Pleiotropy as a mechanism to stabilize cooperation

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Most genes affect many traits[1–4]. This phenomenon, known as pleiotropy, is a major constraint on evolution because adaptive change in one trait may be prevented because it would compromise other traits affected by the same genes[2,4]. Here we show that pleiotropy can have an unexpected effect and benefit one of the most enigmatic of adaptations—cooperation. A spectacular act of cooperation occurs in the social amoeba *Dictyostelium discoideum*, in which some cells die to form a stalk that holds the other cells aloft as reproductive spores[5,6]. We have identified a gene, *dimA*[7], in *D. discoideum* that has two contrasting effects. It is required to receive the signalling molecule DIF-1 that causes differentiation into prestalk cells. Ignoring DIF-1 and not becoming prestalk should allow cells to cheat by avoiding the stalk. However, we find that in aggregations containing the wild-type cells, lack of the *dimA* gene results in exclusion from spores. This pleiotropic linkage of stalk and spore formation limits the potential for cheating in *D. discoideum* because defecting on prestalk cell production results in an even greater reduction in spores. We propose that the evolution of pleiotropic links between cheating and personal costs can stabilize cooperative adaptations.

Acts of cooperation such as stalk formation in *D. discoideum* are a challenge for evolutionary biologists because of the potential for disruptive cheaters[8–12]. When starving, the normally solitary amoebae of *D. discoideum* aggregate together to form a migratory slug and then a fruiting body in which most cells become spores but around one-fifth die to form a supporting stalk[5,6]. Genetically different clones of *D. discoideum* will aggregate together and form chimaeric fruiting bodies[5] and chimaerism seems to be common in nature because multiple clones co-occur in tiny soil samples[13]. This suggests that a defector[12] that produces fewer stalk cells can gain a selfish advantage[5] and raises the question of how stalk formation is maintained[9,11]. Around half of prestalk cells in *D. discoideum*, known as the prestalk O cells, are induced to differentiate by the signalling molecule DIF-1 (ref. 14), that is released by the neighbouring prespore cells[15] (Fig. 1a). A viable cheating strategy, therefore, would be to ignore DIF-1 and overproduce spore cells in chimaeras. This is supported by the observation that cells with lowered DIF sensitivity, resulting from growth with glucose, cheat and are over represented in the spores when mixed with cells grown without glucose[16,17].

We used a knockout mutant to examine the effects of ignoring DIF-1. *dimA* encodes a central component of the DIF response pathway. A mutant with this gene disrupted (*dimA*$^-$) ignores DIF-1 and produces prespore cells in place of prestalk O cells[7] (Fig. 1a). We examined the behaviour of *dimA*$^-$ cells as they aggregated with equal numbers of their parental wild-type strain (AX4). The *dimA*$^-$ cells co-aggregated normally with AX4: *dimA*$^-$ and AX4 cells entered aggregations in equal numbers, and there was no loss of *dimA*$^-$ from aggregations during the migratory slug stage (mean percentage of *dimA*$^-$ cells in *dimA*/AX4 chimaeras from two time points in two independent experiments = 48%; 1 petri plate per mixture in each experiment, cells counted $N = 1{,}168$; Chi-squared

