The surface ciliation of anuran amphibian embryos and early larvae: Patterns, timing differences and functions

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Abstract
Embryonic and early larval surface ciliation patterns are described stage by stage, using scanning electron microscopy and live specimen observations, in 20 species of anuran amphibians from six families including the first detailed description of surface ciliation in a direct-developing eleutherodactylid: four species are from temperate regions and 16 are tropical. The study asks whether differences in pattern relate to ciliation functions, or show any phylogenetic features, or demonstrate heterochrony. It also asks whether the timing of ciliated cell regression is fixed or can vary with the environment. Respiration appears to be the main function of surface ciliation, both pre- and post-hatching, regressing when internal gills become functional. Substratum gliding after hatching may also be a valuable role. Late stage persistence of ciliation around the external nares suggests a sensory function. Localized early regression of ciliated cells is linked to lateral line development. Dramatic changes in ciliated cell shape are described, though functions are unclear. Ciliation patterns, density and duration vary considerably between species, with no obvious phylogenetic or environmental correlates, nor is there a clear relationship with egg size or hatching stage. Ciliation patterns also vary with body region, with density and duration consistently highest round the nostrils and adhesive glands (when present) compared to other body regions.

Keywords: amphibian, anuran, cell shape change, ciliary gliding, embryo respiration, embryonic rotation, embryos, external nares, heterochrony, polyphenism

Introduction
The presence of ciliated cells on the outer surface of many kinds of invertebrate embryos and larvae is well known. They have roles in swimming and in filter feeding and their patterns are claimed to have phylogenetic significance (Nielsen 1987). Amongst the vertebrates, significant embryonic/larval surface ciliation has been reported only for lungfish (Budgett 1901; Whiting and Bone 1980) and amphibians, though occasional isolated cilia are seen transiently in other kinds of embryos such as the dogfish (\textit{Squalus})
Ciliated cells have also been reported on early stages of protochordates such as amphioxus (Stokes and Holland 1995a, 1995b) and ascidians (Crowther and Whittaker 1994). Surface ciliation on the embryos of urodeles and anurans (we know of no reports of ciliation on caecilian embryos) has been known since the late 19th century and was first investigated in detail by Assheton (1896) using embryos and larvae of *Rana temporaria* Linnaeus. The advent of scanning electron microscopy made it feasible to map the distribution of ciliated cells on embryonic/larval surfaces relatively easily, and a number of studies on different amphibian species soon appeared (Billett and Courtenay 1973; Lofberg 1974—*Ambystoma mexicanum* (Shaw); Steinman 1968—*Xenopus laevis* Daudin; Kessel et al. 1974—*Rana piniens* Schreber). Subsequent studies (e.g. Nishikawa et al. 1991, 1992; Deblandre et al. 1999) have investigated aspects of ciliated cell differentiation, patterning and disappearance: essentially, ciliated cells originate from deep cells in the epidermis and migrate to the surface; when ciliated cells disappear from the surface, they resorb their cilia and transform into mucus-secreting cells, rather than being shed or dying.

In recent years, the study of comparative patterns of development has become a major focus of developmental biology, with the general aim of elucidating how alterations in development produce the morphological changes in adults that are selected for in evolution. Less attention has been given to embryo/larval specific structures that have no adult successors, though Haeckel, in his original formulation of the Biogenetic Law (which asserted that development is a short recapitulation of phylogeny) accepted that embryonic and larval stage adaptations provided one of the main distorters of recapitulatory sequences (see Gould 1977, for review). There is a growing interest, however, in examining the interactions between embryos and their environment and in studying the adaptations and responses that allow embryos of different types to develop successfully under different conditions (Gilbert 2001).

Previous work (Kessel et al. 1974; Landstrom 1977) on surface-ciliated cells in anurans and urodeles has shown that they begin to appear during neurulation and disappear from the epidermis soon after hatching (though Assheton 1896 claimed that ciliated cells persisted on the tails of *Rana temporaria* tadpoles until close to metamorphosis). This pattern of development suggests that the surface cilia have a functional role restricted to early development. Several functions, not necessarily mutually exclusive, have been suggested (Assheton 1896; Kessel et al. 1974; Burggren 1985; Altig and McDiarmid 1999). These are:

- preventing micro-organisms and debris from attaching to the epidermis;
- movement of the body, including pre-hatching rotation and post-hatching gliding;
- respiratory gas exchange;
- movement of surface mucus films.

An additional function, which Assheton (1896) suggests very briefly and which we amplify here, is the generation of water currents that can provide chemical information about the surrounding environment.

Two other occurrences of surface ciliation in vertebrates are worth mentioning, though they are clearly distinct from the numerous multi-ciliated cells reported from amphibian embryo and larval surfaces.

First, in early mammal and bird embryos, the mono-ciliated cells found in the region of Hensen’s node have been shown to have a role in left-right patterning (Brueckner 2001;
Nonaka et al. 2002). Essner et al. (2002) reported short-lived localized monociliated cells in early *Xenopus* and zebra fish embryos, which they suggest have a similar role in left-right patterning.

Second, Webb and Northcutt (1991) reported the presence of epidermal ciliated cells in halos around the canal pores and pit lines of the lateral line system in two actinopterygian fish, *Polypterus* and *Acipenser*: the individuals examined were juveniles or adults and Webb and Northcutt were therefore only able to speculate on their developmental origins; the function of the ciliated cells was also unclear, though their location suggested some relationship with the lateral line system.

There has been remarkably little experimental investigation of these functions, particularly in the context of different types of embryos and larvae. The main aims of the work reported here are to document the distribution of surface-ciliated cells both morphologically and in time over a wide range of anuran species, chosen to represent a diversity of taxonomic and adaptive types, with the intention of testing hypotheses on the roles of these cells in development.

The measurement of developmental timing has been the subject of extensive theoretical analysis (e.g. Hall and Miyake 1995; Smith 2001). Developmental biologists have long relied on so-called “normal tables” such as that for *Xenopus laevis* (Nieuwkoop and Faber 1967) which sub-divide the continuum of development into a sequence of recognizably distinct morphological stages: these inevitably rely on externally obvious characters such as somite number, or appearance of digits. Normal tables are a considerable convenience for experimentalists, but are of limited use for the measurement of timing, since the times between stages are variable. Normal tables are also problematic for inter-specific comparisons: a traditional view, extending back to Von Baer, is that early stages of development are highly conserved, and that developmental trajectories for closely related species only tend to diverge later in a sequence. Richardson et al. (1997) provided empirical evidence against this view and showed that the notion of a series of highly conserved early stages in the development of related animals is a myth. Smith (2001) emphasized the difference between developmental “stages” and developmental “events”. Because development is a process which occurs over time, events inevitably occur in a sequence, but different events (e.g. lens induction, tail bud formation) may be independent of one another and therefore their relative timing can alter as species diverge: this means that a developmental “stage”, defined, say, by the state of tail bud development, may not show a common morphology across related species because of divergence in the timing of other developmental events.

The difficulty with timing changes in development (heterochrony) is how to measure them. For amphibian early development, Chipman et al. (2000) used a “normalization” technique to circumvent the problems caused by temperature differences. Their “normalized time” for any developmental event is a dimensionless ratio derived from the timing of the event relative to a standard. Their results, for 12 species and a considerable number of developmental events (assessed both externally and internally) extending from fertilization to stages just beyond hatching, showed considerable inter-specific differences in timing, as great between closely related as distantly related species. However, the sequences of external events were relatively conserved, though significant differences in normalized time did occur. An interesting conclusion of their work was that, given the relatively conservative morphologies of the feeding stage tadpoles they studied, considerable divergence in developmental timing at embryonic stages resulted in remarkably little alteration in the end result.
In the work on ciliation patterns reported here, we have used a combination of real time and Gosner (1960) staging to assess heterochrony. For most of the species studied, temperature is not a confounding factor, because embryos were from a single tropical environment (Trinidad) and incubated in a laboratory at a temperature within the normal environmental range. We used Gosner’s staging system because it is the system most commonly used by ecologists studying comparative aspects of amphibian development: Gosner devised it and tested its usefulness for a wide range of amphibian families. It is based entirely on external characters, and for a comparative study of the timing and patterns of an external character (ciliation) it seems appropriate, given the finding of Chipman et al. (2000) that external features are well conserved in sequence and despite general concerns about the use of staging tables in comparative studies.

Ciliation patterns on amphibian embryos/larvae have previously been reported in any detail for only three species. We provide descriptions for 18 new species, and more detailed descriptions of two that have been previously reported on, *Xenopus laevis* and *Rana temporaria*. Our intention was to investigate variation between closely related species (e.g. we have four *Bufo* species) but also to look for differences related to embryonic adaptive types. Given that external ciliation is generally thought to serve a range of functions in the embryo/early larva, we might expect to find differences related to factors such as egg size and deposition site. Our sample therefore includes species with eggs in water (in strings, clumps or singly), eggs in foam (on the surface of water, or in burrows) and eggs out of water, and a considerable range of egg size.

Finally, we can ask whether the timing of the ciliation pattern for a species is a fixed programme, or whether it can be adjusted, depending on circumstances. There is considerable interest in examples of polyphenism in development, where morphology can be varied and adapted to environmental circumstances, such as predation levels (see Gilbert 2001, for review). Given that external ciliation is mainly a pre-hatching feature, the scope for polyphenetic adaptation is probably limited. In this study, we report on one case, *Leptodactylus fuscus*, where adaptation of the ciliation pattern might be expected, dependent on the post-hatching circumstances of different egg batches.

Drawing on this introductory discussion, we can ask the following questions about the surface ciliation patterns of anuran embryos and larvae:

- Do ciliation patterns show any clear relationships to functions? For example, are ciliated cells associated with particular regions of the body surface? Does their development and regression show a consistent relationship to life stages such as hatching and the onset of feeding? Do ciliated cell patterns show any relationship to egg size or incubation habitat?
- Do ciliation patterns show any phylogenetic features, i.e. are patterns within families more similar than these between families?
- Do ciliation patterns show any heterochronic features, and if so, why?
- Is the timing of ciliation pattern development fixed, or can it vary with ecological factors?

Materials and methods

*Egg collection, incubation and fixation*

All spawn was collected from field sites in Trinidad, Scotland and Iran as soon after spawning as was practicable. The exception was *Xenopus laevis*, provided from a captive population maintained at the University of St Andrews.
The 20 species studied are shown in Table I. Ecological information on Trinidad species was extracted from Murphy (1997). Spawn was incubated at temperatures appropriate to the normal habitat (Trinidad 27–29°C; Scotland 15–17°C; Iran 25–27°C; *Xenopus laevis*, 25°C) either in water (most species) or in air (foam-nesting species; terrestrial spawners). At appropriate times, samples were taken for fixation, in order to examine the ciliation patterns of most stages from around neurulation to early feeding tadpoles. The normal stage at which hatching occurred in each species was also noted.

