Original Article

**Generation of animal form by the Chordin/Tolloid/BMP gradient: 100 years after D’Arcy Thompson**

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The classic book “On Growth and Form” by naturalist D’Arcy Thompson was published 100 years ago. To celebrate this landmark, we present experiments in the *Xenopus* embryo that provide a framework for understanding how simple, quantitative transformations of a morphogen gradient might have affected evolution and morphological diversity of organisms. D’Arcy Thompson proposed that different morphologies might be generated by modifying physical parameters in an underlying system of Cartesian coordinates that pre-existed in Nature and arose during evolutionary history. Chordin is a BMP antagonist secreted by the Spemann organizer located on the dorsal side of the gastrula. Chordin generates a morphogen gradient as first proposed by mathematician Alan Turing. The rate-limiting step of this dorsal–ventral (D-V) morphogen is the degradation of Chordin by the Tolloid metalloproteinase in the ventral side. Chordin is expressed at gastrula on the dorsal side where BMP signaling is low, while at the opposite side peak levels of BMP signaling are reached. In fishes, amphibians, reptiles and birds, high BMP signaling in the ventral region induces transcription of a secreted inhibitor of Tolloid called Sizzled. By depleting Sizzled exclusively in the ventral half of the embryo we were able to expand the ventro-posterior region in an otherwise normal embryo. Conversely, ventral depletion of Tolloid, which stabilizes Chordin, decreased ventral and tail structures, phenocopying the *tolloid* zebrafish mutation. We explain how historical constraints recorded in the language of DNA become subject to the universal laws of physics when an ancestral reaction-diffusion morphogen gradient dictates form.

Key words: dorsal-ventral patterning, evolution of body form, morphogen gradient, morphogenesis, reaction-diffusion.

Introduction

One hundred years ago, in 1917, D’Arcy Thompson published a very influential book entitled “On Growth and Form”. He proposed that in order to understand the evolution of the myriad animal forms a more mathematical approach would be required which took into account physical and chemical aspects of morphogenesis. For D’Arcy Thompson, the evolution of body shapes was not solely explicable by pure “functionalist” random variation followed by natural selection and survival of the fittest leading to ever increasing optimization (Darwin 1859). He suggested that morphology should also consider underlying constraints that are imposed by the laws of physics and mathematics. The idea that natural selection was insufficient to completely explain animal form perdured in “structuralist” theories, which incorporated historical constraints that channeled evolution into the framework of Darwinism (Gould 2002). Thompson’s treatise was concerned with the geometry of corals, the logarithmic spiral of molluscan shells and ruminant horns, the diversity of foraminiferan shells and other geometric forms amenable to mathematical explanations. But by far the most striking was his theory of transformed coordinates.

The most famous of his illustrations was the case of the ocean sunfish *Orthogoriscus mola* (now called *Mola mola*) in which the posterior part of the body is dramatically expanded and truncated (see Fig. 1 below). The sunfish resembles close relatives (porcupine fish of the genus *Diodon*) from which it must have
evolved not so long ago, yet the form of the body was reshaped in a surprisingly harmonious manner. D’Arcy Thompson proposed that this change in morphology could have involved adjustments in an imaginary underlying system of Cartesian coordinates in which changes in mathematical parameters could change the form of the body. Of course, the mechanisms that coordinate these morphological changes were entirely unknown.

In this study, we present experiments on depleting Tolloid and Sizzled in the ventral side of the embryo. They illustrate, in a simple way, D’Arcy Thompson’s proposal that structural transformations between related species could reveal underlying physical and chemical mechanisms that might be interpreted mathematically, in this case through the application of the universal law of diffusion to the Chordin morphogen. In particular, our experiments specifically reduce expression of Sizzled and Tolloid in the ventral half of the embryo, preserving anterior development while modifying posterior body morphogenesis to mimic the transformations of coordinates imagined by D’Arcy Thompson. The results support the idea that morphological diversity among animals could be achieved by simple changes in the parameters of morphogen gradient dynamics.

