Morphometric study of the regeneration of individual rays in teleost tail fins

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ABSTRACT

The results obtained using morphometric variables which describe fin ray regeneration patterns are reported for individual fin ray amputations in the goldfish (*Carassius auratus*) and zebrafish (*Brachydanio rerio*). Classical and updated experiments are compared to verify previous morphogenetic models of cell tractions (Oster et al. 1983) or epidermis-mesenchyme induction (Saunders et al. 1959) applied to the limb of other vertebrates. Position-dependent patterns within the fin of *Carassius auratus* are analysed under a comparative protocol using morphometric methods. Conditions in which the apical epidermis is separated from blastema may differentiate small fin rays, thus suggesting this epidermis is involved in blastemal formation. Blastemal cells differentiating as lepidotrichia forming cells (LFCs) may also be related to morphological changes in covering epidermis. Long-range interactions from neighbouring fin ray blastemas or short-range interactions within the blastema, may be postulated through the analysis of segmentation.

Key words: Fin ray regeneration; induction; pattern formation.

INTRODUCTION

Both vertebrates and invertebrates possess regenerative capabilities which allow them to restore their morphology following amputations or grafts of particular parts of their bodies. Morphological regeneration is effective in adapting these populations to changing/competitive local environment. Extensive embryological and experimental studies have been carried out to reveal the underlying cellular and genetic control mechanisms. Vertebrate limbs have also been extensively analysed to generate operative models of cellular interactions controlling both development and regeneration (Hinchliffe & Johnson, 1980; Wallace, 1981). Fin development and regeneration have recently been the subject of genetic analysis, and a number of mutants are now available for the study of these processes (Johnson & Weston, 1995; van Eeden et al. 1996; Haffter et al. 1996 *a*, *b*; Akimenko et al. 1996). Experimental embryology may contribute significantly to unravelling the underlying cellular interactions which are active during the process.

The fin of teleosts is composed of a structural unit, the fin ray, made of dermal bones, the lepidotrichia, each tapered distally by a group of actinotrichia (long rigid rods of a collagen-like protein called elastoidin). Lepidotrichia are segmented and each segment is formed of 2 hemisegments (right and left, in the tail fin) joined by ligaments in a parenthesis-like structure, which surrounds nerve bundles and blood vessels. The whole structure is covered by a typical epidermis (Becerra et al. 1983). After partial ablation of the fin, the stump of each ray regenerates by an epimorphic process. Restoration of the epidermis, blastema formation, cell proliferation and differentiation, are all events that follow excision. In many teleosts, actinotrichia appear after the 5th day at the distal end of the regenerate, and remain in that position during the whole process, which lasts 40–50 d (Becerra et al. 1996).

The present study deals with partial excisions and morphometric studies of the regenerative response, in order to verify our hypothesis of cell and tissue interactions: 'the fin ray is the regenerative unit of the fin, and depends on cell-to-cell interactions to com-

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pletely restore its original pattern'. The regenerating pattern was experimentally disturbed in the goldfish (*Carassius auratus*) and the zebrafish (*Brachydanio rerio*).

MATERIALS AND METHODS

Animals

Thirty-eight specimens of the goldfish *Carassius auratus* L (Cyprinidae, Teleostei) and 46 of the zebrafish *Brachydanio rerio* (Hamilton-Buchanan) (Cyprinidae, Teleostei), have been studied for their capacity for morphological restoration following partial ablations. The morphology of the regenerate of 28 goldfish and 16 zebrafish was studied following the drawing of their profiles in a camera lucida. Fish

Fig. 1. (*a*) Schematic drawings of surgical operations in goldfish and zebrafish fin rays. Control: 70% of the fin was removed in each lobe. 1st group: 3 individual fin rays were ablated in one lobe. The other lobe was cut in some instances as an internal control (see text). 2nd group: following transverse amputation of the fin, some ray blastemas were isolated by daily excision of the interray tissue, shown in the figure as longitudinal lines. (*b*) The drawings shows fin morphogenetic variables measured in the experiment. On the left side, a schematic representation of sequential bifurcations of a single fin ray, from the tail peduncle (lower region) proceeds distally (top); B1 and B2 are 1st and 2nd bifurcation distances from the base of the fin; different segments are magnified to the right. The more proximal segment (lower region) shows *length* (L) corresponding to the proximodistal axis and *width* (w) corresponding to the dorsoventral axis. Mandelbrot diameter (Δ) was measured after D1, D2 and D3 of the bifurcating segment. Bifurcates were considered additively in some instances (Σ areas).