In the case of *Leptodactylus fuscus*, after hatching at Gosner stage 19, larvae may remain in the foam nest (which is in a burrow close to a temporary pool site) for several weeks. The original foam of the nest breaks down and is replaced by a new kind of foam made by the larvae. During this time, the larvae progress to stage 27 after 3 days and then enter a period of developmental arrest where progressive development largely ceases (Downie 1994a, 1994b). We tested whether or not ciliated cell regression occurs in these arrested larvae by transferring samples of larvae to water with food at stages 20, 23 and 25 while keeping the

| Family      | Species                          | Country          | Habitat | Spawning place                      |
|-------------|----------------------------------|------------------|---------|-------------------------------------|
| Bufonidae   | *Bufo beebei* Gallardo           | Trinidad         | S       | Temporary pools                     |
|             | *Bufo bufo* (Linnaeus)           | UK               | WT      | Pools, ponds                        |
|             | *Bufo marinus* Linnaeus          | Trinidad         | R, E, S | Pools, ponds, rivers                |
|             | *Bufo viridis* (Laurenti)        | Iran             | WM      | Pools, ponds                        |
| Pipidae     | *Xenopus laevis* Daudin          | South Africa     | S       | Ponds                               |
| Hylidae     | *Hyla crespitans* Wied-Neuwied    | Trinidad         | E, S    | Pools, ponds                        |
|             | *Hyla boans* (Linnaeus)          | Trinidad         | R       | Pools at river edge                 |
|             | *Hyla geographica* Spix          | Trinidad         | R, E    | Edge of streams                     |
|             | *Hyla microcephala misera* Fouquett | Trinidad     | S       | Temporary pool                      |
|             | *Hyla minuta* Peters             | Trinidad         | R, E, S | Forest and road-side pools          |
|             | *Hyla minuscula* Rivero          | Trinidad         | S       | Ponds, swamps                       |
|             | *Phyllomedusa trinitatis* Mertens | Trinidad       | R, E, S | Leaves above water                  |
|             | *Phrynohyas venulosa* (Laurenti) | Trinidad         | E, S    | Temporary pools                     |
| Leptodactylidae | *Leptodactylus fuscus* (Schneider) | Trinidad       | S       | Burrow in ground*                   |
|             | *Physalaemus pustulosus* (Cope)  | Trinidad         | S       | Burrow in ground*                   |
|             | *Leptodactylus validus* Garman   | Trinidad         | R, E, S | On water surface*                   |
|             | *Leptodactylus bolivianus* Boulenger | Trinidad    | S       | On water surface*                   |
|             | *Eleutherodactylus urichi* (Boettger) | Trinidad | R, E  | Leaves on ground                    |
| Microhylidae | *Elachistocleis ovalis* (Schneider) | Trinidad       | S/R     | Temporary pool, ditches             |
| Ranidae     | *Rana temporaria* Linnaeus       | UK               | WT      | Pools, ponds                        |

R, rainforest; E, forest-edge; S, savanna; WT, widespread temperate; WM, widespread Mediterranean. *In foam.
remainder in the foam nest. Samples from water and foam were fixed over the next few days.

All specimens were fixed in 2.5% glutaraldehyde in phosphate buffer for about 5 h, then rinsed in 0.1 M phosphate buffer (pH 7.4) and stored in buffer at 5°C, until required for electron microscopy.

**Egg size and developmental rate**

We chose at least three fixed eggs of each species from the first day of development at Gosner stage 10 or earlier to measure the mean diameter of the eggs. To estimate developmental rates, we assumed eggs had been fertilized at midnight of the night prior to collection of fresh spawn. Hatching stage and time were assessed for at least five specimens of each species.

**Specimen staging and examination**

The jelly and vitelline membrane were removed from stored specimens using forceps or filter paper. They were then staged using Gosner’s (1960) system. We use Gosner staging throughout this paper, and when referring to stages using other systems in the literature, have converted them to Gosner, using the table in McDiarmid and Altig (1999). An exception is the only *Eleutherodactylus* species we report on: development has been so modified in this directly developing genus that Gosner staging is inappropriate. We use the system devised by Townsend and Stewart (1985).

Specimens were rinsed in several changes of buffer and then postfixed in 1% osmium tetroxide for 1 h. They were washed in distilled water, then immersed in 0.05% aqueous uranyl acetate in the dark for 1 h. After a brief rinse in distilled water, specimens were dehydrated in an acetone series (30%, 50%, 70%, 90%, 100%, and dried 100%). They were then critical point dried for 40 min, coated with gold using a Polaron SC 515 then examined using a Philips 500 scanning electron microscope. Images were examined over a range of magnifications $\times 24–\times 3200$, and recorded by Imageslave for Windows (Meeco Holdings, Australia).

**Distribution, shape and density of ciliated cells**

At each stage for each species, we examined three specimens when they were available. We assessed the presence/absence and density of ciliated cells at 10 locations on the body surface of embryos/larvae: nostrils, mouth, adhesive glands, ventral side of head, dorsal side of head, external gills, dorsal side of trunk, ventral side of trunk, lateral side of trunk, and tail (Figure 1). Examples of ciliated cell patterns at these different locations are shown in Figure 2.

Cell size was measured on scanning electron photomicrographs (at magnification of $\times 800$) using a cursor connected to a computer program (Cherry Digitiser), which converts the space enclosed by any perimeter into an area measurement. In each field total ciliated cell area and total non-ciliated cell area were measured, as well as the ratio (number) of ciliated to non-ciliated cells.

At early stages of development, ciliated cells tend to be small in size compared to non-ciliated cells. Fully developed ciliated cells tend to be much larger and therefore to occupy a larger proportion of the body surface than before, without necessarily increasing in number. Since our interest is largely in the functions of surface ciliation, the most relevant measure is of proportionate area.
Ciliated cell density, based on the ratio of ciliated cell area to non-ciliated cell area was classified into the six categories below. In the data presentation tables, we use a star system to denote the different density categories, as shown.

1. Very dense: the ratio of ciliated cells to non-ciliated cells is greater than or equal to 3:1 (*****).
2. Dense: the ratio is between 2:3 and 3:1 (****).
3. Intermediate: the ratio is between 1:3 and 2:3 (**).
4. Dispersed: the ratio is between 1:5 and 1:3 (*).
5. Very dispersed: the ratio is less than 1:5 (*).
6. Zero: no ciliated cells (0).

Examples of these different density categories are shown in Figure 3. We also classified ciliated cells by their shape, as follows: four- or five-sided; six- or more sided, circular or oval and elongated. Examples of ciliated cells of these different shapes are shown in Figure 4.

**Data analysis**

In order to subject the individual species data to comparative analysis, we carried out the following computations:
For each species and each body region, we took all stages (between Gosner stages 14 and 27) where ciliated cell density was intermediate (*** or over. From this we generated two parameters: the total number of stages at these higher densities, and the total number of stars per body region during these stages. This allowed us to calculate two measures of ciliation abundance: (a) a high density duration index—the mean
Figure 3. The range of ciliated cell densities from “very dispersed” to “very dense”. (A) *H. minuta*, very dispersed, stage 20, ventral side of the body; (B) *R. temporaria*, dispersed, stage 18, dorsal side of the head; (C) *L. fuscus*, intermediate density, stage 20, ventral side of the trunk; (D) *L. fuscus*, dense, stage 24, ventral side of the trunk; (E) *Phrynophyus venulosa*, very dense, stage 17, adhesive gland. Scale bars: 35 μm (A, D); 30 μm (B, C, E).
Figure 4. The range of ciliated cell shapes. (A) *B. viridis*, five-sided, stage 15, lateral side of the head; (B) *P. venulosa*, six-sided, stage 17, ventral side of the trunk; (C) *H. boans*, multi-sided, stage 20, ventral side of the trunk; (D) *L. fuscus*, circular, stage 23, ventro-posterior side of the trunk; (E) *L. fuscus*, oval, stage 23, ventral side of the trunk; (F) *P. trinitatis*, elongated, stage 20, lateral side of the trunk. Scale bars: 30 μm (C, D, F); 35 μm (A, B); 60 μm (E).
number of stages per organ or per species where ciliation density was high; (b) a high cell density index—the mean number of stars (three or above) per stage, per species, per body region. Since ciliated cell density at a stage and region can range from 0 to 5, this index has a maximum value of 5. For comparative analysis, it was necessary to have data points for all body regions and all stages. Unfortunately, some stages and some views of particular regions were not available. Where this was the case, we estimated the likely density by examining the density figures for the stages before and after. From a total of 180 data points, only 37 included small contributions from such estimates.

Two species were excluded from this analysis: *L. bolivianus*, because two complete stages were missing; *E. urichi* because of its highly modified pattern of development.

2. For each species, the period of maximum ciliation was computed, by summing the total star count for each stage. Where a lower density stage differed by only one star from the maximum, the period of maximum ciliation is given as extending over both stages. As above, *L. bolivianus* and *E. urichi* had to be excluded from this analysis.

*Ciliary movements, embryo rotation, gliding, and twitching*

For several of the species in our sample (*R. temporaria, B. bufo, X. laevis, H. minuta, P. trinitatis, P. pustulosus, L. fuscus, B. beebei, P. venulosa,* and *E. ovalis*) we made observations on living embryos and larvae, both pre- and post-hatching. Embryos and larvae were observed using a stereomicroscope at ×40 magnification. Currents generated by ciliated cells could often be detected by observing the movements of particles (within the vitelline fluid before hatching or in the medium after hatching). In a few cases, we used a carmine particle suspension to provide better resolution. This was injected into the vitelline fluid on the tip of a fine tungsten needle or micropipette, or added to the water close to hatched larvae.

Prior to hatching, embryos may rotate within the confines of the vitelline membrane. The time to complete a full rotation was measured in several species. Embryos also begin to show spontaneous twitching movements a little before and for some time after hatching. Frequency of twitching was assessed in some species. After hatching, larvae lying on their sides at the bottom of the dish often glide at a perceptible rate: speed, distance and direction of gliding were measured in some species.

**Results**

*Egg sizes and developmental rates*

Egg sizes, hatching times and developmental rate data for all species are shown in Table II.

*Species accounts, ordered by Family*

Results for each species are given as a table. We were unable to collect every stage for every species. For consistency, these are shown as “no case” in the tables. In addition, even when we had examples of a stage, it was occasionally not possible to obtain views of every body region. These are also shown as “no case”. Examples of the ciliated cell patterns found are shown in Figure 2.
Table IIIa–d shows detailed ciliary pattern results for four bufonid species: *B. beebei*, *B. bufo*, *B. marinus*, and *B. viridis*. Common features of all four species were: at stage 14, ciliated cells appeared on the ventral side of the trunk and except in *B. viridis* they disappeared at stage 24/25. Ciliated cells on the adhesive glands and ventral side of the head were dense to very dense. Features, which differed between the species, were ciliated cell density, which on *B. beebei* was the lowest, and on *B. bufo* the highest among the four species. Ciliated cells remained after stage 26 on the ventral side of the trunk on *B. viridis*, while they disappeared at stage 24 on *B. bufo*. The maximum density of ciliated cells on the body surface occurred at stage 22 on *B. marinus* but at stage 19 on the other species.