Materials and methods

Embryo manipulations

*Xenopus laevis* eggs were collected in 1× High Salt solution (0.12 mol/L NaCl, 2.4 mmol/L KCl, 2.5 mmol/L MgCl₂, 3.1 mmol/L CaCl₂, 1.8 mmol/L HEPES, adjusted to pH 7.6 with NaOH) in a bacteriological plastic Petri dish, and saline solution removed as much as possible. Eggs were fertilized *in vitro* using 300 µL of sperm suspension obtained from freshly dispersed testis fragments in 1× MMR (Marc’s Modified Ringers: 0.1 mol/L NaCl, 2.0 mmol/L KCl, 2 mmol/L MgCl₂, 2.7 mmol/L CaCl₂, 5 mmol/L HEPES, pH 7.4) and allowed to adsorb for 5 min, after which 0.1× MMR was added gradually. After one hour, eggs were dejellied in 2% Cysteine solution in 0.1× MMR adjusted to pH 7.8 with NaOH. At four-cell stage, embryos with regular D-V cleavage in which the two dorsal blastomeres were lightly pigmented and the ventral ones dark, were selected (Klein 1987). These were microinjected into two ventral blastomeres with various MOs.

Antisense morpholinos

Antisense morpholinos are an effective way of depleting specific gene products in *Xenopus* embryos (Blum et al. 2015). The *sizzled* morpholino sequence was 5′-GAGGAGCAGGAAGACTCCGGTCATG-3′ as described previously by others (Collavin & Kirschner 2003). *Xenopus laevis* has 3 Tolloid metalloproteases: BMP1 (also known as Procollagen C-Peptidase in mammals), Xolloid-related (Xlr, called Tolloid-like 1 in mouse and Tolloid in zebrafish) and Xolloid (designated Tolloid-like 2 in mouse) (Goodman et al. 1998; Dale et al. 2002). The sequence of the Tolloid metalloprotease antisense morpholinos used in this study were: BMP1 5′-CAGCCTGTGGGATATCATTGTGTCC-3′ and Xlr 5′-AGATGAGCCAAGAAAGCATGTTCAT-3′ (Inomata et al. 2008), and Xolloid 5′-CAGCTCATTGCCGGCACGAGGT-3′ (this study). MOs from GeneTools LLC were dissolved in sterile water to 1 mmol/L concentration to generate stock solutions. Working dilutions were prepared by diluting the Morpholino solution in nuclease-free water, followed by denaturation at 65°C for 5 min, cooled down to room temperature, and centrifuged for 5 min at 15.7 × 1000 g. MOs were usually injected at 8 ng per blastomere, although in some experiments 6 and 4 ng were used to show dose dependency. Embryos were cultured to the desired stages in 0.3× MMR, scored for phenotype, and fixed in MEMFA (0.1 mol/L MOPS, pH 7.4, 1 mmol/L EGTA, 2 mmol/L MgSO₄, 3.7% formaldehyde) before photography.

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Results

Background on the Chordin/Tolloid/BMP pathway

We start by providing necessary background information on the Chordin/Tolloid/BMP biochemical pathway. Chordin is a bone morphogenetic protein (BMP) antagonist that is expressed in the dorsal region of the embryo called the Spemann organizer. Chordin was cloned by Yoshiki Sasai only one month after arriving at UCLA as a postdoctoral fellow (Sasai et al. 1994). Sasai was a very accomplished molecular biologist who had previously cloned the Notch downstream gene HES-1 with Shigetada Nakanishi, and would then go on to coax stem cells into making brains, eyes and pituitary glands (De Robertis 2014). Chordin is expressed in very high amounts in the Xenopus gastrula. If present uniformly in the extracellular space Chordin would reach a concentration of 33 nmol/L (Lee et al. 2006), while the BMPs it inhibits are expressed at much lower amounts in the pico molar range. However, Chordin is not uniform and is secreted into the extracellular matrix separating the ectoderm from the endomesoderm, where it forms a smooth long-range gradient over a distance of 2 mm (Plouhinec et al. 2013). From this strategic location Chordin is able to pattern multiple germ layers with a single gradient as cells undergo the morphogenetic movements of gastrulation.