weights ranged between 1 and 4 g (goldfish) and between 0.4 and 0.8 g (zebrafish). A small group of goldfish alevins of different sizes was used to measure the Mandelbrot diametral exponent of the zebrafish (see below). Three one-month old zebrafish embryos were also used.

The animals were kept in tanks of charcoal-filtered water of 100 l. Dark/light periodicity of 12/12 h and temperatures of 21 °C (goldfish) and 28.5 °C (zebrafish) were also controlled during the experiment. Animals were fed with Sera Vipan (Sera, Heinsberg, Germany). Handling and processing of fish were carried out according to principles approved by the Council of the American Physiological Society and National laws in Spain (B.O.E., 67, 1988).

Experiments

Figure 1 shows the experimental groups studied in both species and the morphometric variables measured. Different conditions were obtained in which lateral interactions are affected at the blastema. Animals anaesthetised with Tricaine (MS222, Sigma, St Louis, MO, USA) in a concentration of 62 μg/ml were operated on, following 3 different protocols (Fig. 1*a*).

The experiments performed in goldfish were as follows (Fig. 1*a*). *1st group*: individual fin ray regenerates. Partial ablation of one lobe of the tail fin by cutting individual rays (3rd, 6th and 9th rays, dorsal or ventral) (Goss & Stagg, 1957). The remaining lobe was completely ablated and used as an internal control. Eight specimens were used. *2nd group*: isolated fin ray regenerates. Following complete ablation of the tail fin over approximately 30% of its total length, some selected regenerating rays were separated daily from their neighbouring regenerating rays by indentations made with scissors. The selected ray blastema was thus separated from both the dorsal and ventral neighbouring blastemas during the first 10 d of regeneration. 20 specimens were used. *Controls*. To act as controls, the tail fins of 10 animals were amputated for approximately 30% of their total length and undisturbed regeneration of the fins was allowed.

Experiments in zebrafishes followed similar operations. Eleven specimens were used for the control group and 22 for the 1st group. In addition, individual tail fin ray ablations were performed in *long fin* (*lof*) mutants (7 specimens), as well as in pectoral fins of wild-type individuals (6 specimens).

Statistical analysis was carried out independently in each species, and case study and comparison of
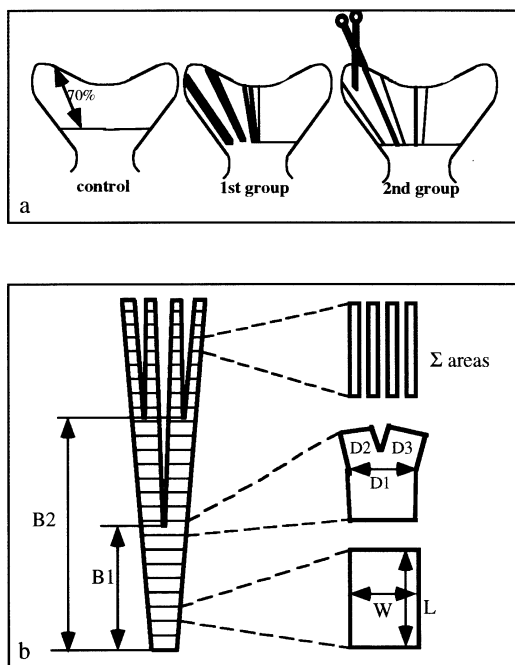
variable behaviour and statistical level of significance were examined in terms of the null hypothesis of equal response.