**Pipidae**

Table IV shows detailed ciliary pattern results for *X. laevis*. At stage 15, ciliated cells appeared on the dorsal and ventral side of the head and the ventral side of the trunk. At stage 26 cilia disappeared from the ventral side of the trunk. While the ciliated cells around the nostrils were very dense, they were very dispersed on the rest of the body, except for the external gills.

**Hylidae**

Table V shows detailed ciliary pattern results for eight hylid species: *H. crepitans*, *H. geographica*, *H. microcephala*, *H. minuta*, *H. minuscula*, *P. trinitatis*, *P. venulosa*, and
Table III. Ciliated cell distribution in the Bufonidae: *B. beebei*, *B. bufo*, *B. marinus*, and *B. viridis*.

| Stage | Nostril | Mouth | Adhesive gland | Ventral head | Dorsal head | External gill | Dorsal trunk | Ventral trunk | Lateral trunk | Tail |
|-------|---------|-------|----------------|-------------|-------------|---------------|--------------|---------------|---------------|------|
| (a) *B. beebei* |
| 12/13 | p       | p     | p              | p           | p           | p             | p            | *             | –             | *    |
| 14    | p       | p     | p              | p           | p           | p             | p            | ***           | ***           | ***  |
| 15    | p       | p     | –              | p           | –           | p             | p            | ***           | ***           | ***  |
| 16    | p       | –     | –              | –           | p           | –             | –            | –             | –             | –    |
| 17+   | p       | ***   | *****          | –           | –           | p             | ***          | ***           | ***           | ***  |
| 18    | ***     | ***   | *****          | *****       | –           | –             | ***          | ***           | ***           | ***  |
| 19    | ****    | ***   | ****           | ****        | *           | –             | ***          | ***           | ***           | ***  |
| 20    | ****    | **    | ***            | ***         | *           | *             | *            | *             | *             | *    |
| 21    | **      | *     | **             | **          | **          | **            | *            | *             | *             | **   |
| 22    | **      | *     | **             | **          | **          | **            | *            | *             | *             | *    |
| 23    | **      | *     | *              | –           | **          | **            | *            | **            | **            | **   |
| 24    | **      | *     | *              | ***         | *           | **            | **           | *             | **            | **   |
| 25    | *       | 0     | 0              | *           | d           | *             | 0            | *             | –             | –    |
| 26    | –       | –     | –              | –           | –           | –             | –            | –             | –             | –    |
| 27    | –       | –     | –              | –           | –           | –             | –            | –             | –             | –    |
| (b) *B. bufo* |
| 13    | p       | p     | p              | p           | p           | p             | 0            | 0             | 0             | p    |
| 14/15 | p       | p     | *****          | –           | ***         | p             | ***          | ***           | ***           | **** |
| 16    | p       | p     | *****          | *****       | **          | p             | ***          | ***           | ***           | **** |
| 17    | p       | p     | *****          | *****       | ***         | p             | ***          | ***           | ***           | **** |
| 18+   | ***     | ***   | *****          | ****        | ***         | ***           | ***          | ***           | ***           | **** |
| 19    | ****    | ****  | ****           | ****        | ***         | ***           | ***          | ***           | ***           | **** |
| 20    | ****    | ****  | ****           | ****        | ***         | ***           | ***          | ***           | ***           | **** |
| 21    | ****    | ****  | ****           | ****        | ***         | ***           | ***          | ***           | ***           | **** |
| 22    | ****    | ****  | ****           | ****        | ***         | ***           | ***          | ***           | ***           | **** |
| 23    | ****    | **    | ***            | ****        | ***         | ***           | ***          | ***           | ***           | ***  |
| 24    | ****    | **    | **             | ***         | ***         | ***           | ***          | ***           | ***           | ***  |
| 25    | *****   | *     | *              | ****        | d           | **            | 0            | *             | **            | **   |
| 26    | *****   | *     | *              | ***         | –           | **            | 0            | *             | –             | –    |
| 27    | *****   | –     | d              | 0           | ***         | d             | ***          | 0             | –             | *    |
| (c) *B. marinus* |
| 14    | p       | p     | p              | p           | p           | p             | –            | –             | –             | p    |
| 15    | p       | p     | p              | p           | p           | p             | –            | –             | –             | p    |
| 16    | p       | p     | –              | –           | p           | –             | –            | –             | –             | p    |
| 17    | p       | p     | –              | –           | p           | –             | –            | –             | –             | p    |
| 18+   | **      | **    | *****          | –           | **          | –             | ***          | ***           | ***           | –    |
| 19    | **      | **    | ****           | ****        | **          | **            | ***          | ***           | ***           | –    |
| 20    | ****    | ****  | ****           | ****        | **          | **            | ***          | ***           | ***           | –    |
| 21    | ****    | **    | ***            | ****        | ***         | ***           | ***          | ***           | ***           | –    |
| 22    | ****    | **    | ***            | ****        | ***         | ***           | ***          | ***           | ***           | –    |
| 23    | ****    | **    | ***            | ****        | ***         | ***           | ***          | ***           | ***           | –    |
| 24    | ****    | *     | ***            | ****        | **          | **            | ***          | ***           | ***           | –    |
| 25    | *****   | *     | **             | ****        | d           | **            | *            | *             | *             | –    |
| 26    | *****   | *     | *              | ****        | d           | **            | 0            | *             | *             | –    |
| 27    | ***     | 0     | d              | 0           | **          | d             | 0            | –             | 0             | –    |
H. boans. Common features of all eight species were: ciliated cells appeared around stage 15 and, except in H. minuscula, a very dense pattern of ciliated cells could be seen around the nostrils, on the external gills and on the ventral side of the head. Features which differed between species were: the ciliated cells disappeared from the body surface at stage 20 in P. venulosa; at stage 22 in H. boans and H. microcephala; at stage 23 in H. crepitans and H. minuta; at stage 24 in H. geographica and after stage 27 in P. trinitatis and H. minuscula. Hyla geographica had the highest density of ciliated cells while H. minuta and H. microcephala had among the lowest density of ciliated cells.

Table IV. Ciliated cell distribution in Xenopus laevis.

| Stage | Nostril | Mouth | Adhesive gland | Ventral head | Dorsal head | External gill | Dorsal trunk | Ventral trunk | Lateral trunk | Tail |
|-------|---------|-------|----------------|--------------|-------------|---------------|--------------|---------------|---------------|------|
| 14    | p       | p     | p              | p            | p           | p             | 0            | *             | *             | p   |
| 15    | p       | p     | p              | p            | p           | p             | 0            | *             | *             | p   |
| 16    | p       | *****| ****           | **           | ***         | ***           | ***          | ***           | ***           | p   |
| 17    | p       | *****| *****          | ***          | ***         | ***           | ***          | ***           | ***           | p   |
| 18+   | p       | *****| *****          | *            | ***         | ***           | ***          | ***           | ***           | p   |
| 19    | ****    | **** | *****          | *            | ***         | ***           | ***          | ***           | ***           | p   |
| 20    | ****    | **** | *****          | *            | ***         | ***           | ***          | ***           | ***           | p   |
| 21    | ***     | ***  | ****           | *            | ***         | ***           | ***          | ***           | ***           | p   |
| 22    | ***     | ***  | ****           | *            | ***         | ***           | ***          | ***           | ***           | p   |
| 23/24 | ****    | **** | *              | *            | **          | ***           | ***          | ***           | ***           | p   |
| 24    | *****   | *****| 0              | d            | ***         | ***           | ***          | ***           | ***           | p   |
| 25    | *****   | *****| 0              | p            | ***         | ***           | ***          | ***           | ***           | p   |
| 26    | *****   | *****| 0              | p            | ***         | ***           | ***          | ***           | ***           | p   |
| 27    | 0       | 0     | 0              | 0            | 0           | 0             | 0            | 0             | 0             | 0   |

+, In stage column shows hatching stage; –, no case; 0, no ciliated cell; *very dispersed; **dispersed; ***intermediate density; ****dense; *****very dense; d, structure regressed or covered; p, structure not yet developed.

All symbols as in Table III.
Table V. Ciliated cell distribution in the Hylidae: *H. crepitans*, *H. boans*, *H. geographica*, *H. microcephala*, *H. minuta*, *H. minuscula*, *P. trinitatis*, and *P. venulosa*.

| Stage | Nostril | Mouth | Adhesive gland | Ventral head | Dorsal head | External gill | Dorsal trunk | Ventral trunk | Lateral trunk | Tail |
|-------|---------|-------|---------------|-------------|-------------|--------------|--------------|--------------|--------------|------|
| (a) *H. crepitans* |
| 14    | p       | p     | p             | p           | p           | p            | 0            | 0            | 0            | p    |
| 15    | p       | p     | p             | p           | p           | p            | 0            | 0            | 0            | p    |
| 16    | p       | p     | *****         | ****        | ***         | p            | ***          | ***          | ***          | p    |
| 17    | p       | p     | *****         | ****        | ***         | p            | ***          | **           | ***          | **** |
| 18    | ***     | ***   | *****         | ****        | ***         | ***          | ***          | ***          | ***          | ***  |
| 19+   | ***     | ***   | *****         | ****        | ***         | ***          | ***          | ***          | ***          | ***  |
| 20    | ****    | ****  | ****          | ****        | **           | ***          | ***          | ***          | ***          | ***  |
| 21    | ****    | ***   | **            | ****        | **           | ***          | ***          | ***          | ***          | ***  |
| 22    | *****   | **    | **            | ****        | ***          | ***          | ***          | ***          | ***          | ***  |
| 23    | ****    | *     | *             | *           | *           | *            | *            | 0            | *            | *    |
| 24    | ****    | *     | *             | *           | *           | *            | *            | 0            | *            | *    |
| 25    | ****    | *     | *             | *           | *           | *            | d            | 0            | *            | *    |
| 26    | –       | –     | –             | –           | –           | –            | –            | –            | –            | –    |
| 27    | –       | –     | –             | –           | –           | –            | –            | –            | –            | –    |
| (b) *H. boans* |
| 13/14 | p       | p     | p             | p           | p           | p            | **           | *            | 0            | 0    |
| 14/15 | p       | p     | p             | p           | p           | p            | p            | 0            | –            | p    |
| 16    | p       | p     | p             | p           | p           | p            | 0            | –            | –            | –    |
| 17/18 | p       | p     | ****          | ***         | ***         | p            | ***          | ***          | ***          | ***  |
| 18    | p       | p     | *****         | ***         | ***         | p            | ***          | ***          | ***          | ***  |
| 19+   | **      | ***   | ***           | ***         | ***         | ***          | ***          | ***          | ***          | ***  |
| 20    | ***     | ***   | ***           | ***         | ***         | ***          | ***          | ***          | ***          | ***  |
| 21    | ****    | ***   | ****          | ***         | ***         | ***          | ***          | ***          | ***          | ***  |
| 22    | ****    | *     | *             | ***         | *           | ***          | *            | 0            | –            | **   |
| 22/23 | **      | **    | *             | *           | **          | *            | 0            | 0            | 0            | *    |
| 24    | **      | 0     | 0             | *           | *           | –            | *            | 0            | 0            | 0    |
| 25    | **      | 0     | 0             | *           | *           | d            | 0            | 0            | –            | –    |
| 26    | –       | –     | –             | –           | –           | –            | –            | –            | –            | –    |
| 27    | –       | –     | –             | –           | –           | –            | –            | –            | –            | –    |
| (c) *H. geographica* |
| 14/15 | p       | p     | ****          | ****        | ****        | p            | *            | *            | **           | p    |
| 16    | p       | p     | ****          | ****        | ****        | p            | ***          | ***          | ****         | **** |
| 17    | p       | p     | ****          | ****        | ****        | p            | ***          | ***          | ****         | **** |
| 18    | p       | p     | ****          | ****        | ****        | p            | ***          | ***          | ****         | **** |
| 19    | p       | p     | ****          | ****        | ****        | ****         | ****         | ****         | ****         | **** |
| 20+   | p       | p     | ****          | ****        | ****        | ****         | ****         | ****         | ****         | **** |
| 21    | p       | p     | ****          | ****        | ****        | ****         | ****         | ****         | ****         | **** |
| 22    | p       | p     | ****          | ****        | ****        | ****         | ****         | ****         | ****         | **** |
| 23    | p       | p     | ****          | ****        | ****        | ****         | ****         | ****         | ****         | **** |
| 24    | p       | p     | ****          | ****        | ****        | ****         | ****         | ****         | ****         | **** |
| 25    | p       | p     | ****          | ****        | ****        | ****         | ****         | ****         | ****         | **** |
| 26    | –       | –     | –             | –           | –           | –            | –            | –            | –            | –    |
| 27    | –       | –     | –             | –           | –           | –            | –            | –            | –            | –    |
### Table V. (Continued).