Chordin is part of a self-organizing network of secreted proteins that bind to each other in the extracellular space (De Robertis & Moriyama 2016). As shown in Figure 2, the Chordin biochemical pathway comprises the entire embryo. On the ventral side, which we call the ventral center, BMP4 and BMP7 are expressed, while on the dorsal side two other BMPs are expressed, ADMP and BMP2. The dorsal BMPs, however, are unable to signal due to inhibition by Chordin and other BMP antagonists. The rate-limiting step in this biochemical pathway is provided by a zinc metalloproteinase called Tolloid which we found, in a collaboration with Leslie Dale, could cleave Chordin at two very specific sites, releasing active BMPs (Piccolo et al. 1997) from previously inactive Chordin-BMP complexes (Piccolo et al. 1996). There are three Tolloids in Xenopus and other vertebrates, of which the highest expressed at gastrula is Xolloid-related (Xlr). Known as Tolloid-like-1 in mouse and Tolloid in zebrafish, Xlr is expressed at highest levels in the ventral center where its transcription is activated by BMP4 (Dale et al. 2002) (Fig. 2). The Chordin gradient is maintained by the degradation of Chordin by Tolloid, so that when Tolloid activity is depleted by antisense morpholinos (MO) in the whole embryo, Chordin levels rise and the gradient extends into the ventral side (Plouhinec et al. 2013). In other words, Tolloid acts as a sink for Chordin, promoting peak BMP signaling on the ventral side. Crossveinless-2 (CV2) is a molecule with similarities to Chordin expressed on the ventral center that is unable to diffuse yet binds with high affinity to Chordin-BMP complexes, concentrating them in the ventral center for cleavage by Tolloid and the release of BMPs (Ambrosio et al. 2008; Serpe et al. 2008).

Ventral center genes are turned on transcriptionally by high BMP signaling and are part of the BMP4 syn-expression group (Niehrs & Pollet 1999). Sizzled is a
BMP4 target gene (Collavin & Kirschner 2003) that, like Chordin, is secreted at very high amounts (reaching 30 nmol/L in the extracellular space if it were uniform) (Lee et al. 2006). Sizzled is an sFRP (secreted frizzled related protein) that lost its ability to bind Wnt and functions as a dedicated competitive inhibitor of Tolloid (Lee et al. 2006; Muraoka et al. 2006). Tolloid binds Sizzled but is unable to cleave it. On the dorsal side, Crescent serves a similar function to that of Sizzled, inhibiting Tolloid (Ploper et al. 2011) (Fig. 2).

The dorsal and ventral centers secrete proteins of similar structure and function, for example, Chordin and CV2; BMP2/ADMP and BMP4/BMP7; Crescent and Sizzled. These feedback loops result in a self-regulating system as ventral center genes are transcribed at high BMP signaling levels and dorsal genes at low BMP levels (Reversade & De Robertis 2005) (Fig. 2). For every action on the dorsal side there is a reaction on the ventral side, resulting in a constant BMP signaling gradient during gastrulation, even as the blastopore becomes smaller during the movements of epiboly that enclose the yolk (Little & Mullins 2006; De Robertis & Moriyama 2016).

There are many other important components in the Spemann organizer that participate in embryonic patterning, such as the BMP antagonists Noggin (Smith & Harland 1992) and Follistatin (Hemmatti-Brivanlou et al. 1994; Fainsod et al. 1997; Iemura et al. 1998), the Wnt antagonists Frzb (Leyns et al. 1997) and Dickkopf-1 (Glinka et al. 1998), and the multiple inhibitor Cerberus (Bouwmeester et al. 1996). RNA-seq analyses have revealed many more D-V genes (Ding et al. 2017a,b). These genes were not addressed here because the present work is concerned with the Chordin morphogenetic pathway.