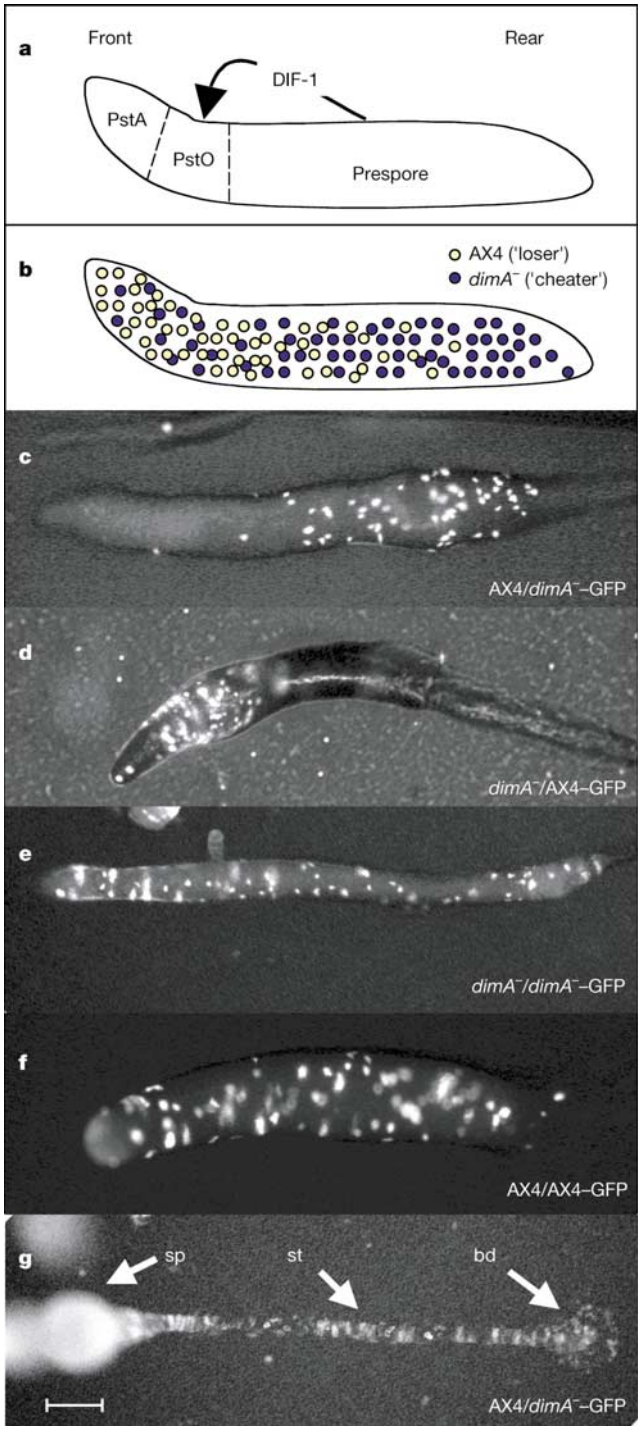

Figure 1 *dimA*$^-$ cells occupy the prespore zone and behave like a cheater in chimaeras. **a**, A schematic diagram of a slug of the slime mould *Dictyostelium discoideum* showing three of the major cell types: prestalk A (pstA), prestalk O (pstO) and prespore cells. Prespore cells release DIF-1, which induces pstO cell differentiation. **b**, Schematic diagram showing the distribution of *dimA*$^-$ cells in a chimaeric slug with the wild-type AX4. **c**, *dimA*$^-$ cells are over represented in the rear of the slug: chimaeras of 50% AX4, 48% *dimA*$^-$ and 2% *dimA*$^-$–GFP. Some *dimA*$^-$ cells are present in the prestalk zone (see text) but are not visible with 2% GFP expressing cells. **d**, AX4 cells are over represented in the front of the slug: 50% *dimA*$^-$, 48% AX4 and 2% AX4–GFP. **e**, Control: 98% *dimA*$^-$ and 2% *dimA*$^-$–GFP. **f**, Control: 98% AX4 and 2% AX4–GFP. **g**, A chimaeric fruiting body (50% AX4, 48% *dimA*$^-$ and 2% *dimA*$^-$–GFP): sp, sorus; st, stalk; bd, basal disc. There are more *dimA*$^-$ cells in the stalk than basal disc. Scale bar is 0.1 mm.