| Stage | Nostril | Mouth | Adhesive gland | Ventral head | Dorsal head | External gill | Dorsal trunk | Ventral trunk | Lateral trunk | Tail |
|-------|---------|-------|----------------|--------------|-------------|---------------|--------------|---------------|---------------|------|
| (d) H. microcephala | p | p | p | p | p | p | – | – | – | p |
| 14 | p | p | p | p | p | ** | p | * | ** | ** | p |
| 15 | p | p | p | ** | p | – | ** | ** | – | p |
| 16 | p | ** | ** | ** | ** | ** | ** | ** | – | p |
| 17 | p | – | ** | ** | ** | p | ** | ** | ** | p |
| 18 | – | ** | ** | ** | ** | ** | ** | ** | – | p |
| 19 | – | ** | ** | * | ** | * | ** | ** | ** | ** |
| 20+ | *** | * | ** | ** | ** | ** | ** | ** | ** | ** |
| 21+ | *** | * | ** | ** | ** | ** | ** | ** | ** | ** |
| 22 | *** | * | ** | ** | ** | ** | ** | ** | ** | ** |
| 23 | **** | 0 | 0 | 0 | ** | 0 | * | 0 | 0 | ** |
| 24 | **** | 0 | 0 | 0 | ** | 0 | – | 0 | * | ** |
| 25 | – | – | – | – | – | d | – | – | – | – |
| 26 | *** | 0 | – | 0 | 0 | d | 0 | 0 | * | ** |
| 27 | – | – | – | – | – | – | – | – | – | – |
| (e) H. minuta | p | p | p | 0 | 0 | p | – | 0 | – | p |
| 14 | p | p | * | * | * | * | p | ** | * | ** |
| 15 | p | p | ** | ** | * | p | ** | ** | * | p |
| 16 | p | ** | ** | * | p | ** | ** | ** | ** | ** |
| 17 | p | – | ** | ** | ** | ** | ** | ** | – | p |
| 18 | – | *** | * | ** | ** | ** | ** | ** | – | p |
| 19/20+ | – | *** | * | ** | ** | * | ** | ** | ** | ** |
| 20 | ** | ** | ** | * | *** | * | ** | ** | ** | ** |
| 21 | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** |
| 22 | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** |
| 23 | **** | * | 0 | 0 | ** | 0 | **** | 0 | * | ** |
| 24 | **** | 0 | 0 | 0 | ** | 0 | – | 0 | * | ** |
| 25 | **** | * | 0 | * | 0 | d | 0 | – | ** | ** |
| 25/26 | – | 0 | 0 | 0 | * | d | * | 0 | * | – |
| 27 | – | – | – | – | – | – | – | – | – | – |
| (f) H. minuscula | p | p | p | p | p | p | – | – | – | p |
| 14 | p | p | p | p | p | p | – | – | – | p |
| 15 | p | p | p | p | p | p | – | – | – | p |
| 16 | p | p | – | – | p | – | – | – | – | p |
| 17/18 | – | **** | *** | *** | ** | – | ** | *** | ** | ** |
| 19 | – | **** | *** | *** | ** | – | ** | *** | ** | ** |
| 20+ | – | – | **** | *** | *** | ** | – | ** | *** | ** |
| 20/21 | **** | * | ** | ** | ** | **** | ** | *** | ** | ** |
| 22 | **** | * | ** | ** | ** | **** | ** | *** | ** | ** |
| 23 | **** | ** | ** | ** | ** | **** | ** | *** | ** | ** |
| 24 | **** | ** | ** | ** | ** | **** | ** | *** | ** | ** |
| 25/26 | **** | * | ** | ** | * | ** | d | ** | ** | ** |
| 26 | **** | – | – | ** | ** | d | *** | **** | ** | ** |
| 27 | – | – | – | – | – | – | – | – | – | – |
Leptodactylidae

Table VI shows detailed ciliary pattern results for four foam-nesting leptodactylids: *L. fuscus*, *P. pustulosus*, *L. validus*, and *L. bolivianus*. Common features for all species were: except in *L. fuscus*, external gills in these species had a very dense pattern of ciliated cells. Adhesive glands had a dispersed or intermediate density of ciliated cells where this structure developed (*L. fuscus* lacked adhesive glands). Features which differed between species were: ciliated cells appeared on the ventral side of the trunk on *P. pustulosus*, *L. validus* and *L. fuscus* at stage 16, 17 and 18, respectively (ciliated cells were missing at early stages in *L. bolivianus*). Ciliated cell density on the dorsal side of the head was lowest in *L. fuscus* and highest in *L. bolivianus*. Ciliated cells disappeared from the ventral side of the trunk in *P. pustulosus* at a stage later than 27, in *L. fuscus* by stage 27, and in *L. bolivianus* and *L. validus* at about stage 25/26.

In *L. fuscus*, after hatching at stage 19 (2.5 days after deposition), embryos in foam nests progressed to stage 22/23 by day 3.5, stage 25/26 by day 4.5, stage 27 by day 5.5, and stage 27/28 by day 6.5, by which time all ciliated cells had disappeared. When embryos were transferred to water at stage 20 or 23, their development progressed at the same rate as those in foam, including the progressive loss of ciliated cells, up until stage 26. Embryos transferred to water with food at stage 25, when feeding begins, grew rapidly in size.
Table VI. Ciliated cell distribution in the Leptodactylidae: *L. fuscus* kept in foam nests, *P. pustulosus*, *L. validus*, and *L. bolivianus*.

| Stage | Nostril | Mouth | Adhesive gland | Ventral head | Dorsal head | External gill | Dorsal trunk | Ventral trunk | Lateral trunk | Tail |
|-------|---------|-------|----------------|-------------|-------------|---------------|-------------|--------------|---------------|------|
| (a) *L. fuscus* | | | | | | | | | | |
| 14 | p | p | p | p | | p | | | | |
| 15 | p | p | p | | p | | | | | |
| 16 | p | p | | | | | | | | |
| 17 | p | p | | | | | | | | |
| 18 | | | | | | | | | | |
| 19 | | | | | | | | | | |
| 20+ | | | | | | | | | | |
| 21+ | | | | | | | | | | |
| 22 | | | | | | | | | | |
| 23 | | | | | | | | | | |
| 24 | | | | | | | | | | |
| 25 | | | | | | | | | | |
| 26 | * | 0 | | | | | | | | |
| 27 | * | 0 | | | | | | | | |
| 27/28 | 0 | 0 | | | | | | | | |
| (b) *P. pustulosus* | | | | | | | | | | |
| 14 | p | p | p | p | | p | | | | |
| 15 | p | p | | | | | | | | |
| 16 | p | | | | | | | | | |
| 17 | p | | | | | | | | | |
| 18 | | | | | | | | | | |
| 19/20 | | | | | | | | | | |
| 21+ | ** | ** | *** | ** | *** | *** | *** | *** | * | ** |
| 22 | *** | *** | *** | *** | *** | *** | *** | *** | ** | *** |
| 23 | *** | *** | *** | *** | *** | *** | *** | *** | ** | *** |
| 24 | *** | *** | *** | *** | *** | *** | *** | *** | ** | *** |
| 25 | | | | | | | | | | |
| 26 | * | 0 | | | | | | | | |
| 27 | * | 0 | | | | | | | | |
| 28 | *** | 0 | d | * | 0 | d | * | 0 | 0 | |
| (c) *L. validus* | | | | | | | | | | |
| 13 | p | p | p | p | | p | | | | |
| 14 | p | p | p | | p | | | | | |
| 15 | p | p | | | | | | | | |
| 16 | p | p | | | | | | | | |
| 17 | | | | | | | | | | |
| 18 | | | | | | | | | | |
| 19 | | | | | | | | | | |
| 20+ | **** | **** | **** | **** | **** | **** | **** | **** | **** | **** |
| 21+ | **** | **** | **** | **** | **** | **** | **** | **** | **** | **** |
| 22 | **** | **** | **** | **** | **** | **** | **** | **** | **** | **** |
| 23 | **** | **** | **** | **** | **** | **** | **** | **** | **** | **** |
| 24 | **** | **** | **** | **** | **** | **** | **** | **** | **** | **** |
| 25 | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** |
| 26 | | | | | | | | | | |
| 27 | | | | | | | | | | |
| 28 | *** | 0 | d | * | 0 | d | * | 0 | 0 | |
compared to those kept in foam (21–22 mm long after 4 days, compared to 12–13 mm), but there was no discernible difference in ciliated cell regression which was already well advanced by stage 25 in the foam nest.