**Targeted sizzled and Tolloid depletion in the ventral side**

To address whether modifications of a morphogen gradient could elicit transformations in the body plan of the *Xenopus* embryo, we performed ventrally targeted morpholino-mediated knockdown experiments. In particular, we focused on downregulating two key components of the Chordin long-range morphogenetic pathway: Sizzled and Tolloid. Reducing the levels of these proteins has opposite effects on Chordin. Sizzled MO reduces Chordin protein levels and increases BMP signaling indirectly through an increase in Tolloid enzyme activity (Lee et al. 2006; Inomata et al. 2013). Tolloid depletion promotes Chordin stabilization and decreases BMP signaling (Plohinec et al. 2013). To limit the phenotypes to the posterior part of the embryo, the two ventral blastomeres were exclusively targeted by MO injections at the four-cell stage (Fig. 3A). This prevented the generalized ventralization or dorsalization that occurred when the entire embryo is microinjected (Collavin & Kirschner 2003; Lee et al. 2006). Targeted depletion of Sizzled promoted an enlargement of the ventro-posterior part of the embryo, while leaving the most anterior structures such as the head, eyes and trunk largely unaffected and similar in size to those of control siblings (Fig. 3B, C). Specifically, we observed that injected embryos displayed an expanded posterior region, especially in proximity to the blastopore (Fig. 3C). Embryos from at least five independent experiments were analyzed, showing a phenotypic penetrance of 94% ($n = 117$). Embryos died off gradually, with survivors reaching the stage shown in Figure 3C, which is analogous to D’Arcy Thomson’s sunfish transformation (Fig. 1).

To deplete Tolloid metalloproteinase, we microinjected a combination of two (Xr/BMP1) or three (Xr/ BMP1/Xolloid) morpholinos into the two ventral blastomeres of four-cell stage *Xenopus* embryos (Fig. 4A). Both morpholino combinations produced similar results in terms of phenotype and penetrance. Despite showing a slight dorsalization at early stages, the dorso-anterior structures of injected embryos recovered and developed into well-proportioned head and trunk structures comparable to those of uninjected siblings (Fig. 4B–E). However, posterior development was profoundly affected and this was mostly evident at tailbud stages (stage 34 and later). Unlike wild-type embryos, the anus of Tolloid knockdown embryos was enlarged (“flared”) and displaced to a more posterior location closer to the tailbud (Fig. 4C, E). The tail was much shorter than in controls, and presented reduced ventral fin formation (Fig. 4C). In the most strongly affected cases, a few embryos with posterior truncation were observed (Fig. 4E). The observed phenotypes showed a range of severity, with most of the embryos having the milder phenotype shown in Figure 4C, which were observed in five independent experiments in at least 66% ($n = 121$) of the injected embryos that reached tailbud stage 34. The phenotype was reminiscent of zebrafish *minifin* mutant, in which the affected locus encodes the *tolloid* gene (the homologue of Xr) (Connors et al. 1999). In the strongest *minifin* mutant phenotypes, ventral fin development is completely lost, leading to adult fishes lacking the tail fin (Fig. 4F–G) (Connors et al. 1999). In conclusion, the experiments show that targeted perturbations in the Chordin/Tolloid/BMP pathway induce coordinated transformations in the posterior body form of a vertebrate animal, and provide a mechanistic interpretation for the quantitative transformations of D’Arcy Thompson. For example, one would only need lower activity
of a ventral enhancer of Sizzled, decreasing its expression levels, to transform body form into a shape resembling a sunfish. These results are discussed in the context of animal evolution in Figures 5–7 (see below).