Morphometric procedures

Regeneration rate was studied on photographs taken every 2 d during the first 45 d of regeneration in goldfish and 10–15 d in zebrafish. Length variation of fin ray regenerates (R) through time (t), as measured from the photographs, was approached by least squares regression to generalised hyperbolic curves of the type $R(t) = Rm(1+wt)(t^c/(b+t^c))$, where Rm (*maximum*) corresponds to the length of the ray before amputation, 'b' and 'c' are also constants and 'w' is the slope corresponding to the growth rate of each ray. An indirect estimation of the value of 'w' was obtained using measurements from 24 non-manipulated specimens of goldfish obtained in advance of our experiment, with a standard body length ranging from 30 to 110 mm. The average values for growth anisometries 'w' obtained from intact 3rd, 6th and 9th tail fin rays (as compared with whole body growth) were the following: ray 3: 0.1484 ± 0.0868; ray 6: 0.1223 ± 0.08221; ray $9 = 0.09757 \pm 0.05513$. b, c, th (th $= b^{1/c}$) constants were obtained by this procedure, defining regeneration rates; where 'th' corresponds to the time needed to attain half the length of whole regeneration. Previous work, showing the relationship between regeneration rates and the underlying control mechanisms, has been published on this procedure (Voit et al 1985; Voit, 1987).

Fin ray segments were drawn in a camera lucida. Perimeter and shape analyses were carried out in an image analyzer device (IBAS 1 computer system). Bifurcation length, measured as the number of segments or as the cumulative length of all segments located before the first bifurcation process, was also estimated. In each case study a univariate comparison of means was also performed, using the *t* test.

Proximodistal series of several morphometric variables, such as segment *length* (proximodistal axis in the tail fin), *width* (dorsoventral axis in the tail fin), and its corresponding *area* (which was estimated by measuring the digitised outline of each hemisegment printed as a negative on photographic paper) were approached by Fourier harmonic analysis. In order to make the variables *width* and *area* independent of the bifurcation processes we measured them additively in neighbour segments once bifurcation had occurred in our proximodistal series (Σ areas in Fig. 1). This methodology of time-series analysis is commonly used to determine if a set of data shows natural periodicities

or cycles. Any time-series data set can be expressed as a combination of fundamental harmonics that are fitted to the data by the method of least squares. In brief, Fourier series consist of trigonometric equations incorporating sines and cosines, which can be used to describe and reproduce, as precisely as needed, any bidimensional curve, provided that only one value in the y-axis corresponds to any value in the x-axis (i.e. the curve must be continuous and single-valued). The shape of the curve (e.g. segment length, width and corresponding area, y-variable) is defined as a function of the position of the segment in the proximodistal axis of the tail fin. Y-variable is estimated from the following equation, which adjusts to expansion of the y-variable as a function of the cumulative length (x-variable) of the curve (x is adjusted from 0 to 2π):

$$y(x) = y_0\left[1 + \sum_{n=1}^{\infty} A_n \cos(nx) + \sum_{n=1}^{\infty} B_n \sin(nx)\right],$$

an equation which is normally used in the following transformation:

$$y(x) = y_0\left[1 + \sum_{n=1}^{\infty} C_n \cos(nx - P_n)\right],$$

with

$$C_n = (A_n^2 + B_n^2)^{1/2} \quad \text{and} \quad P_n = \arctan(B_n/A_n),$$

where 'y_0' is the mean value of the y-variable in the series, 'n' is the harmonic order number, 'c_n' is the harmonic amplitude of the nth-order harmonic and 'P_n' is its phase angle, which determines the starting position of harmonics within the curve (see Palmqvist et al. 1996 for details on the least squares adjustment of these equations).

The precision of Fourier analysis in characterising the shape of a curve is such that this morphometric procedure allows the curve to be split into its geometric components, regardless of its total length and without the necessity of having homologous points (i.e. this methodology allows comparison of regeneration processes between long rays with many segments and short rays composed by a small number of segments). Particularly, the amplitudes of low order harmonics estimate the overall geometric properties of the analysed curve, since the 2nd harmonic represents a periodicity of 2 maxima and 2 minima within the curve, the 3rd describes a 3-fold pattern, and so on (for review and applications, see Ehrlich & Weinberg, 1970; Fox, 1987; Rohlf, 1990; Palmqvist et al. 1996). Such cycles could be related to morphogenetic events (e.g. changes in segment length related to bifurcations).