Eleutherodactylus urichi was the fifth leptodactylid species we examined. Members of this direct-developing genus lay relatively large terrestrial eggs with no foam, and hatch as fully metamorphosed froglets. Their highly modified pattern of development makes direct comparison with conventional species difficult. Figure 5 shows the body regions we examined in E. urichi; Table VII shows the distribution of ciliated cells by Townsend and Stewart (1985) (TS) stages; Figure 6 gives some representative images. Townsend and Stewart (1985) estimated that Eleutherodactylus stage 4 is equivalent to Gosner (1960) stage 17–18, when many conventional anurans hatch. It is worth noting here that whereas levels of ciliation peak in most species at or just after hatching, in E. urichi, the time of greatest ciliated cell abundance was TS stages 6–9, much later than the equivalent stages in anurans with tadpoles. Ciliated cells in E. urichi did decline prior to hatching, but in eleutherodactylids hatching is equivalent to metamorphosis in other anurans, as the time when the embryonic environment is exchanged for terrestrial conditions, and a terrestrial integument is required.

In E. urichi, highest ciliated cell densities were found on the lateral side of the head, essentially the pharyngeal region, and on the tail “fins”. In eleutherodactylid embryos, the tail fins are thin-walled highly vascularized and laterally expanded structures, thought to have a respiratory function. An unexpected finding was the occurrence of ciliated cells on the limbs, with forelimbs having denser populations than hind limbs. In anurans with tadpoles, the hind limb buds make their first appearance when surface-ciliated cells have generally regressed, and forelimbs undergo their development covered by the operculum. The only other species where we found ciliated (hind) limb buds were L. fuscus, R. temporaria and B. viridis (Figure 2e).

Microhylidae

Table VIII shows detailed ciliary pattern results for E. ovalis. At stage 13 the cilia appeared on the surface of some cells. At stage 26, the cilia disappeared from all body regions.
Embryos at stage 18 had the highest density of ciliated cells. The ventral side of the head and dorsal side of the trunk were regions where the highest and lowest density of ciliated cells, respectively, occurred.

**Ranidae**

Table IX shows detailed ciliary pattern results for *R. temporaria*. At stage 15, ciliated cells appeared on the ventral side of the trunk and disappeared at a stage after 25/26. At stage 20, the highest density of ciliated cells could be seen on the surface. Nostrils and adhesive glands were among regions where the ciliated cell pattern was very dense.
Tables X and XI show mean ciliated high cell density and duration indices for each species (where data were adequate) and each body region, respectively. Indices were calculated as described in Materials and Methods. A combined index (product of the first two) is also shown.

From Table X, it is clear that species vary considerably in overall ciliation patterns and their durations, and that this is as true between related species (within families) as unrelated species. The combined index, by giving a measure of both ciliated cell density and duration across stages, probably provides the best indication of variability in ciliation across species.

With this index, species can be divided into three groups:

1. low (less than 10): three species, X. laevis and two small hylid.
2. medium (10–20): 11 species.
3. high (over 20): the three temperate species plus H. geographica, which spawns in cool rivers.

Using the egg size data from Table II, we tested whether there was any relationship between the combined index values and egg size, but the correlation was not significant ($r=0.324; P=0.190$).

From Table XI, there is clearly considerable variation in ciliation patterns across the body regions we measured. Again, we have constructed a combined density/duration index. Nostrils came highest; anterior regions were generally higher than posterior; and ventral were higher than dorsal. The persistence of nostril ciliation into post-hatching stages was a very general result. Figures 2a and 12 show nostril ciliation in a range of species and stages.

It is noteworthy that the interspecific variability in the combined index is greater (range: 4.6–35.6; mean ± SD: 17.2 ± 9.2) than the variability between body regions (range: 10.5–25.5; mean ± SD: 17.0 ± 5.3).

Ciliated cell shape

Ciliated cell shape varied with stage, body region and species. Examples of the different cell shapes we recorded are shown in Figure 4.

Table VII. Distribution of ciliated cells by body region and Townsend Stewart stage in E. urichi.

| Region        | 4 | 5 | 6 | 7 | 8 | 9 | 11 | 12 | 14 |
|---------------|---|---|---|---|---|---|----|----|----|
| Head          |   |   |   | * | * | * |    |    |    |
| Dorsal        | 0 | 0 | 0 | * | * | * | 0  | 0  | 0  |
| Lateral       | * | **| ***|***|***|***|***|***|***|
| Trunk         |   |   |   |   |   |   |    |    |    |
| Dorsal        | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  |
| Lateral       | * | * | * | * | * | * | *  | *  | *  |
| Tail          |   |   |   |   |   |   |    |    |    |
| Stem          | 0 | * | * | * | * | * | *  | n  | a  |
| Fins          | 0 | 0 | **|**|**|**|**|n|a|
| Forelimbs     | 0 | * | * | * | * | * | *  | 0  | 0  |
| Hindlimbs     | 0 | * | * | * | * | * | *  | 0  | 0  |
| Yolk-sac      | * | n | * | * | * | * | *  | n  | a  |

Body regions defined in Figure 5. 0, ciliated cells; *low density; **medium density; ***high density; n, not available; a, structure absent.
Figure 6. Scanning electron micrographs to show distribution of ciliated cells on the surface of *E. urichi* at different stages and locations. The need to open up the yolk sac before processing accounts for the somewhat crumpled appearance of some embryos. (A) Stage 5, overall dorsal view; (B) stage 6/7, overall dorsal view; (C) stage 8/9, anterior end, dorsal view; (D) stage 14, overall dorsal view; (E) stage 5, dorso-lateral side of the head; (F) stage 6/7, dorso-lateral side of the head and forelimb bud. *Forelimb. Scale bars: 500 μm (A, C); 1 mm (B, D); 150 μm (E, F).
In general, at the earlier stages, ciliated cells were mostly four- or five-sided. Only in later stages did elongated cells become common and these were not seen in some species. As a semi-quantitative summary of the data, Table XIII shows the distribution of the different cell shapes in each species and at a selection of sites in each species.

Where elongated cells occurred, they were commonest on the lateral and ventral side of the trunk. Since elongated ciliated cells have not previously been reported, we present a selection of images of these cells in Figure 7.

In most cases, elongated cells were orientated perpendicular or slightly oblique to the body axis, but in a few cases, they were parallel to the body axis. On the external gills, orientation of these cells was parallel or oblique to the axis of the gill in some species, but at right angles to the axis in others.

Table VIII. Ciliated cell distribution in *E. ovalis*.

| Stage | Nostril | Mouth | Adhesive gland | Ventral head | Dorsal head | External gill | Dorsal trunk | Ventral trunk | Lateral trunk | Tail |
|-------|---------|-------|----------------|-------------|-------------|---------------|--------------|---------------|---------------|------|
| 13    | *p      | *p    | *p             | *p          | *p          | *p            | *p           | *p            | *p            | p    |
| 14/15 | *p      | *p    | *p             | *p          | *p          | *p            | *p           | *p            | *p            | p    |
| 16    | *p      | *p    | *p             | *p          | *p          | *p            | *p           | *p            | *p            | p    |
| 17    | *p      | *p    | *p             | *p          | *p          | *p            | *p           | *p            | *p            | p    |
| 18    | ***     | ***   | ***            | ***         | ***         | ***           | ***          | ***           | ***           | ***  |
| 19    | **      | **    | ***            | ***         | ***         | ***           | ***          | ***           | ***           | ***  |
| 20+   | ***     | ***   | ***            | ***         | ***         | ***           | ***          | ***           | ***           | ***  |
| 21/22 | ***     | ***   | ***            | ***         | ***         | ***           | ***          | ***           | ***           | ***  |
| 23    | ***     | ***   | *              | ***         | ***         | ***           | ***          | ***           | ***           | ***  |
| 24    | **      | ***   | *              | ***         | ***         | ***           | ***          | ***           | ***           | ***  |
| 25    | d       | d     | d              | d           | d           | d             | d            | d             | d             | d    |
| 26    | d       | d     | d              | d           | d           | d             | d            | d             | d             | d    |
| 27    | -       | -     | -              | -           | -           | -             | -            | -             | -             | -    |

All symbols as in Table III.

In general, at the earlier stages, ciliated cells were mostly four- or five-sided. Only in later stages did elongated cells become common and these were not seen in some species. As a semi-quantitative summary of the data, Table XIII shows the distribution of the different cell shapes in each species and at a selection of sites in each species.

Where elongated cells occurred, they were commonest on the lateral and ventral side of the trunk. Since elongated ciliated cells have not previously been reported, we present a selection of images of these cells in Figure 7.

In most cases, elongated cells were orientated perpendicular or slightly oblique to the body axis, but in a few cases, they were parallel to the body axis. On the external gills, orientation of these cells was parallel or oblique to the axis of the gill in some species, but at right angles to the axis in others.

Table IX. Ciliated cell distribution in *R. temporaria*.

| Stage | Nostril | Mouth | Adhesive gland | Ventral head | Dorsal head | External gill | Dorsal trunk | Ventral trunk | Lateral trunk | Tail |
|-------|---------|-------|----------------|-------------|-------------|---------------|--------------|---------------|---------------|------|
| 15    | *p      | *p    | *p             | *p          | *p          | *p            | *p           | *p            | *p            | p    |
| 16    | *p      | *p    | *p             | *p          | *p          | *p            | *p           | *p            | *p            | p    |
| 17    | *p      | *p    | *****          | **          | **          | ***           | ***          | ***           | ***           | ***  |
| 18    | ***     | ***   | *****          | ***         | ***         | ***           | ***          | ***           | ***           | ***  |
| 19+   | *****   | ***** | *****          | ***         | ***         | ***           | ***          | ***           | ***           | ***  |
| 20+   | *****   | ***** | *****          | ***         | ***         | ***           | ***          | ***           | ***           | ***  |
| 21    | *****   | ***** | *****          | ***         | ***         | ***           | ***          | ***           | ***           | ***  |
| 22    | *****   | ***** | *****          | ***         | ***         | ***           | ***          | ***           | ***           | ***  |
| 23    | *****   | ***** | *****          | ***         | ***         | ***           | ***          | ***           | ***           | ***  |
| 24    | *****   | *     | ***            | ***         | ***         | ***           | ***          | ***           | ***           | ***  |
| 25/26 | *****   | 0     | **            | ***         | **          | d             | **          | **            | **            | 27   |

All symbols as in Table III.
In general, cilia appeared one stage earlier on the dorsal side of the body than on the ventral. As development proceeded, cilia on individual surface cells increased in number and in length, until around the stage of hatching. The pattern of cilia resorption appeared to differ in different species. For example, amongst the bufonids, in *B. beebei* and *B. marinus*, cilia were lost first from the periphery of each cell and later from the centre; whereas in *B. bufo* and *B. viridis*, cilia loss occurred evenly all over the cell. In some hylids,

**Table XI. Mean ciliated cell density and duration indices for each body region.**

| Body region        | Mean ciliated cell high density index | Mean ciliated cell high density duration index | Combined density and duration index |
|--------------------|--------------------------------------|-----------------------------------------------|-------------------------------------|
| Nostrils           | 3.8                                  | 6.7                                           | 25.5                                |
| Mouth              | 3.5                                  | 3.4                                           | 11.9                                |
| Adhesive gland     | 4.2                                  | 4.9                                           | 20.6                                |
| Ventral head       | 3.5                                  | 6.9                                           | 24.2                                |
| Dorsal head        | 3.5                                  | 3.0                                           | 10.5                                |
| External gills     | 3.5                                  | 4.8                                           | 16.8                                |
| Dorsal trunk       | 3.4                                  | 4.3                                           | 14.6                                |
| Ventral trunk      | 3.5                                  | 5.2                                           | 18.2                                |
| Lateral trunk      | 3.5                                  | 4.7                                           | 16.5                                |
| Tail               | 3.5                                  | 3.1                                           | 10.9                                |

For each body region, the density and duration indices for each species were summed, and the means then calculated. The absence of adhesive glands in two species has been taken into consideration. The combined index is the product of the density and duration indices.