Discussion

D’Arcy Thompson 100 years later

The experiments presented here provide a proof of concept that modifying the gradient of Chordin/Tolloid/BMP can change body form in the Xenopus embryo along the lines of the transformed coordinates imagined by D’Arcy Thompson (1917). Evolution between closely related species may have proceeded along orderly lines by tweaking a long-distance morphogenetic gradient. This was achieved here by depletion of the ventral center signaling molecules Sizzled and Tolloid, which have opposite activities, specifically by microinjecting the two ventral blastomeres at the four-cell stage. By limiting the treatment to the ventral half of the embryo, the development of the anterior half including the head was kept relatively normal. Sizzled depletion results in higher Tolloid activity, less Chordin and more ventral-posterior BMP signaling (Plouhinec et al. 2013). This explains the expansion of ventral tissues observed (Fig. 3), which is reminiscent of the transformed Cartesian coordinates shown for the sunfish in Figure 1. The opposite phenotype, reduced tailbud and ventral-posterior structures, was obtained when the three (or just two) of the Xenopus Tolloids were depleted. When Tolloid is depleted, more Chordin is present and this leads to less BMP signaling (Plouhinec et al. 2013).

“On Growth and Form”, a treatise written in magnificent Victorian prose, left a lasting impact in biology for those yearning for a more mathematical interpretation of the generation of form, i.e., morphogenesis (Meinhardt 1998; Niehrs 2010). A Theoretical Biology Club was formed at Cambridge University by biology luminaries such as Conrad Waddington, Joseph Needham and J. B. S. Haldane in an attempt to bring the laws of physics into biology (Ball 2015). However, the most productive insight was to come from brilliant mathematician Alan Turing.

Turing’s morphogens and diffusion-reaction chemical reactions

Alan Turing proposed a simple yet powerful theory to explain biological form in 1952. He suggested that anatomical structure might result from the diffusion of hypothetical substances that he designated morphogens (Turing 1952). He took the complex embryonic system and rendered it much simpler, in the mold of D’Arcy Thompson’s book (one of only six citations in his 35-page paper). He proposed that universal physical and chemical laws were able to explain many of the facts of morphogenesis. He imagined that a system of chemical substances capable of reacting with each other and diffusing through a tissue would
be able to generate a pattern (Turing 1952). “The systems actually to be considered consist therefore of masses that are not growing, but within which certain substances are reacting chemically, and through which they are diffusing. These substances will be called morphogens, the word being intended to convey the idea of a form producer.” The D-V morphogenetic biochemical pathway discovered in the frog *Xenopus* does indeed contain many interacting secreted protein molecules, which are able to diffuse over long distances in the embryo (Fig. 2).

The reactions between morphogens depend on their concentration (law of mass action) and on their diffusion (Fick’s law of diffusion in fluid medium). Turing formulated a general partial differential equation to describe quantitatively the changes in concentration of a morphogen (C) over time (\(\frac{\partial C}{\partial t}\)), shown in Figure 5. The right side of the equation describes that, following Fick’s law of diffusion, the change in concentration of the morphogen (C) over time is proportional to its diffusion rate (D) and to the second derivative in space of the morphogen concentration (\(\nabla^2 C\)). In addition, the change in morphogen concentration is also a function (F) of all the chemical reactions it undergoes (such as synthesis, degradation, and association and dissociation with other proteins such as antagonists). From this initial insight, a large number of “reaction-diffusion” computer models to explain the behavior of developing systems have been derived (Meinhardt 1998, 2008; Lee et al. 2009; Genikhovich et al. 2015).

A second important advance was the realization by Gierer and Meinhardt (1972) that in theory a pair of morphogens composed of an Activator and an Inhibitor originating from the same cellular source could generate stable patterns. If the Activator turns on its own production as well as the synthesis of an Inhibitor that interacts with the Activator, a field of cells can be patterned into two different zones, provided the inhibitor diffuses faster than the activator. The Activator and Inhibitor are produced and secreted near the source,
Reaction-diffusion equations define how morphogen concentration \( C \) changes over time \( t \):

\[
\frac{\partial C}{\partial t} = D \cdot \nabla^2 C + F(C)
\]

Fick’s law of diffusion
\( D \) = Diffusion rate
\( \nabla^2 = 2^{nd} \) derivative in space

Function \( F \) describing all chemical reactions of a component of the morphogen gradient: synthesis, degradation, association/dissociation with inhibitors