from 50% = 2.23; $P = 0.13$). As predicted, *dimA*$^-$ defected by preferentially sorting to the prespore zone (Fig. 1b–f). Cell counts of the prestalk region of slugs (front 25%) confirmed there were significantly fewer *dimA*$^-$ cells than AX4 cells (mean proportion *dimA*$^-$ in chimaeras with AX4 cells in two experiments = 34%; 10 slugs per mixture in each experiment; cells counted $N = 1{,}239$; Chi-squared versus 50% = 126.93; $P < 0.0001$). The localization of *dimA*$^-$ cells at the back of slugs is not because they are weakened cells with low motility. Low motility cells would be expected to be preferentially sloughed during slug migration. In contrast, we found equal numbers of *dimA*$^-$ and AX4 cells in slugs that had migrated (above) and did not observe an excess of *dimA*$^-$ cells being left behind in slug trails. In addition, *dimA*$^-$ cells in the back of the slug are true prespore cells because they express the prespore marker *cotB–lacZ*[7]. Finally, *dimA*$^-$ cells did not seem to be simply impaired in their ability to differentiate. Mutants which exhibit general differentiation defects are typically greatly enriched in the basal disc of chimaeric fruiting bodies with wild-type cells[18]. In contrast, *dimA*$^-$ cells contributed to all terminally differentiated cell types and were not enriched in the basal disc of chimaeras (Fig. 1g).

To test whether the defection of *dimA*$^-$ in slugs resulted in a competitive advantage, we examined the spores in the fruiting bodies that form after the slug stage. We were surprised to find that *dimA*$^-$ cells ultimately lose out with about half as many present as AX4 cells in the spores (percentage of green fluorescent protein (GFP) spores in AX4/*dimA*$^-$–GFP chimaeras = 34.7%; spores counted = 1,256; Student's *t*-test versus 50%: $t = -6.4$; $N = 6$ petri plates; $P < 0.002$; percentage GFP spores in *dimA*$^-$/AX4–GFP chimaeras = 63%; spores counted = 1,279, Student's *t*-test versus 50%: $t = 4.2$; $N = 6$ petri plates; $P < 0.009$; Fig. 2). In contrast, controls of *dimA*$^-$/*dimA*$^-$–GFP chimaeras and AX4/AX4–GFP chimaeras demonstrated no significant biases from 50:50 owing to GFP labelling (percentage of GFP spores from both controls = 52%; spores counted = 1,275; Student's *t*-test versus 50%: $t = 1.164$; $N = 12$ petri plates; $P = 0.27$). The low number of *dimA*$^-$ spores is not simply because *dimA*$^-$ cells present in the spore head fail to make spores and remain as amoebae: there was no reduction in total spore number in the *dimA*$^-$/AX4 chimaera compared with AX4 alone (Fig. 3). Additional evidence that *dimA*$^-$ losing is not because of an intrinsic sporulation deficit comes from clonal *dimA*$^-$ development, which shows that *dimA*$^-$ is effective at producing spores: *dimA*$^-$ produces around 82% of the spore number of the wild type (mean spores per plate: *dimA*$^-$ = 1.70×10^7; AX4 = 2.08×10^7; $N = 36$ plates). Taken together, these results suggest that in chimaeras *dimA*$^-$ cells are replaced in the spore population by AX4 late in development.

To test whether AX4 prestalk cells replace *dimA*$^-$ prespore cells, we used a prestalk specific marker *ecmAO–lacZ*[19]. This marker is expressed in about one-fifth of prestalk cells (data not shown) but is rarely expressed in spore cells. However, prestalk cells that express the gene and then transdifferentiate into spores will retain expression owing to the inherent stability of the β-galactosidase enzyme produced by the *lacZ* gene[20]. The number of *lacZ* positive AX4 spores increased sixfold when developed in chimaera with *dimA*$^-$ cells (Fig. 4). This result is consistent with the majority of AX4 prestalk cells switching fate and becoming spores late in development and replacing *dimA*$^-$ cells in the spores (for example, if 12.5% of cells are AX4 prestalk and one-fifth of these express lacZ and all transdifferentiate, we expect about 3% of cells to be labelled spores, not far above the percentage shown in Fig. 4).