**Growth and resorption of cilia: patterns and timing**

In general, cilia appeared one stage earlier on the dorsal side of the body than on the ventral. As development proceeded, cilia on individual surface cells increased in number and in length, until around the stage of hatching. The pattern of cilia resorption appeared to differ in different species. For example, amongst the bufonids, in *B. beebei* and *B. marinus*, cilia were lost first from the periphery of each cell and later from the centre; whereas in *B. bufo* and *B. viridis*, cilia loss occurred evenly all over the cell. In some hylids,

**Table X. Mean ciliated cell high density and duration indices for each species.**

| Species          | Mean ciliated cell high density index | Mean ciliated cell high density duration index | Combined duration and density index |
|------------------|--------------------------------------|-----------------------------------------------|-------------------------------------|
| *B. beebei*      | 3.3                                  | 3.8                                           | 12.5                                |
| *B. bufo*        | 4.0                                  | 8.8                                           | 35.2                                |
| *B. marinus*     | 3.6                                  | 4.7                                           | 16.9                                |
| *B. viridis*     | 3.6                                  | 8.3                                           | 29.9                                |
| *X. laevis*      | 3.8                                  | 1.2                                           | 4.6                                 |
| *H. crepitans*   | 3.5                                  | 5.3                                           | 18.6                                |
| *H. boans*       | 3.5                                  | 4.8                                           | 16.8                                |
| *H. geographica* | 4.4                                  | 8.1                                           | 35.6                                |
| *H. microcephala*| 3.1                                  | 1.5                                           | 4.7                                 |
| *H. minuta*      | 3.4                                  | 1.6                                           | 5.4                                 |
| *H. minuscula*   | 3.4                                  | 4.4                                           | 15.0                                |
| *P. trinitatis*  | 3.5                                  | 4.1                                           | 14.4                                |
| *P. venulosa*    | 3.9                                  | 4.7                                           | 18.3                                |
| *L. fuscus*      | 4.1                                  | 3.3                                           | 13.5                                |
| *P. pustulosus*  | 3.6                                  | 3.6                                           | 13.0                                |
| *L. validus*     | 3.9                                  | 5.0                                           | 19.5                                |
| *E. ovalis*      | 3.2                                  | 3.3                                           | 10.6                                |
| *R. temporaria*  | 3.8                                  | 6.4                                           | 24.3                                |

For each species, the density and duration indices for each body region were summed, and the mean then calculated. The absence of adhesive glands in two species has been taken into consideration. The combined index is the product of the density and duration indices.
Cilia resorption began at the anterior side of the cell, before progressing to the posterior. Examples of resorption patterns are shown in Figure 8.

Ciliated cell regression occurred early along the pathways of neuromast migration and appearance, an observation previously made by Smith et al. (1988) and confirmed in the species examined here (Figure 9).

Table XIV collates data on the timing of ciliated cell expression at one location (ventral trunk) in the different species studied. We used *X. laevis* as a reference point from which to assess changes in timing of ciliated cell onset and offset and classified timing changes (heterochrony) using the terms in Alberch et al. (1979). We chose *X. laevis* as reference point because of the basal position of the pipid frogs in anuran evolution, but we are not seeking to make any phylogenetic inferences from these data.

**Ciliated cell size**

In all species, ciliated cells started small in area, then grew, and finally declined. Figure 10a, b shows the pattern of size changes in a selection of species.

**Ciliary movements, embryo rotation and gliding**

Water currents generated by ciliary movements were observed closely in three species (Table XV; Figure 11) and less systematically in a few others. Prior to hatching, particles were moved rapidly from the anterior to the middle of the embryo, and then outwards to the vitelline membrane, then back to the anterior end. This was the basic pattern in the three species studied in detail, and in the few others where observations were possible. In *P. trinitatis* which hatches late (stage 23 onwards), ciliary movements within the capsule were very strong and particularly obvious in a proximo-distal direction along the elongated external gills.
Currents declined over the body after hatching, but at different times on different regions. The current around the nostrils was difficult to detect once buccal movements began around stage 25.

Embryo rotation results are shown in Table XVI. Rotation was by no means universal and even where it occurred in a species, was not seen in all individuals. In general, rotation rate slowed as embryos developed.

Post-hatching gliding movement results are shown in Table XVII. Gliding occurred only over a narrow range of stages, and was not detected in all species or all individuals within a species. The results suggest a general decline in gliding rate as development proceeds. Gliding was seen in larvae lying on the bottom of a dish. However, most larvae after hatching were attached to the sides of their container by their adhesive glands and hung vertically and motionless; alternatively, they hung from the water surface film. An exception is *L. fuscus*, which has no adhesive glands, but it normally remains in the foam nest till beyond the stages when gliding occurs. *P. trinitatis* does not hatch till after the stages when gliding occurs in other species, and post-hatching *P. trinitatis* were never observed to glide.

**Discussion**

The results section details the surface ciliation patterns of 20 species of embryos and larvae from six anuran families. We have not been able to collect every relevant stage from every species, owing to the problems of field collections, especially in the tropics, where amphibians pass through their embryonic stages very quickly. However, we feel that the results are complete enough to allow some firm conclusions.

To discuss the significance of the data, we have divided this section into four parts, relevant to the questions outlined at the end of the Introduction.
Figure 7. Elongated ciliated cells at different locations. (A) *B. viridis*, stage 18/19, ventral side of the trunk before cell elongation; (B) *B. viridis*, stage 23, ventral side of the trunk after cell elongation; (C) *P. trinitatis*, stage 22, tail; (D) *H. crepitans*, stage 20, ventral side of the trunk; (E) *P. trinitatis*, stage 20, lateral side of the trunk; (F) *B. viridis*, stage 22, ventral side of the trunk. Scale bars: 500 μm (A–D); 120 μm (E); 15 μm (F).
As we noted in the Introduction, a recently identified function for ciliated cells in mammal and bird embryos, and possibly also in fish and amphibians (Essner et al. 2002), is the generation of left–right patterning. However, this appears to be a function of short-lived and highly localized mono-ciliated cells, which are distinct from the multi-ciliated cells we have described. We will not discuss this function further.

A function suggested from the literature on fish is some relationship to the lateral line system. Webb and Northcutt (1991) found halos of ciliated cells round lateral line organs.
in *Polypterus* and *Acipenser*. However, in studies on the development of topography of the lateral line system in anurans and urodeles, Lannoo (1987a, 1987b) and Smith et al. (1988) found an inverse relationship between ciliated cells and lateral line organs. The appearance of lateral line organs at the skin surface was preceded by localized disappearance of ciliated cells, and even when ciliated cells persisted into later stage larvae, as in pond-dwelling urodeles, ciliated cell patches were always between lines of neuromasts, never within the lines. Smith et al. (1988) regarded the functional significance of these observations as “relatively clear”: since lateral line organs act to detect movements in the surrounding water, any movements generated by nearby ciliated cells would provide confusing information. Our observations on the loss of ciliated cells as lateral line organs develop are in accordance with those of Smith et al. The possible role of the ciliated cells described by Webb and Northcutt (1991) is puzzling in this light.
Functions previously suggested for amphibian embryo surface-ciliated cells are the movement of surface mucus films; the prevention of attachment of micro-organisms and debris; enhancement of respiratory gas exchange by the reduction of “dead-space” in surface fluid films; movement of the body in the form of pre-hatching rotation and post-hatching gliding; and assessment of the quality of water flowing over the external gills (Assheton 1896; Kessel et al. 1974; Burggren 1985; Altig and McDiarmid 1999). Do the patterns we have observed help to elucidate these and any other possible functions?

Table XIV. Differences in onset and offset times for ciliated cells.

| Species            | Type of heterochrony | Duration of ciliated cell distribution (ventral side of the trunk) for developmental stages 12–29 |
|--------------------|----------------------|---------------------------------------------------------------------------------------------------|
|                    |                      | 12  13  14  15  16  17  18  19  20  21  22  23  24  25  26  27  28  29                           |
| Bufo beebei        | Pre-displacement     | ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** |
| Bufo marinus      |                      | Missing                                                                                           |
| Bufo bufo         | Pre-displacement/progenesis | ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** |
| Bufo viridis      | Pre-displacement/hypermorphosis | ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** |
| Xenopus laevis    | Model                | ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** |
| Hyla boans        | Post-displacement/progenesis | ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** |
| Hyla crepitans    | Progenesis           | ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** |
| Hyla geographica  | Pre-displacement/progenesis | * ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** |
| Hyla microcephala | Progenesis           | ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** |
| Hyla minuta       | Post-displacement    | ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** |
| Hyla minuscula    | Post-displacement/hypermorphosis | ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** |
| Phyllomedusa trinitatis | Pre-displacement/hypermorphosis | * ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** |
| Phrynohyas venulosa | Progenesis          | ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** |
| Leptodactylus fuscus | Post-displacement  | ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** |
| Leptodactylus validus | Post-displacement | ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** |
| Leptodactylus bolivianus |                | Missing                                                                                           |
| Physalaemus pastosus | Hypermorphosis    | * ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** |
| Elachistocleis ovalis | Pre-displacement | * ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** |
| Rana temporaria  | Hypermorphosis       | ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** |

In assigning heterochronic changes, *X. laevis* is taken as the model. *Specimens examined were at an intermediate stage; **specimens clearly at a particular Gosner stage.

