold for flow separation and stall. What protects insects from the disastrous consequences of this flagrant disregard of sound aerodynamic design?

A critical concept in predicting the behavior of fluid is the Reynolds number (Re), a dimensionless quantity that is formally defined as the ratio of inertial to viscous forces. (From a physical perspective, both liquids and gases are considered fluids because the force required to push against them is proportional to how fast you push, not how far you push.) Each tiny volume of fluid has density and velocity and therefore also momentum,

and thus can exert an inertial force on adjacent volumes or an immersed solid such as a wing. For an object moving in a fluid, the Re is equal to $UL/\nu$, where $U$ is velocity, $L$ is a linear dimension and $\nu$ is the kinematic viscosity of the fluid. Values for insect wings fall in the range of so-called 'intermediate' Re ($10 < Re < 10,000$), where inertial forces still dominate, but viscous effects are large enough to exert a significant influence that qualitatively alters the flow relative to what would be expected for the high Re flows around an airplane wing. Although aerodynamic performance drops with decreasing Re, it may be the increased importance of viscosity that allows insects to exploit a peculiar aerodynamic mechanism that is not feasible for large aircraft.

With a few noteworthy exceptions, like dragonflies, most insects flap their wings back and forth, not up and down. In each stroke, the wings move propeller-like around their base before flipping over, reversing direction, and sweeping back in the opposite direction. As expected from the high angle of attack, flow separates at the sharp leading edge at the start of motion,
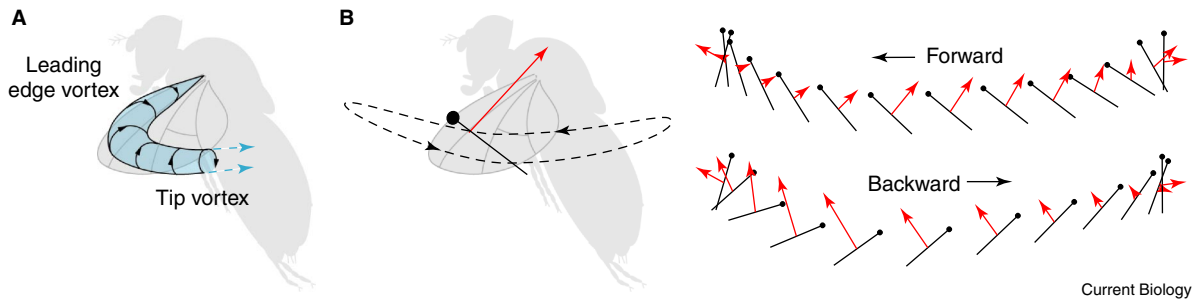
Figure 2. The aerodynamics of insect wings.

(A) Insect wings create a complex but stable flow pattern called a leading edge vortex (LEV) as they sweep propeller-like through the air. The tubular region of revolving flow is responsible for the high forces required for flight. At the wing tip, the LEV bends backward to form a tip vortex that traces the path of the wing during the stroke. (B) Most insects flap their wings back and forth in a 'u-shaped' pattern in a horizontal plane. The red vector shows the direction of total force, which acts perpendicular to the surface of the wing. Between strokes the wing rapidly flips over and reverses direction, creating additional forces and adopting the proper angle of attack for the next stroke.

forming a large swirling structure called a leading edge vortex (LEV) (Figure 2A). While attached to the top surface of the wing, the LEV creates a substantial suction force that acts perpendicular to the surface creating both elevated lift and drag. Translating wings also develop LEVs at high angles of attack, but they quickly grow unstable and are shed, resulting in stall. On the short rotating wings of insects, however, the leading edge vortex remains attached as the wing sweeps through the stroke. This stability is unexpected and means that the flow of air around an insect wing approaches a steady-state pattern, albeit one that is much more complicated than on an airplane wing. As the wing rotates, its leading edge creates vorticity (basically, vorticity is a measure of local fluid rotation), which rolls up into the LEV. To reach the observed equilibrium, this source of vorticity must be balanced by a sink, i.e. transport of vorticity out of the vortex.