The *dimA* gene therefore has two contrasting effects. Cells that do not express *dimA* behave like a cheater in chimaeric slugs by increasing their representation in the prespore population, presumably because they are able to ignore the signal of prestalk cell induction, DIF-1. In the terminology of Velicer[12], *dimA*$^-$ cells are defectors that under-invest in the cooperative resource of prestalk cells. However, they do not benefit from defection because of a second phenotype of *dimA*$^-$ cells that causes them to be competitively excluded from the spores late in development. This second pleiotropic effect of *dimA* makes ignoring the DIF-1 signal unprofitable and helps to limit cheating and ensure fair contribution to the stalk.

We do not expect all cheating to be controlled by pleiotropy and single-gene mutants have been found that cheat under laboratory conditions[12]. Nevertheless, we predict that pleiotropy will be a common form of cheater control because it is so ubiquitous. It has been neglected because the organisms studied by most sociobiologists are genetically intractable, but additional evidence can be found from microorganisms. For example, another potential cheating strategy in *D. discoideum* is to reduce adhesion and slide to the back of the slug, where the prespore zone is located (Fig. 1a). This is supported by the *csA*$^-$ knockout mutant, which lacks a key adhesion molecule and is a cheater in chimaeras with the wild type on agar[21,22]. However, its reduced adhesion means that it has trouble getting into aggregations on soil and incurs a great fitness cost[21]. Another example comes from the fluorescent bacterium *Vibrio fischeri*, that is used for bio-illumination by the squid *Euprymna scolopes*. Bacteria that defect by not producing the light production enzyme luciferase should gain a selfish advantage. However, a luciferase knockout strain grows poorly in the squid light organ[23] suggesting a pleiotropic link between light production

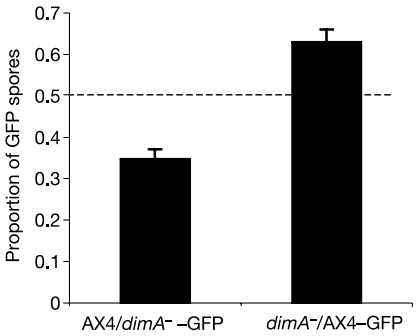

Figure 2 The *dimA*$^-$ mutant loses out in the fruiting body. In the spore head of *dimA*$^-$/AX4 chimaeras, there are almost twice as many AX4 spores as *dimA*$^-$ spores. Data are from two independent experiments. Bars are standard errors calculated using each petri plate as a single replicate. In addition, we have independently confirmed the losing behaviour of *dimA*$^-$ without GFP expression, using blasticidin S resistance (C.R.T. and L. Santorelli, unpublished data) to distinguish between AX4 and *dimA*$^-$ cells[7].

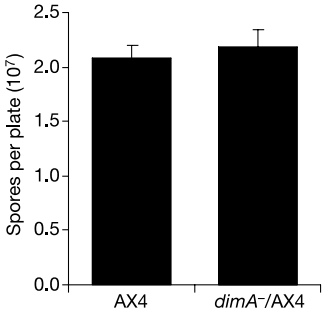

Figure 3 AX4/*dimA*$^-$ chimaeras produce the same number of spores as wild type (AX4) alone. Total number of spores produced per plate by AX4 clones and chimaeras of AX4/*dimA*$^-$ (AX4 = 2.08×10^7 spores, *dimA*$^-$/AX4 chimaeras = 2.18×10^7, $N = 30$ petri plates, *t*-test = −0.523, $P = 0.61$). Bars are standard errors calculated using each petri plate as a single replicate. In addition, spore viability assays demonstrated no significant difference between AX4 and *dimA*$^-$/AX4 chimaeras (*t*-test, $t = 1.264$, $N = 30$ petri plates, $P = 0.22$).

and growth. Finally, in *Escherichia coli* bacteria, a single-gene GASP (growth advantage under stationary phase) mutant has been identified that defects from the coordinated developmental process that naturally occurs under low nutrients[24]. Instead of reducing reproduction to form resistant spore-like cells, it rapidly divides and replaces the wild type. However, defection again results in a cost because the resulting GASP cells do not tolerate acidity and cannot survive passage through their mammalian hosts' stomachs.