Functions previously suggested for amphibian embryo surface-ciliated cells are the movement of surface mucus films; the prevention of attachment of micro-organisms and debris; enhancement of respiratory gas exchange by the reduction of “dead-space” in surface fluid films; movement of the body in the form of pre-hatching rotation and post-hatching gliding; and assessment of the quality of water flowing over the external gills (Assheton 1896; Kessel et al. 1974; Burggren 1985; Altig and McDiarmid 1999). Do the patterns we have observed help to elucidate these and any other possible functions?
Respiration. In an investigation of respiration in gelatinous egg masses of the pickerel frog *Rana palustris* Le Conte, Burggren (1985) found, contrary to previous reports, that the centres of egg masses did not become hypoxic. He measured the patterns of convection of fluid within the egg capsules, generated by embryonic surface ciliated cells, and concluded that these fluid movements were important in assisting gas exchange. The fluid convection pattern Burggren figured, essentially from anterior to posterior along the body, then back to the anterior end further from the body, is similar to what we observed but we additionally noted a current along the length of the external gills. We did not attempt to measure changes in rate of fluid currents, but Burggren found some differences with stage, with a peak at stage 20 and a slight decline to stage 21, when hatching occurred. Burggren comments that the cilia are lost subsequent to hatching and he did not attempt to measure post-hatching fluid movements. However, as Kessel et al. (1974) noted for *Rana pipiens* and we confirm for many more species, hatching does not actually mark the decline of surface ciliation: our Table XII shows that maximum surface ciliation usually occurs in the stage of hatching or in one or two subsequent stages, and Tables III–IX show that loss of ciliation occurs differentially over the body surface, but mainly at stages 24/25, well after hatching in most species. Table XV shows that fluid currents continue to be generated from hatching to around the time of operculum closure (stage 25).
Seymour and Bradford (1995) reviewed the respiratory problems of amphibian egg masses: they noted a number of mechanisms for generating fluid convection movements throughout the egg mass, or alternative egg mass designs, such as foam nests—these help aerate the mass as a whole. Once oxygen reaches to the surface of an individual egg, it has to diffuse through the jelly layer but once in the perivitelline fluid, it is moved by the cilia-generated convection currents. Strathmann and Chaffee (1985) report cilia-generated convection currents in the large jelly masses of some marine invertebrates and also suggest a respiratory function.

Table XV. Water currents generated by ciliary movements in three species of embryos/larvae.

| Species      | Stage | Patterns of currents                                                                 |
|--------------|-------|--------------------------------------------------------------------------------------|
| *R. temporaria* | 17    | Anterior–posterior current, strongest at the anterior end and oblique/ventral at distal end of trunk. |
|              | 18    | Tail current now begins: proximo-distal.                                             |
|              | 19    | Current strongest over the head, especially ventral and lateral sides.               |
|              | 20    | Developing external gills now have a current from base to tip.                      |
|              | 21    | Current on posterior trunk and proximal tail now oblique/ventral.                   |
|              | 22    | A new current now occurs around the recently formed nostrils. Its direction is toward the outside of the embryo. |
|              | 23–26 | Two current directions now on the tail: on the ventral side, strong oblique posterior/ventral; dorsal weaker, oblique posterior/dorsal. |
|              | 27    | Current only visible, weak and intermittent, at proximal end of tail.               |
| *X. laevis*  | 15    | Weak anterior–posterior current around head.                                        |
|              | 16    | Anterior–posterior current around head, and oblique ventral anterior–posterior on lateral sides of the body. |
|              | 17    | Similar currents, but stronger, especially around the head.                          |
|              | 18    | Anterior–posterior currents now over head, body and tail, strongest on the ventral side of the trunk. |
|              | 19    | Current on the lateral side of the trunk now oblique dorso-ventral and posterior.   |
|              | 21    | Strong current around head, including nostrils and over gills from base to tip. On lateral sides of trunk, current still oblique. Current weak on tail, still proximo-distal. |
|              | 22    | On tail, two currents, oblique ventrodorsal on dorsal side, and dorsoventral on ventral side. |
|              | 23    | Currents still strong around head, including nostrils. Weaker but persistent on tail. |
|              | 24    | Currents weak on the body.                                                          |
|              | 25    | Currents now weak around the head, and no longer occur elsewhere on body.           |
| *Bufo bufo*  | 14    | No currents visible.                                                                |
|              | 15    | Anterior–posterior current already detectable.                                      |
|              | 18    | Anterior–posterior current at anterior end, oblique/ventral at posterior end.        |
|              | 20    | Ventral currents now stronger than previously; now oblique dorso–ventral on lateral side of trunk and proximal part of tail. |
|              | 21    | Very strong currents on lateral side of head and around the nostrils.               |
|              | 22    | Currents on tail now weak.                                                          |
|              | 24    | No current now on ventral side of trunk; currents persist elsewhere, but very weak on tail. |
|              | 25    | Current now visible between eyes, on lateral sides of the trunk and proximal part of tail. |
|              | 27–28 | Proximal end of tail still shows a weak intermittent current.                       |
Figure 11. Ciliary water currents in different stages of *R. temporaria* embryos and larvae. (A) stage 16; (B) stage 17; (C) stage 18; (D) stage 19; (E) stage 21; (F) stage 22; (G) stage 24; (H) dorsal side; (I) ventral side, stage 26; (J) stage 27. Not all to the same scale. Arrows indicate the observed currents.
The existing literature and our observations clearly support a respiratory function for cilia prior to hatching, although as Burggren (1985) notes, there has been no cost analysis of ciliary function: not only do they require energy, they must also consume oxygen.

Post-hatching, Kessel et al. (1974) suggest that surface ciliation may have a respiratory value still since the hatchlings are generally quiescent, their external gills regress and the internal gill apparatus does not become functional till around stage 25. In addition, Bradford and Seymour (1985) and Seymour and Bradford (1995) report that oxygen consumption rises immediately after hatching in several amphibian species: they suggest

### Table XVI. Embryo rotation rate, frequency and stages in different species.

| Species (temperature) | Stage | Proportion showing rotation (%) | Time for complete rotation ± SD, s (n) |
|-----------------------|-------|---------------------------------|----------------------------------------|
| *R. temporaria* (22–24°C) | 16 | 40 | 50 ± 5 (5) |
|                        | 17 | 30 | 90 ± 4 (4) |
|                        | 18 | 30 | 170 ± 13 (5) |
| *X. laevis* (22–24°C) | 16 | None | |
|                        | 17 | None | |
|                        | 18 | 40 | 86 ± 60 (5) |
|                        | 19 | 80 | 127 ± 63 (4) |
| *B. bufo* (22–24°C) | 13 | None | |
|                        | 14 | 85 | 85 ± 15 (4) |
| 15–16 | Not measured | |
| 17 | 40 | 90 ± 12 (4) |
| *H. minuta* (28°C) | 16 | None | |
| 17 | 25 | 169 ± 38 (3) |
| 18 | None | |
| *P. trinitatis* (28°C) | 16 | None | |
| *P. pustulosus* (28°C) | 15 | None | |
| 18 | None | |
| *B. beebei* (28°C) | 15 | None | |

### Table XVII. Post-hatching gliding movements.

| Species (temperature) | Stage | Speed of gliding ± SD, mm min⁻¹ (n) |
|-----------------------|-------|-------------------------------------|
| *R. temporaria* (22–24°C) | 21 | 20 ± 6 (3) |
|                        | 22 | 3 ± 1 (3) |
|                        | 23 | None |
| *X. laevis* (22–24°C) | 19 | 13 ± 9 (4) |
|                        | 21 | 4 ± 1 (12) |
|                        | 22 | 3 ± 3 (9) |
| *B. bufo* (22–24°C) | 18 | 10 ± 8 (6) |
|                        | 19 | 28 ± 10 (5) |
|                        | 20 | 20 ± 11 (8) |
|                        | 22 | 17 ± 8 (4) |
| *H. minuta* (28°C) | 19–20 | Negligible |
| 22–23 | Occasional |
| *L. fuscus* (28°C) | 20 | 8 ± 1 (2) |
| 22 | 3 ± 2 (3) |
| *B. beebei* (28°C) | 19 | 30 ± 13 (3) |
| 20 | 37 ± 16 (4) |
| 21 | Negligible |
| *P. venulosa* (28°C) | 20 | 9 ± 1 (3) |
| 21 | None |
| *E. ovalis* (28°C) | 20 | None |
Figure 12. Ciliated cell patterns around the nostrils. (A) *B. viridis*, stage 25; (B) *X. laevis*, stage 23/24; (C) *B. bufo*, stage 24; (D) *P. venulosa*, stage 27; (E) *L. bolivianus*, stage 23; (F) *R. temporaria*, stage 23. Scale bars: 500 $\mu$m (A, E); 250 $\mu$m (B); 35 $\mu$m (C); 120 $\mu$m (D, F).
that oxygen consumption just prior to hatching has probably been limited by the physical characteristics of the egg mass, but that this constraint is removed by hatching. Given the factors noted above and the fact that most amphibians hatch into still water, an increase in oxygen consumption would seem to require a ventilatory mechanism, and surface ciliation could provide it.

An important factor to consider here is the effect of differences in egg size. On conventional surface area/volume ratio arguments, we would expect large eggs to have more difficulty in obtaining adequate oxygen than small eggs, since they are dependent on diffusion through the egg surface, and the relative surface area is smaller in larger eggs. However, Einum et al. (2002) have challenged this argument in the case of salmonid eggs. They found that in low oxygen conditions, small eggs had higher mortality than large eggs. They also found that the scaling factor for the relationship between egg size and oxygen consumption was a low 0.44, rather than the value (1.0) expected if oxygen consumption simply increases proportional to egg mass. A possible interpretation of this result is the presence of non-metabolizing yolk: in addition, the living tissue in a fish egg is a relatively thin surface layer surrounding the yolk mass. With holoblastic cleavage and intracellular yolk, we might expect amphibian eggs to scale somewhat differently. For amphibian eggs, Seymour and Bradford (1995) also found a low scaling factor (0.52) in an interspecific comparison. They concluded that the rate of development of larger amphibian eggs is limited by the rate of diffusion of oxygen through the capsule, and that for amphibians, there is a trade-off as eggs increase in size between the respiratory need for a thin capsule and the structural integrity requirement for a relatively thick one.

How do these considerations affect surface ciliation in eggs of different size? We suggest that denser ciliation could produce stronger currents and increase embryo surface ventilation rate, but, if the main limitation in large eggs is the oxygen diffusion rate through the capsule, then increased ciliation is unlikely to make much difference. To test this hypothesis, we correlated the egg size data from Table II with the combined ciliation duration and density index data from Table X but there was no relationship, suggesting that there is no simple link between egg size and levels of surface ciliation. Another possible factor that may interact with surface ciliation is incubation environment. Most amphibian eggs develop in still water, but some are on land in foam nests or in burrows. Seymour and Bradford (1995) suggest that foam nests, in surrounding eggs with thin-walled air-filled bubbles are providing an abundant and immediate oxygen supply. This might suggest that foam-nesting species could have less need of cilia than aquatic spawners. Table X shows that the two foam-nesting species for which data were most complete, L. fuscus and P. pustulosus, had relatively low ciliation indices. It is therefore frustrating that our data for the two other foam-nesters, L. bolivianus and L. validus, were so incomplete.

Another exceptional species in our sample is P. trinitatis. Its eggs form a rather solid mass enfolded in a leaf above water. Jelly capsules lie between eggs, rather than the jelly forming a thick layer around each egg (Agar 1910). Although the egg mass is out of water it is enclosed by the leaf and by top-and-bottom jelly plugs: it might therefore be expected to suffer some oxygen limitation, and the unusual arrangement of the jelly capsules may be designed to alleviate this. Unfortunately, in considering the likely effects on ciliation density, other factors are the rather large size of P. trinitatis eggs, their late hatching stage and rather long incubation period (Table II). Table X shows a middle-of-the-range ciliation density index for this species.