This approach was used to obtain the mean shape

variation in each experimental situation as described by the first 16 harmonics, after determination of the minimal number of harmonics needed to accurately reproduce curves of fin regeneration.

Diameter relationships of the fin rays before and after bifurcation were also estimated in order to calculate diametral exponents (Mandelbrot, 1983). This relation is of the type $D(1)^\delta = D(2)^\delta + D(3)^\delta$ according to the drawing in Figure 1b. The exponent δ is a functional dimension, suggesting the form of the cell assembly controlling morphogenesis, and it was estimated after $\delta = \ln 2/(\ln D1 - \ln D_M)$, whereas $D_M = (D2 + D3)/2$ and $D2 \approx D3$.

Histology

Whole mount preparations were obtained following staining with Picrosirius red (Becerra et al. 1983). Histological slides were also used to verify 3-dimensional variables. Changes in the form of blastemas were approached to variations in height, width and thickness measured in serial sections of each blastema, as described before for lepidotrichial hemisegments. TEM preparations were obtained according to previous standard protocols (Becerra et al. 1996).

RESULTS

Our results are presented as anatomical descriptions of the resulting regenerates obtained in each operation, and as morphometric analyses of anatomical and histological variables selected as representative of the previous inspection. Both species of teleost used in the present study conformed to the general pattern described below. In order to prevent repetition of similar descriptions throughout the paper, the general morphogenetic pattern of individual fin ray regen-

eration will be described based mainly on our observations in the goldfish; the comparative aspects regarding specific characteristics of the zebrafish will be emphasised when necessary.

The tail fin holds a variable number of *long rays*, ranging from 16 to 20. There also exist about 4–5 *short* dorsal and ventral unbifurcated fin rays. Each *long ray* is segmented and bifurcates twice in the adult individual. All the segments of a ray are of approximately equal length. Fin rays grow both by addition of new apical segments and by segment widening. The first long (dorsal and ventral) rays are unbifurcated.

The zebrafish tail fin is similar to the one of the goldfish. The number of long fin rays is 18 and the bilobed profile arises from the different lengths of each ray.

The regenerates obtained from each operation are described as follows. *Controls*. Complete regenerate of excised fin material of the goldfish is restored in 50 d. Morphological restitution is complete, each fin regenerating approximately its original dimensions, and the bifurcation and segmentation patterns are both restored. The first segment shows an erratic segmentation process, with right-left asymmetry, and is of longer length, caused by a delay in the segmentation process. During the first few days of regeneration, actinotrichia could not be detected under polarised light. *Individual fin ray regenerates.* Following individual fin ray ablations, restitution of final fin ray size is obtained earlier in regenerating rays than in controls (see Table). The regenerated pattern is different, being characterised by a narrower width of each segment, by unperturbed segment lengths, by initial right-left asymmetries, and also by important delays in bifurcation. Actinotrichia do not appear until almost the complete length of the regenerate is restored.

Table. *Mean values of the morphogenetic variables measured in each regenerate. Morphometric and kinetic parameters obtained from animals under each protocol referred to in the text. Regeneration growth curve constants were obtained following linear regression to linearised curves, for a comparison. Mean and standard deviation are also included for each morphometric parameter*

	n	c	Lnb	th	l (%)	W (%)	A (%)	B (%)	Δ
Goldfish									
Control	5	1.5±0.2	4.3±0.5	18.7±4.2	94±16	93.5±29	87±38	23±24	1.01±0.05
1st group	7	2.6±0.7*	6.7±1.1*	14.2±6.7	99±9	42±19*	44±22*	69±48°	N.D.
2nd group	8	1.7±0.2	4.6±0.6	18±7	97±10	90±11	86±13	74±23*	N.D.
Zebrafish									
Control	7	1.9±0.1	4.7±0.1	12.1±1.2	108±10	63±8	66±16	29±9	1.1±0.1
1st group	5	3.3±0.8*	6.3±0.4*	6.7±2.6*	113±10	50±11*	57±9	70±32*	N.D.