A form of cheater control related to pleiotropy occurs by tight linkage of genes in colicin production in bacteria[25]. Colicins are produced by bacteria through a plasmid that contains genes for both colicin production and resistance to their toxic effects. This linkage of genes prevents loss of colicin production by plasmid loss because this would also cause loss of resistance[26]. However, it is unknown if colicin genes exhibit pleiotropic constraints and loss of production may occur without loss of resistance through mutation of the colicin production gene alone[25].

Pleiotropy may be shown as important in more familiar social organisms such as the social insects, once molecular mechanisms are uncovered. In several species, the queen releases a pheromone that suppresses worker ovary development, suggesting chemical control of reproduction[27]. The control hypothesis has been criticized on theoretical grounds because workers should evolve to ignore any chemical control agent[27]. However, workers could be prevented from ignoring the queen pheromone if, analogous to DIF-1, it targets a pleiotropic gene that is required for both response to the queen pheromone and ovary development itself.

The spread of social genes will be promoted by mechanisms that make their loss costly ('intrinsic defector inferiority')[26]. Our study demonstrates that pleiotropy is one such mechanism. The complex interrelationships of developmental and biochemical processes mean that genes have innumerable idiosyncratic side effects that constrain adaptation[1–4]. Paradoxically, we have found that these constraints can benefit cooperative adaptations by limiting the potential for individual selfishness. Social innovations will persist only when disruptive cheaters cannot invade[28,29]. As a result, the successful innovations will often be tied through pleiotropy to other essential functions, limiting the number of cheating mutations with a net advantage. Pleiotropy provides one way to limit individual rebellion and allow stable cooperation to evolve. Where pleiotropic costs are strong, other means of control like high relatedness[30] and policing[11] may be less necessary. □

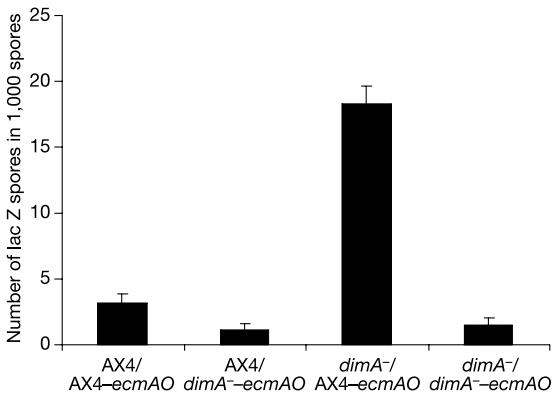

Figure 4 The majority of AX4 prestalk cells switch fate and become prespore cells when in chimaeras with *dimA* $^-$. There is a sixfold increase in the number of spores expressing the prestalk marker *ecmAO–lacZ* in chimaeras of *dimA* $^-$/AX4–*ecmAO–lacZ* compared to other mixtures (analysis of variance between groups (ANOVA), $N = 24$, $F = 70.9$, $P < 0.001$). Data are from two counts of 1,000 spores from each of three independent experiments. Bars are standard errors calculated using each count as a single replicate.

Methods

Cell growth, maintenance and chimeric development

Strains were maintained in association with *Klebsiella aerogenes* or in liquid medium[7]. *Actin15–GFP* and *ecmAO–lacZ* transformants were generated as described[7] with initial selection in 20 μg G418 and maintenance in 2.5 μg G418. Before development, cells were grown for 48 h without G418. For development, cells were collected by centrifugation, washed with KK2 (16.1 mM KH_2PO_4 and 3.7 mM K_2HPO_4) and developed at a density of 6.4×10^5 cells per cm^2 on KK2 plates containing 1.5% purified agar (Oxoid)[7].