An important topic in current research concerns the physical basis of this transport and why it perfectly balances the creation of vorticity during propeller-like motion. Evidence from large insects such as hawk moths (operating at a Re of about 5000) suggested that a tip-to-base flow within the core of the vortex is responsible for this transport, in analogy with a mechanism known to operate on swept wing aircraft such as the Concorde. The explanation is not universally sufficient, however, because wings revolving at lower Re create stable

LEVs in the absence of outward flow within the vortex core. Other possible pumps for transporting vorticity include the influence of the tip vortex — the rearward extension of the LEV after it peels off the wing surface, and the centrifugal forces acting on revolving objects.

If creating a leading edge vortex is such a clever trick, why don't engineers build flapping airplanes? For one thing, the ability for a propeller to create a stable LEV appears to depend on Re, and the increasing influence of turbulence and other factors interferes with the delicate balance. In addition, there is nothing particularly efficient about the insect's strategy. At high angles of attack, the mean force created by a wing is perpendicular to its surface. This means that at an angle of 45° a wing creates as much drag as lift. Insects would be better off using a much lower angle of attack, forgoing the LEV altogether, but to create sufficient force they would need to flap their wings very quickly, well beyond the physiological capability of even stretch-activated muscle. Thus, insects appear to be making the best of a bad situation, living with the energetic consequences of high drag and high flight costs, but living within the constraint space of biological motors.

Although the formation of the LEV by propeller-like motion is the most important means by which flapping wings create forces, it is not the only way. Unlike propellers, insect wings reverse direction twice each stroke as they flap back and forth. During stroke reversal, the wings flip around their long axis

in order to adopt an appropriate angle of attack for the next stroke (Figure 2B). As a consequence, the wings function 'upside-down' during the backward stroke. The rapid rotation causes additional shear at the leading edge that can transiently increase (or decrease) the size of the LEV.

Another consequence of the back-and-forth pattern of motion stroke is that the wings start from rest and thus must rapidly accelerate at the beginning of each stroke. As a consequence, the wing encounters so-called 'acceleration-reaction' or 'added mass' force as it accelerates the air in its path. The situation is further complicated by the fact that the wings do not start each stroke by moving through still air, but rather encounter the quite complicated wake of the previous stroke, which can alter force production just as if the wings encountered a brief gust. All these effects make the forces created during stroke reversal quite complicated and extremely sensitive to small changes in wing motion.

Whereas the steady forces created by the LEV dominate on sweeping, propeller-like strokes, the complicated time-dependent stroke reversal forces become increasingly significant in brief short strokes such as those used by honeybees and their kin. Stroke reversal forces are also likely to play a particularly important role in flight control. Because they are created when the wing is far in front or far behind the animal's center of mass, they contribute disproportionately to pitching

moments that, if not well-balanced, would send the animal spinning antennae-over-abdomen. The role of aerodynamic forces is not simply to offset body weight, but also to provide the forces and moments that maintain the proper flight speed and orientation. As discussed in the next section, a critical feature of the animal's brain is its ability to detect perturbations and make quick, subtle adjustments to wing motions and force production.

## Control

The very specializations that enable the asynchronous power muscle to generate mechanical power at high frequency render them ill-suited for regulating wing motion. They are indirectly attached to the wing and only loosely controlled by the nervous system. However, insects using asynchronous power muscles also have a complimentary set of 12 or so flight control muscles that insert directly onto apodemes at the base of the wing (Figure 1B). These tiny muscles have a conventional twitch-type physiology, in which each contraction is controlled by a motorneuron spike. Incapable of generating large forces or high power because of their extensive SR, these tiny steering muscles act to adjust the gearing of the wing hinge, thus determining how the mechanical strain generated by the indirect flight muscles is transformed into wing motion. In controlling the wing hinges, the steering muscles act, not as motors, but rather as variable-stiffness springs. Although this goes against the classic image of muscles as motors, recent comparative work in many animals suggests that, in addition to their role as force generating motors, muscles can perform many different mechanical tasks within an animal, including roles as struts, brakes, and springs.