The Neperian logarithm of b (Lnb) corresponds to the ordinate origin, c (slope) and th (half regeneration time, expressed as days), n, means number of specimens studied. Δ, Mandelbrot exponential diameter; L, segment length; W, segment width; A, segment area; B, bifurcation point in the proximodistal axis, expressed as percentages of normal fin ray segment values. * and °, significant differences ($\alpha = 0.05$, and 0.1 respectively) with control; N.D., not determined.

The anatomical outcome is as follows. Epidermal wound healing is extensive during the first 2–3 d, sealing the complete hole all along the fin anatomy. This re-epithelisation of the whole wound fills, as a wedge, the space of the missing tissue, thus leaving the apical epidermal fold at the tip of the fin (a long distance away from the blastema formed over the remaining ray stump). This blastema is very particular (probably due to the fact that it has no apical epidermal cap, being in contact with the left and right epithelial coverings, which display a rather conspicuous basement membrane): it is smaller than control blastemas and, as regeneration progresses, it induces a change in the neighbouring epithelium, this change being displaced along the epithelium adjacent to the level where the blastema is located (see below).

This process results in 2 epidermal coverings (left and right), apposed by their basal laminae, with no connective tissue between them. This 'fin fold' is characterised by the absence of nerves, blood vessels, or actinotrichia in between the apposed basal laminae all along the structure of the 'fin fold'. Its formation precedes the regeneration of the connective tissue, which is somewhat delayed as compared with the regeneration that follows complete fin amputation. The regenerating connective tissue (Fig. 2) differentiates blood vessels, which grow from the remaining neighbouring rays towards the blastema. A rapidly distalising blastema is characterised by flattened, peripherally-located fibroblast-like cells (Fig. 3), enclosing a centrally-located extracellular matrix, as observed under transmission electron microscopy. When this rapid process of regeneration is subjected to an increased lag period it differentiates abnormally segmented rays (Fig. 4). The width of these segments is greatly reduced (Fig. 5). In some instances (2 of 24), complete regeneration did not occur, thus suggesting that distal elements had not been determined. In any case, the first segments are very poorly differentiated, showing weakly arranged collagenous fibres as compared with unexcised fin rays, which may be due to disorganisation of the scleroblasts (lepidotrichia forming cells; LFCs) (Fig. 6). There also exist differences in the segmentation process of each hemiray, which only occurs along the first 5–8 segments. Coincident segmentation is an important clue that may permit an analysis of right-left interactions among the cell populations in the distalising blastema (these different populations would correspond to the dorsoventral axis in paired fins).

In general, similar results were obtained in zebrafish. Individual fin ray regenerates were wider than those obtained in the goldfish (although thinner than control regenerates of the zebrafish) and showed normal segmentation (Fig. 7). A process of isolation of rays regenerating out of the fin has naturally occurred in single fin ray regenerates in zebrafish (Fig. 8).

The regeneration of individual tail fin rays shows an interesting effect in zebrafish *long fin* mutants. Whereas individual fin ray segments are narrower than normal regenerates and show normal length, total length was reached only in 1 case out of 7, after 1 mo (although it reached 3 cases out of 7 when a period of 5 mo had elapsed). Previous studies showed that normal regeneration never reaches complete length in this mutant, even after 5 mo of regeneration (Géraudie et al. 1995). Thus individual fin regeneration may show properties which allow the regenerate to complete its total length.

In 3 cases we have observed ray regeneration out of the fin in the zebrafish individual fin ray regenerates. Individual fin ray regeneration in *long fin* mutants also differentiates a larger 'fin fold' than in wild type fishes. Ray regenerates out of the fin may also spontaneously appear in these operated mutants (Fig. 8).

Individual fin ray regeneration in zebrafish pectoral fins showed a slow re-epithelisation, leading to a continuous connection between apical epidermis and blastema (data not shown). Thus, in the pectoral fin, the apical epithelial cap directly covers the blastema during the whole regeneration process. In these cases, the rays differentiate normal bifurcations.