Disaggregation, spore counts and lacZ staining of spores

GFP labelling was used to distinguish between *dimA* $^-$ and AX4 cells. To count cells in chimaeras during development, multicellular structures were disaggregated at two time points: mounds and migrated slugs. The structures were washed from plates with KK2 and separated from loose cells using a 77 μm cell sieve. The structures were then transferred from the sieve into 20 mM EDTA and passed several times through a 20-gauge needle in order to disaggregate the cells for counting by fluorescence microscopy. For counting of spores, structures were harvested from plates after 48 h of development into 20 mM EDTA containing 0.1% NP-40 to lyse amoebae. Spore density was counted using a haemocytometer. Fluorescence microscopy was used to count GFP spores (Fig. 2), and pure *dimA* $^-$–GFP and pure AX4–GFP controls used to adjust counts for non-glowing cells. For the *lacZ* labelled strains, slugs of pure strains of AX4–*ecmAO–lacZ* and *dimA* $^-$–*ecmAO–lacZ* were disaggregated as described above and stained to give percentage of expressing cells. Spore staining was performed as described[20]. To measure spore viability, the colonies that resulted from the plating of 100 spores were counted. All experiments were performed at least twice and data are combined because there were no significant differences between experiments.

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Migratory neural crest-like cells form body pigmentation in a urochordate embryo

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The neural crest, a source of many different cell types in vertebrate embryos, has not been identified in other chordates[1–3]. Current opinion therefore holds that neural crest cells were a vertebrate innovation[4–7]. Here we describe a migratory cell population resembling neural crest cells in the ascidian urochordate *Ecteinascidia turbinata*. Labelling of embryos and larvae with the vital lipophilic dye DiI enabled us to detect cells that emerge from the neural tube, migrate into the body wall and siphon primordia, and subsequently differentiate as pigment cells. These cells express HNK-1 antigen and *Zic* gene markers of vertebrate neural crest cells. The results suggest that migratory cells with some of the features of neural crest cells are present in the urochordates. Thus, we propose a hypothesis for neural crest evolution beginning with the release of migratory cells from the CNS to produce body pigmentation in the common ancestor of the urochordates and vertebrates. These cells may have gained additional functions or were joined by other cell types to generate the variety of derivatives typical of the vertebrate neural crest.

Neural crest cells delaminate from the dorsal neural tube in an anterior to posterior sequence, migrate through stereotypical pathways, and differentiate into a variety of cell types in vertebrate embryos, including neuronal, glial, endocrine, skeletal and pigment cells[1,2]. Migratory neural crest cells have not been described in non-vertebrate chordates. In ascidian urochordates and amphioxus, however, cells at the border of the neural plate or within the neural tube are often proposed as evolutionary precursors of the neural crest[3,7–10].

The life cycle of the ascidians is usually separated into distinct larval (or embryonic) and adult developmental phases[11]. The tadpole larva consists of a trunk (or head) with a dorsal central nervous system (CNS) containing two melanized sensory organs (otolith and ocellus) and a tail with a notochord, spinal cord and muscle cells. During metamorphosis, the larval tail is destroyed and the head is extensively reorganized into a sessile filter-feeding adult. Previous searches for neural crest cells were restricted to the embryonic phase in ascidian species such as *Ciona intestinalis*, whose small larvae exhibit the conventional mode of development. These studies did not use cell-tracing methods, which originally defined the neural crest in vertebrates[1,2]. We have investigated the possibility of neural crest-like cells in urochordates using the colonial ascidian *Ecteinascidia turbinata* (Fig. 1a), which exhibits a giant tadpole that is suitable for cell-tracing approaches to detect migratory cells (Fig. 1b). In contrast to more commonly studied ascidians, the *Ecteinascidia* tadpole initiates adult development in its head during the embryonic phase, where adult features such as the pharynx, heart, siphons and stellate body pigment cells are formed precociously (Fig. 1c, d)[12,13].