How does the fly's nervous system control the stiffness of the steering muscles? The fly cannot rely on motor unit recruitment or spike frequency, as most vertebrates would, because each muscle is innervated by a single motor neuron, which due to the high wing beat frequency, can fire no more than a single spike
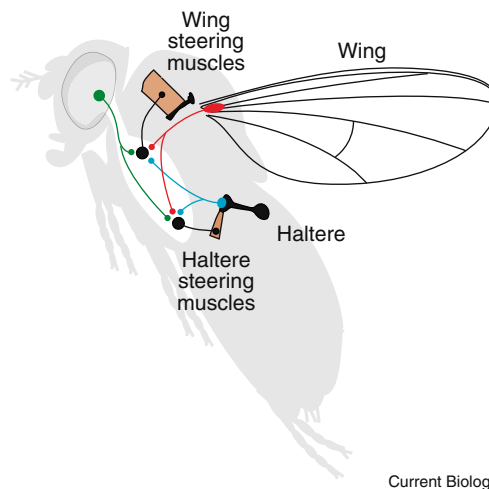


Figure 3. Simplified cartoon of sensory motor pathways used in flight control.

Mechanoreceptors on the wing (red) and haltere (blue) make direct connections with steering muscles of the wing and haltere. Descending visual information (green) converges on steering motor neurons, but the means by which this information is integrated with wing and haltere sensors is not well understood.

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within each stroke. Thus, the fly's nervous system is limited to just two control parameters: whether a particular muscle fires in a given wing stroke and the phase at which it does. Single spikes in quiescent muscle cause large transient changes, whereas phase changes in a continuously active muscle cause graded changes in muscle stiffness, hinge gearing, and thus wing motion. This phase-based motor code is one of the reasons that the fly can generate such subtle and impressive flight maneuvers with so few motor neurons. The ability to create subtle changes is essential, because the animal's body dynamics are such that small changes in flight force have large effects on body motion.

The fact that the motor system employs a phase code places important constraints on the neural circuits that process sensory information, because ultimately the command signals to steering motorneurons must be carefully synchronized with the wingbeat (Figure 3). This sensory-to-motor transformation is relatively simple for a fast inner control loop that maintains flight stability. Mechanosensors at the base of the wing and the halteres (the modified hind wings of flies) provide reflexive cycle-by-cycle input to steering motorneurons, thereby entraining them to specific phases of the wingbeat. The tiny halteres are subject to Coriolis forces that deflect them from their back-and-forth motion when the body rotates during flight. Specialized sensors encode this deflection and provide

strong monosynaptic drive that is capable of entraining steering muscles to different phases in the stroke cycle, thereby affecting compensatory changes in wing motion that counter the imposed rotation. The robustness of this relatively simple feedback loop is responsible for the remarkable stability and maneuverability of flies, which can sustain large losses in wing area or the temporary lack of visual input without crashing. Ablation of the halteres, however, causes an immediate catastrophic failure, which indicates the importance of these structures in active flight control.

The translation of sensory signals into the phase code of the motor system is more complicated for the outer control loop that uses visual and olfactory information to guide the animal towards particular features in the environment. The visual and olfactory systems are intrinsically slow due to biochemical transduction cascades and subsequent neural processing. Somehow, these slow signals must be fused with rapid mechanosensory input from the wings and halteres to create a command code that can advance and delay the firing phase of steering motorneurons. Where and how this fusion takes place is unknown, but one intriguing feature of the system is that halteres possess their own set of tiny steering muscles whose motor neurons receive input from the visual system. By altering the motion of the haltere, the visual system might shift the phase of the

feedback from the haltere to wing steering muscles.