Isolated fin ray regenerates. Isolated fin rays regenerate leading to normal fin rays with an oblique or abnormal direction, due to the stretching produced by the manipulations, and do not show any obvious perturbation in terms of segment number or morphology (although bifurcation may not occur and, in some instances, segmentation is completely absent in most of the isolated regenerate). The absence of segmentation is probably due to partial local segmentation that does not succeed in traversing the whole of the segment width; this is observed in both left and right surfaces of the caudal fin (Fig. 9). Modifications of blastema distalisation direction may also occur, probably by the effect of the lack of local tractions from the neighbouring blastemas. This process of local lateral cell tractions may be important for understanding some of the observed morphologies in which normal direction, segmentation and new bifurcations appear following wound healing of interray tissue (Fig. 10). It is interesting to consider that the first evidence of segmentation may be observed in the first 400 μm in the apical blastema
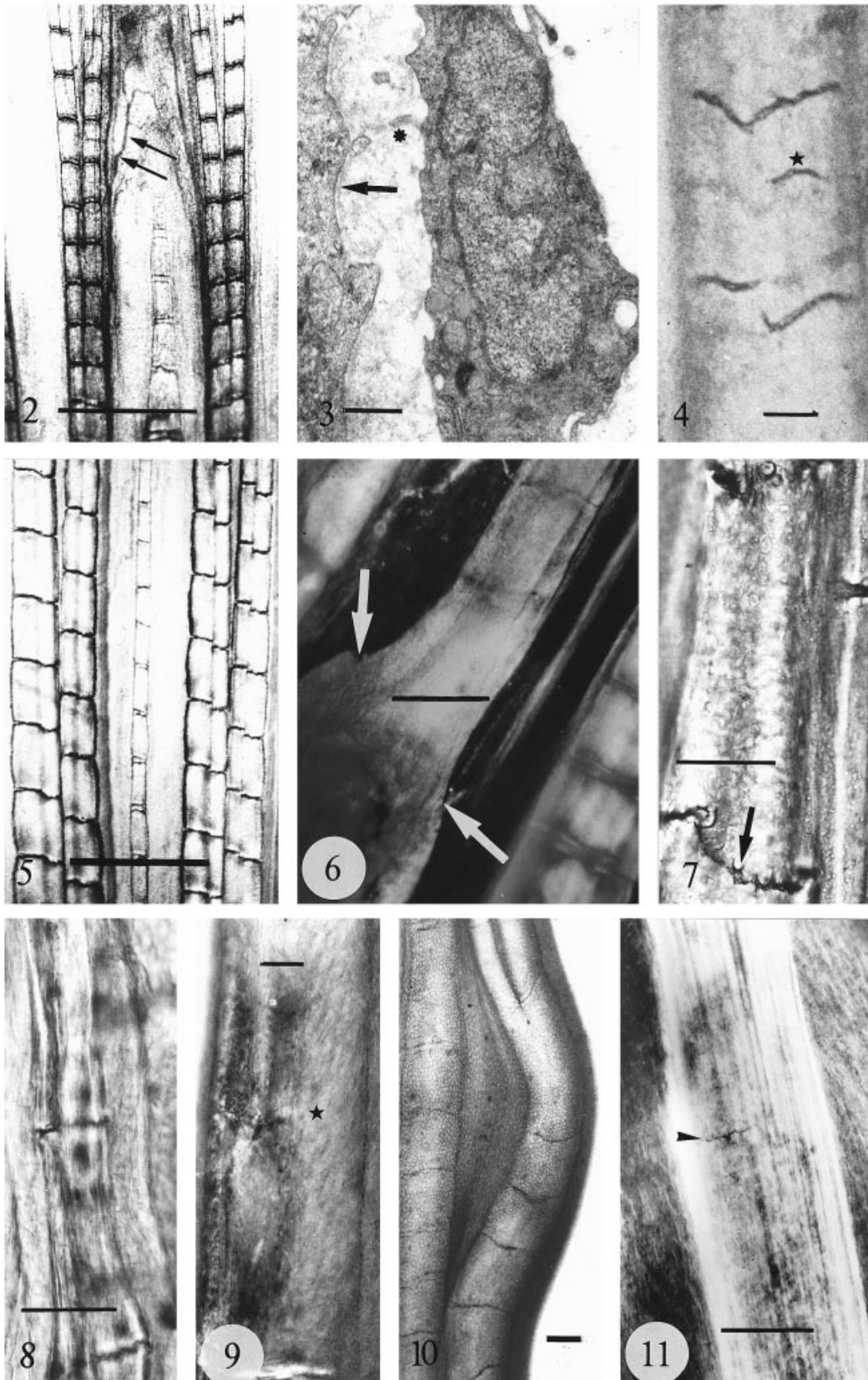
Figs 2–11. For legend see opposite.