Using the otolith and ocellus as landmarks, DiI was injected into the anterior neural tube at the early tailbud stage and the injected embryos were subsequently examined by fluorescence microscopy. There was no movement of DiI or labelled cells immediately after injection (Fig. 2a, b). By 4 h after injection, however, DiI-labelled cells were observed to migrate from the injection site towards the developing siphons (Fig. 2c, d). Migratory cells continued to be observed through the late tailbud stage (Fig. 2e, f). Some of these migrating cells had the stellate morphology typical of body pigment cells (Fig. 2f). At the mid-tailbud stage, DiI was injected into the anterior neural tube, the posterior neural tube, or the ventral head epidermis. As before, migrating cells emerged from the anterior neural tube and moved ventrolaterally into the body wall (Fig. 2g). Sectioning showed that cell migration occurred in two pathways: (1) through the dorsal mesoderm surrounding the neural tube, and (2) between the mesoderm and epidermis (Fig. 2j–l). In contrast, DiI-labelled cells did not migrate from the posterior neural tube (Fig. 2h) or the ventral epidermis (Fig. 2i), although local rearrangements of labelled cells occurred in these regions. The results show that migratory cells emerge from the neural tube during *E. turbinata* embryogenesis.

DiI-labelling experiments were continued during later development. After DiI injection into the posterior neural tube at the late-tailbud stage, labelled cells migrated into the head but were excluded from the tail (Fig. 3a, b). At the same stage, migratory cells were still generated after DiI injection into the anterior neural tube, and many of these cells became associated with the developing siphons (see Fig. 2e, f; data not shown). These results suggest that migratory cells emerge in an anterior to posterior sequence during *Ecteinascidia* development. In contrast to vertebrate neural crest cells, however, the

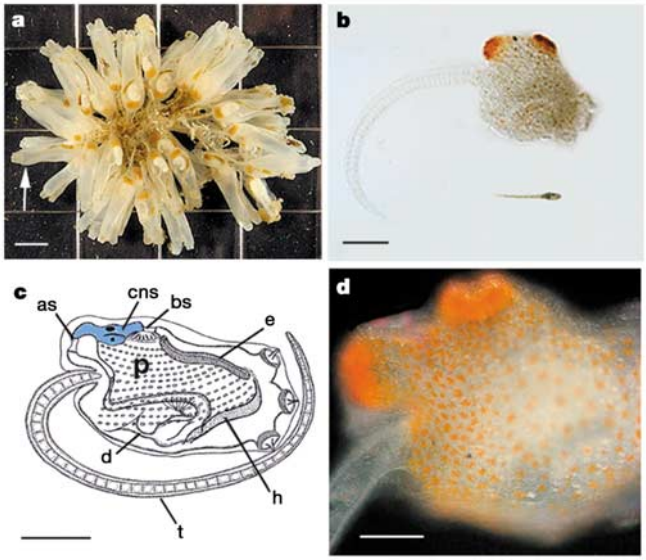

Figure 1 *Ecteinascidia turbinata*. **a**, A colony showing gravid zooids (arrow). **b**, The giant *Ecteinascidia* tadpole (above) compared to a small *Styela clava* tadpole (below). **c**, A diagram of the *Ecteinascidia* tadpole after ref. 12, showing the CNS (blue) with melanized sensory cells (black), branchial siphon (bs), atrial siphon (as), endostyle (e), heart (h), perforated pharynx (p), digestive tract (d), and tail (t). **d**, Orange pigment cells distributed throughout the body wall and concentrated in the siphon primordia of an *Ecteinascidia* tadpole. Scale bars: **a**, 1 cm; **b**–**d**, 800 μm.