## Conclusions

The important message that emerges from a consideration of insect flight is that the peculiar specializations that make flight possible make much more sense when viewed within the context of the system as a whole. Simple scaling laws and the loss of aerodynamic performance at low Reynolds numbers require that small insects flap at high frequency, a physical constraint that drove the evolution of a mechanically gated flight muscle. This, in turn, required the development of a separate steering motor system, which operates using a phase code, thus constraining the final protocol that descending sensory commands must use to guide the animal through its environment. This organization offers us insight into how a complex biological organism works as an intact system. Fortunately, many challenging problems remain, otherwise engineers and toy makers would have already littered the world with tiny mechanical flies.

### Further reading

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## Correspondences

# An ancient Fox gene cluster in bilaterian animals

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Homeobox genes, such as the Hox, Parahox and Nkx genes, are examples of conserved developmental regulatory genes. They are arranged into clusters that have been conserved over hundreds of millions of years of animal evolution [1–3]. Ancient clustering has also been suggested for the Wnt genes [4] but not for other transcription factor genes. Here, we focus on the evolution of Fox genes, which encode winged helix transcription factors with roles in metabolism, development and disease [5,6]. We demonstrate that four genes encoding Fox transcription factors are linked in insects and chordates and were most likely arranged into a gene cluster in basal bilaterians. These genes also show conserved expression in developing endo-mesodermal tissues.

Over 40 Fox genes have been annotated in the human genome, however most of them are dispersed [5,6]. Two exceptions are found at the chromosomal locations 16q24.3 and 6p25. At the former *FOXL1*, *FOXC1* and *FOXF2* are found within 70 kb, while at the latter *FOXC2*, *FOXF1* and *FOXQ1* are found within 325 kb (see Supplemental Data published with this article online; [6]). The presence of *FOXC* and *FOXF* paralogues in both clusters suggests that the current arrangement has evolved by block duplication of a single gene cluster containing four distinct Fox genes in linear array. To establish when these clusters evolved, we first assessed the Fox gene complement of the invertebrate chordate amphioxus — a representative of a lineage believed to have

diverged prior to the genome duplications that mark early vertebrate evolution. We identified single amphioxus orthologues of *FOXL1*, *FoxC*, *FoxF* and *FOXQ1*, and used mapping strategies coupled with chromosomal FISH to demonstrate that they are chromosomally linked (Supplemental Data). This demonstrates that the block duplication was confined to the vertebrate lineage and dates the four gene cluster to before the amphioxus and vertebrate lineages split, over 500 million years ago.

Next, we searched available sequence from other invertebrates for orthologues of all four Fox genes. Previous studies have suggested the presence of *FoxF* and *FoxC* genes in insects, and *FoxF*, *FoxC* and *FoxQ1* genes in urochordates [6,7]. Our analyses also identified distinct *FoxL1* genes in insects and echinoderms, and *FoxF* and *FoxC* genes in a lophotrochozoan, the flatworm *Schmidtea mediterranea* (Figure 1; also see Supplemental Data). We examined the positions of these genes in the genomes that have been subject to whole-genome sequencing and assembly. In the urochordate *Ciona intestinalis*, the three genes are on separate genome scaffolds and thus do not appear to be closely linked.

In *Caenorhabditis elegans*, only *FoxF* has been identified. In drosophilid genomes, all three genes are on the same chromosome, but separated by several megabases. In the mosquito *Anopheles gambiae*, we found close linkage between *FoxC* and *FoxF*, with approximately 45 kb separating these two genes, with *FoxL1* some 14 Mb distant. In the honeybee *Apis melifera*, we found *FoxF*, *FoxC* and *FoxL1* within approximately 140 kb.

These observations show that *FoxF*, *FoxC* and *FoxL1* evolved as distinct genes before the radiation of the bilaterian phyla, as they are found in both protostomes and deuterostomes. The linkage of *FoxQ1*, *FoxF*, *FoxC* and *FoxL1*