**Fig. 5.** Example of a partial differential equation of the type formulated by Alan Turing in 1952 to describe the changes in morphogen concentration \( \partial C \) over time \( \partial t \). The right side of the equation shows that, following Fick’s law of diffusion, the change in concentration of a morphogen at any given point is proportional to its diffusion rate and to the second derivative in space of morphogen concentration. In addition, the change in morphogen concentration is also a function \( F \) of all the chemical reactions it undergoes. This insight brought physics, chemistry and mathematics into developmental biology.

while the activator is turned off by a preponderance of inhibitor in the periphery. Tolloid and Sizzled constitute an example of an Activator and Inhibitor pair that interact with each other and are secreted by the same cells (in which BMP signaling is high, Fig. 2). In the ventral center, Tolloid (Xolloid-related in Xenopus) functions as the Activator (increasing BMP signaling by cleaving Chordin), and Sizzled as the Inhibitor (decreasing BMP signaling by stabilizing Chordin through inhibition of Tolloid).

It is remarkable that mathematicians could provide a theoretical framework for understanding long-range diffusion-reactions of morphogens at a time when the chemical nature of not even a single morphogen was known.

**Conserved patterning systems and evolutionary constraints**

The Chordin/Tolloid/BMP pathway patterns the gastrula of many organisms. Shortly after publication of Xenopus Chordin its homologue short-gastrulation (Sog) was cloned in *Drosophila* by the group of Ethan Bier (Francois et al. 1994). Overexpressed Chordin and Sog were found to have similar functions (Holley et al. 1995; Sasai et al. 1995). Sog was known to interact genetically with *Dpp* (Decapentaplegic, the *Drosophila* homologue of BMP4 and BMP2) (Ferguson...
et al. (1992), prompting the discovery that Chordin/Sog proteins were BMP/Dpp antagonists (Sasai & Anderson 1992), prompting the discovery that Chordin/Tolloid/BMP gradient patterned the D-V axis in the sea anemone Nematostella vectensis which is a cnidarian diploblast that predates bilateral animals (Fig. 6). Nematostella embryos have a phase of bilateral symmetry called the directive axis. As in the case of Xenopus, Nematostella Chordin and BMPs are expressed on the side where BMP signaling is low, and another BMP (called GDF-5) on the side where it is high (Genikhovich et al. 2015). Chordin depletion inhibits peak BMP signaling levels, indicating that its function is to establish a flux of BMPs away from the Chordin source that signal when released by Tolloid on the opposite side, as is the case in Xenopus and Drosophila embryos (Mizutani et al. 2005; Wang & Ferguson 2005; Umulis et al. 2010; Bier & De Robertis 2015). Mathematical reaction-diffusion modeling indicates that Chordin (and Tolloid) are the rate-limiting elements in maintaining a stable BMP gradient, both in Nematostella and Xenopus (Genikhovich et al. 2015). Although cnidarians and bilateral animals diverged an estimated 650 million years ago (Kusserow et al. 2005), they already had in place the Chordin body patterning system.

The urbilaterian ancestor

The discovery of Chordin and Sog revived the old problem of whether a unity of plan existed in animal anatomy. Forty years before Darwin, in 1822, French naturalist Etienne Geoffroy Saint-Hilaire proposed that the ventral side of arthropods was homologous to the dorsal side of vertebrates (Appel 1987). With Yoshiki Sasai we realized that the expression and function of Sog and Chordin on the side where the central nervous system (CNS) is induced (ventral in the fly, dorsal in the vertebrate) greatly supported this view (De Robertis & Sasai 1996). Previously it had been noted that Dpp and BMP4 were expressed on opposite sides of the embryo and that a phenotype of dorsalization could be obtained when BMP signaling was blocked with noggin morpholinos. The importance of the Chordin/Tolloid/BMP is indicated by extensive genetic screens in zebrafish, which identified seven zygotic mutations that affected D-V patterning. Five dorsalized (low BMP) mutants were defective in BMP2 (swirh), BMP7 (snailhouse), the BMP receptor Alk8 (lost-a-fin), Smad5 (somitabun), and Tolloid (mini-fin) (Schier & Talbot 2005; Little & Mullins 2006). Of particular interest to this study is mini-fin (Connors et al. 1999) which is homologous to Xenopus Xfr and was phenocopied by ventral injection of morpholinos in Figure 4. Only two ventralized (high BMP) mutants were found, which affected Chordin (chordino) and Sizzled (ogon/mercedes) (Schulte-Merker et al. 1997; Yabe et al. 2003). These are the same two genes that are so abundantly expressed in Xenopus (Lee et al. 2006).