The largest eggs in our sample were from the direct-developing species E. urichi. Callery et al. (2001) have pointed out that development in Eleutherodactylus involves a complex mix
of features: some larval-specific structures such as adhesive glands, teeth, jaws, and the lateral line have been deleted entirely; others such as the external gills, operculum and tail have been greatly reduced or highly modified. Early reports on ciliation in *Eleutherodactylus* embryos were mixed: Gitlin (1944) saw none in *E. portoricensis* Schmidt (regarded by Townsend and Stewart 1985 as *E. antillensis* Reinhardt and Lütken) but Adamson et al. (1960) reported “numerous” ciliated cells in skin sections of *E. martinicensis* Tschudi (Townsend and Stewart 1985 re-name this as *E. johnstonei* Barbour) and Bayley (1950) observed cilia-generated currents at the surface of embryos removed from their surrounding membranes. More recent studies on the genus have concentrated on *E. coqui* Thomas, where Richardson et al. (1998) figure large numbers of ciliated cells on the dorsal surface of embryos from TS stages 4–7. The study by Richardson et al. (1998) was on the development of limb buds and they made no particular comment on the ciliated cells. As far as we know, our results are the first to map ciliated cell distribution and to assess the stage duration of their occurrence in an eleutherodactylid embryo.

As noted in the results, ciliation in *E. urichi* persisted well past the equivalent to hatching stage in conventional embryos (Gosner stage 17/18, equivalent to TS stage 4). This is not unexpected if a prime function of ciliation is to assist respiration in the pre-hatching embryo. Highest ciliated cell densities were found on the lateral side of the head, essentially the pharyngeal region, and on the tail “fins”. In eleutherodactylid embryos, the tail fins are thin-walled, highly vascularized and laterally expanded structures, thought to have a respiratory function. The occurrence of abundant ciliated cells on the pharyngeal region (external gills are reduced or absent in eleutherodactylids—Townsend and Stewart 1985) and on the tail fins supports the hypothesis that ciliated cells are involved in assisting respiration. Bayley (1950) expected ciliation to decline once the tail became important in respiration, but our results show that the tail itself is ciliated. However, ciliated cells have regressed some time prior to hatching (note that incubation in *E. urichi* is prolonged, about 26 days at 25°C) though the metabolic needs of the embryo would be expected to rise as more and more yolk is converted to metabolizing tissues. A possible explanation lies in Townsend and Stewart’s (1985) observation that from stage 9 onwards, the tail begins to thrash about, increasingly strongly as development proceeds: internal fluid movements therefore no longer require ciliated cells.

**Rotation and gliding.** Kessel et al. (1974) regarded the function of pre-hatching cilia as the movement (rotation) of the embryo within its envelopes. Assheton (1896) was first to measure post-hatching gliding in *R. temporaria* larvae placed on their sides on the bottom of a dish. Our results (Tables XVI, XVII) indicate considerable variability in the occurrence and speeds of these movements. In our view, the very patchy occurrence of rotation suggests that this movement is a by-product of directional patterns of ciliary beating whose actual function is to move fluid for mainly respiratory purposes. Rotation only occurs when the embryo is fully free within its capsule and still essentially spherical. As soon as it elongates, it makes contact with the vitelline membrane, and rotation is blocked. There is no evidence that rotation is essential for development.

Gliding is probably also a by-product of fluid flow, but it may, nevertheless, be useful. On hatching, most amphibian larvae attach using their adhesive glands to the surface film of the water, or to vertical surfaces such as rocks or vegetation. Active movement by rapid muscular twitches can occur even before hatching and, post-hatching, can propel larvae through the water and occurs if they lose their attachments or in response to sudden light dimming (Jamieson and Roberts 2000). But such movement may make larvae highly
conspicuous to predators. We therefore suggest that inconspicuous gliding across the substratum, generated by surface ciliation, has a value in allowing larvae to find good attachment sites.

Twitty (1928) found that tadpoles could reverse the direction of ciliary gliding movement when confronted with an obstacle. However, it is not clear whether gliding movement is generally controllable in its directionality: Twitty regarded the response as localized to the particular ciliated cells that were obstructed.

Mucus and debris movements. Assheton (1896) noticed that ciliary currents were particularly strong in the region of the adhesive glands, and suggested that their function was to propel the mucus secretion away from the gland. A not dissimilar idea is that ciliary currents keep particles from adhering to the epidermis. It is certainly true that the adhesive glands, in all species that have them, are sites of high ciliated cell density (see Table X), though less than the nostrils and the ventral head region. It is not immediately obvious what function moving the adhesive gland secretion could serve: after hatching, these glands help hold the larvae in position.

Similarly, particle movement hardly seems a vital function. Before hatching, any particles within the vitelline membrane must be cellular debris. After hatching, bacteria and fungal spores may attach to the epidermis and could be harmful, but this is the time when cilia decline and for most of the larval period, amphibian larvae lack surface ciliation.

Nostril ciliation. Table X shows that the epithelium surrounding the nostrils was consistently both the most highly ciliated area, and the area where ciliation persisted the longest, well into the post-hatching period. This was the most unexpected result of this study, since previous work on amphibian surface ciliation had not reported anything similar, though Assheton (1896) recorded a well-defined current which dips into the nasal depressions. He remarked that this current was swifter than any other, except that associated with the cement glands, and that it persisted until operculum closure (Gosner stage 25): Assheton concluded: “whether this epithelium (i.e. olfactory) at this stage plays any part in testing the water before it reaches the gills must be left to conjecture”. Figures 2a and 12 show images of nostril ciliation patterns from different species.

Does nostril ciliation have any functional significance? It is well known that tadpoles use mainly water-borne chemical cues, detected by their olfactory organs, to detect predators and conspecifics (in species which form shoals) (Kats et al. 1988; Stauffer and Semlitsch 1993; Hoff et al. 1999). Predator recognition studies have mainly been on well-grown tadpoles: Stauffer and Semlitsch studied stage 30/32 Rana tadpoles; Kats et al. looked at several species, mainly stage 30+ but some as early as stage 25, but they did not look at the ontogeny of predator recognition. Hoff et al.’s review of kin recognition studies show great variability, but at least some species recognize kin via water-borne chemical factors, and in some cases, early ontogenetic factors influence kin recognition—though the mechanism is not known.

Studies on olfactory system development (Klein and Graziadei 1983; Hansen et al. 1998) show that the olfactory epithelium is well developed and connected to the brain soon after hatching in X. laevis (around G21/22). Detailed developmental studies seem not to have been carried out on other amphibian species. Our suggestion is that the olfactory epithelium is capable of receiving and transmitting chemical information from soon after hatching in most anurans. Chemical signals are brought to the olfactory organs by the
Surface ciliation of anuran embryos

Ciliated cell shape, orientation, size, and regression. In the results section, we showed that ciliated cells generally increased in size up to about the stage of hatching and that they often also changed shape, a frequent alteration being from four- or five-sided to highly elongated. Furthermore, elongated cells were not randomly arranged: in general, the long axis of the cell was at right angles to the direction of the current generated along that part of the body. These features have not been pointed out before, though elongated ciliated cells are visible, with no comment, in some published micrographs, for example Smith et al. (1988), Figure 11, cells on the surface of Ambystoma; Kessel et al. (1974), Figure 27, elongated cells on the surface of R. pipiens; Assheton (1896) also showed elongated cells on the tail, where cilia work across the shorter axis of the cells. It is unclear what the functional significance of these size and shape changes is, though the obvious hypothesis is that the changes are related to alterations in the direction and strength of the currents generated by the cilia.

Loss of ciliated cells is clearly developmentally regulated, occurring at a characteristic stage in each species and at each location. Smith et al. (1988) noticed that ciliated cells disappeared just prior to the appearance of lateral line organs and suggested that some kind of inhibitory influence from the neuromasts led to cilia regression. This, however, is a very local effect, and in some embryos/larvae, ciliated cells persist between the lateral lines well after their appearance. Lannoo (1987b) detected a difference in persistence of ciliated cells between still water (lentic) and flowing water (lotic) urodeles, with cilia occurring on the surface of well-advanced lentic larvae, but not on lotic forms.

In our sample, the two species most commonly occurring in flowing water are H. boans (obligate) and H. geographica (rivers and ponds): comparing ciliated cell persistence in these two species with pond-spawning hylids, there is some evidence for ciliated cells remaining till later stages in the pond dwellers, but it is not a major effect. Lannoo (1987b) did not comment on possible reasons for his finding, but it would be consistent with a post-hatching respiratory role for ciliated cells, being more necessary in still than in flowing water. However, as noted earlier, H. geographica has one of the highest ciliated cell indices in our sample.
Ciliation pattern differences: species and family comparisons

Our sample spans six families, three represented by single species and three families represented by four or more species. Unfortunately, missing stages in some cases make comparisons incomplete. We therefore simply note that careful inspection of the data for our best represented family, the Hylidae, shows very considerable inter-specific differences in ciliation patterns.

Heterochrony

Table XIV presents our tentative findings on timing changes in the occurrence of ciliated cells at a single site, the ventral side of the trunk (tentative because we did not always manage to collect the earliest or latest stages where ciliated cells occurred).

There is no agreed phylogeny of tadpole types (see Cannatella 1999 for discussion), but most authors regard the pipids as primitive compared to neobatrachians: we therefore present the data as a comparison with our only pipid, *X. laevis*.

It is clear that the onset and offset of surface ciliation has altered across our range of species, relative to the stage of development of the features used in Gosner’s (1960) staging system. For example, surface ciliation begins early in all the bufonids, and a little late in the leptodactylids and some of the hylids. Both late and early offset occur in a scattering of species. We have already discussed Lannoo’s (1987b) suggestion that late offset of surface ciliation may be environmentally related. Until more complete data are available, further discussion is premature. Lannoo concluded that there was no evidence for heterochronic change in the development of the lateral line in the urodeles and anurans that he examined.

Assheton (1896) reported the persistence of surface ciliation on the tails of *R. temporaria* until just before hatching. We have not made systematic observations on surface ciliation past Gosner stage 27, but we have noticed persistent tail ciliation in some species, though not in others (data not shown). This aspect needs further investigation.

Fixity of ciliation pattern development

We made one attempt to test whether the ontogeny of ciliation patterns could be environmentally modified. *Leptodactylus fuscus* at hatching were kept in their foam nests (as can happen normally) or transferred to water with food (as can also happen normally). Tadpoles kept in the foam nest eventually enter a form of developmental arrest, though some aspects of progressive development continue, such as hatching gland regression (Downie 1994a). Our results showed no difference in persistence and regression of ciliated cells between the two treatments.

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