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seven genes that patterned its antero–Urbilateria studies by other groups led to the conclusion that called HoxC-6 (Carrasco et al. 2001), in addition to the Chordin/Tolloid/BMP and Hox gene systems, Urbilateria expressed otx in the head, pax6 in eyes, tinman and dmef2 in contractile blood vessels, distal-less in appendages and cetera. Furthermore, ancient cell types and tissues present in the ancestor can be identified by the co-expression of unique combinations of transcription factors and microRNAs (Arendt 2008). The common ancestor of invertebrates and vertebrates gave rise to an immense variety of animal forms by the differential use of a conserved ancestral genetic toolkit (De Robertis 2008).

It is very likely that Urbilateria had a complex life cycle in its marine environment (Fig. 7). There are 17 animal phyla that have pelagic (planktonic) larval forms that favor dispersal of the species and benthic (bottom dwelling) adult forms (Jagersten 1972). This includes deuterostomes such as sea cucumbers and hemichordates. As proposed by Jagersten, extant metazoans likely evolved from ancestors with a complex pelagobenthic life cycle. Larval marine forms were readily lost during evolution (sometimes even within the same phylum), with animals developing directly into adult forms specialized for particular environments, at the expense of wider planktonic dispersal (Jagersten 1972). Marine larvae of deuterostomes (e.g., the annelid trochophore larva) and deuterostomes (e.g., the hemichordate tornaria larva) share morphological characteristics (ciliary bands for feeding, an apical organ with a tuft of cilia, an eye spot) and, importantly, homologous regions of expression of brachyury, otx and goosecoid during gastrulation (Arendt et al. 2001). This indicates that a large complexity of life preceded the urbilaterian ancestor.

The use of conserved patterning gene networks during embryonic development must have imposed historical constraints on the type of animal forms that could be evolved by natural selection. Not all forms were possible. The actual results of evolution had to provide not only functional improvements but also be compatible with the structure of the D-V, A-P, and other embryonic gene networks. Our evolutionary history is recorded in an analogue language consisting of A, T, G, and C in DNA. Therein lies the difficulty of applying the general laws of physics and simple mathematical equations to biology. However, once this language is decoded the resulting molecules become subject to the universal laws of physics and chemistry. In the case of the transformations in body form presented in the study, the universal laws of diffusion apply to the Chordin/Tolloid/BMP morphogenetic gradient as
envisioned by the intuition of D’Arcy Thompson and the mathematics of Alan Turing.

**Sizzled and ADMP were lost in mammals**

The self-organizing network of secreted proteins shown in Figure 2 is conserved intact in the embryos of fishes, amphibians, reptiles and birds (De Robertis 2008). However, there have been gene losses as mammals moved into yolk-less embryos that do not require epiboly movements during gastrulation. The platypus (*Ornithorhynchus*) is a mammalian ancestor that lost one of the genes of the network, ADMP, from its genome. Although platypus is a mammal, it still lays eggs in a nest. Although its reptile ancestors had two or three *vitellogenin* (yolk protein) genes, the platypus has retained only one (Warren et al. 2008). As evolution moved into higher mammals, two additional genes of the regulatory network, *sizzled* and *crescent*, were also deleted from the genome. Marsupials such as the opossum (*Monodelphis domestica*) lost *sizzled* but kept a functional copy of *crescent*. The dog (*Canis lupus familiaris*) genome still retains a recognizable *crescent* pseudogene remnant (Ploper et al. 2011). Interestingly, in placental mammals (and also in marsupials) the *vitellogenin* genes were completely lost. The Chordin/Tolloid/BMP network probably evolved to self-organize a morphogen gradient in cells actively migrating in a yolky egg. As soon as yolk disappeared, some genes in the network became dispensable. Another possibility is that the lost genes were important in adjusting to differences in temperature during early development, which became unimportant when embryogenesis took place inside the mother. Gene losses are very common in animal evolution. For example, in a systematic search for genes lost in mammals but present in the chick genome, Kuraku & Kuratani (2011) found 147 losses of entire protein-coding genes.

How can one explain that the Chordin morphogen gradient can still function in the absence of a key component such as the Tolloid inhibitor Sizzled? It has been found that Tolloid activity is also regulated by direct binding of BMP to protein domains, called CUB domains, which lie outside of its catalytic region. If BMP levels become high, it binds to the CUB domains in Tolloid, inhibiting enzymatic activity in a non-competitive fashion (Lee et al. 2009). This additional negative feedback loop provides a molecular explanation for an old mystery in the field. When the first peptide sequences were obtained from extracts with bone-inducing activity (Wozney et al. 1988) the first protein identified, designated BMP1, had the sequence of a Tolloid enzyme containing three CUB domains. The reason why BMP1 was purified together with the BMP2 to 7 growth factors was simple: Tolloids are BMP-binding proteins. *Drosophila* does not contain *sizzled* or any sFRP in its genome yet is able to form a Sog/Dpp gradient by this mechanism in which high Dpp directly inhibits Tolloid non-competitively. This conclusion can be deduced from the existence of antimorphic *tolloid* mutations that inactivate the proteolytic site and display anti-BMP phenotypes (Ferguson & Anderson 1992; Childs & O’Connor 1994). BMP and Tolloid interact at sufficient levels to cause phenotypes in vivo, indicating that the direct binding of BMP to the CUB domains of Tolloid is a very ancient conserved regulatory mechanism. This may explain why mammals and flies and even sea anemones can generate a self-organizing gradient using the Chordin/Tolloid/BMP biochemical pathway. The Tolloid inhibitors Sizzled and Crescent were an evolutionary novelty introduced at the bottom of the vertebrates, and subsequently lost at their top.

**Concluding remarks – 50th anniversary of JSDB**

This study represents an effort to reconcile D’Arcy Thompson’s theory of transformations of Cartesian coordinates to our present knowledge in developmental biology, on the 100th anniversary of its publication. The experimental work on changing body form by manipulating components of the Chordin/Tolloid/BMP gradient reported here was presented by one of us (E.M.D.R) at a lecture celebrating the 50th anniversary meeting of the Japanese Society of Developmental Biologists (JSDB) in Tokyo.

We would like to end with a few comments on JSDB. Scientific societies are crucially important because they are organized by scientists for scientists as part of civil society, independently of governments. They have a key role in training new generations of scientists. Since its foundation JSDB, one of the premier world societies in our discipline, has published Development, Growth & Differentiation, an international journal that reflects the great scientific strength of Japan. Many great names have been associated with JSDB since its foundation such as Hidemichi Oka, Yujiro Hayashi, Katsuma Dan, Tokindo Okada, Masatoshi Takeichi, Makoto Asashima, Shin Aizawa, Kyokazu Agata and current president Naoto Ueno. JSDB contributed two presidents to the International Society of Developmental Biology (ISDB), Tokindo Okada whose life was celebrated at the meeting (ISDB president 1982–1986) and his student Masatoshi Takeichi who discovered Cadherins (ISDB president 2006–2010). E.M.D.R. would like to acknowledge the
essential support of JSDB to ISDB during his presidency (2002–2006). During that time the Asia Pacific Developmental Biology Network was created and the annual meetings of JSDB adopted exclusively the English language. The excellent scientific quality of the young JSDB members was in full display at the 2017 Annual Meeting at Tower Hall Funabori, Tokyo celebrating the 50th anniversary of the foundation of JSDB. We expect that the 100th anniversary will find JSDB even stronger.

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