under polarised light microscopy (Fig. 11). In all cases, actinotrichia were present in the apical blastemas. The process of segmentation shows different degrees of perturbation in the whole set of operations, thus suggesting that manipulation is affecting morphogenetic mechanisms involved in its control.

Morphometric analyses

Camera lucida drawings and serial photographs taken almost every 2 d of regeneration were used to obtain measurements of different morphological variables useful for the quantitative analysis of the observations described above. As it was described in the Material and Methods section, the variables studied were: overall fin ray length, segment length, segment width, segment area and distance of bifurcation.

Regeneration growth curves. Overall fin ray length has been useful for analysing the kinetics of the process, by regression to linearised growth curves (Voit et al. 1985). The values adjusted for 'a', 'b' and 'th' parameters describe the whole process and may comprehensively describe regeneration. The values obtained are presented in the Table. 'c' and 'b' parameters are universal estimators of regeneration curves, and their maximal values in single fin ray regeneration are suggestive of an extensive lag period of blastema formation, in which morphogenetic specifications are probably occurring prior to actual extensive cell growth. 'th' parameter is an estimator of an overall distalisation controlling processes, which probably operate during the whole regeneration.

Bifurcations. The bifurcation distance is included in the Table. Whereas the first bifurcation occurs, for a cut done at 20–30 % of total fin ray length, at a mean of 23 % of total regenerate length in normal control regeneration experiments, bifurcation occurs at a significantly different level of 65–75 % in the single fin ray and isolated ray regenerates.

The Mandelbrot diametral exponent is indicative of effective blastema dimensions during regeneration. If the fin ray blastema is considered as a morphogenetic sphere, the bifurcation process would lead to volume partition. If we measure segment width before and after bifurcation, we should then obtain an allometric relation between both measurements with an exponent value of approximately 2 (i.e. $d_1^2 = d_2^2 + d_3^2$); however, if the behaviour of the morphogenetic fin ray blastema approximates to a surface, as the LFC precursors sheet, the exponent value might be around unity ($d_1^1 = d_2^1 + d_3^1$). Estimation of these values in normal and experimental regeneration lead in all cases to the same value, approximately 1 (Table). This suggests that mechanisms controlling bifurcation may occur in 2-dimensional cellular sheets, probably those involved in the determination of LFCs in the apical blastema.

Segment form correlates. Multiple correlation tests have been performed between the morphometric variable measured in each segment and the values estimated for regeneration rates. Segment width or area are poorly correlated with kinetical parameters

Fig. 2. Whole preparation of individual tail fin ray regenerate in the goldfish. Arrows indicate growing blood vessels in the apical blastema connecting with main vessels in adjacent unoperated fin rays. Bar, 1 mm.

Fig. 3. Transmission electron micrograph of a blastema cell adjoining a well-formed basal lamina (arrow). Small projections (asterisk) may be observed towards the epidermis. An indented nucleus is surrounded by an organelle-rich electron-dense cytoplasm with conspicuous rough endoplasmic reticulum. Secretory granules are not obvious in the interactive cell membrane. Bar, 1 μm.

Fig. 4. Partial segmentations in the initial fin ray regenerates following the procedures performed on the 2nd group in the goldfish (asterisk); observe that the intersegmental region does not completely transect the hemisegment. Bar, 100 μm.

Fig. 5. Single fin ray regenerate of the goldfish (centre) is much thinner than normal unoperated fin rays before their first bifurcation. Bar, 1 mm.

Fig. 6. Regeneration has proceeded from the proximal stumps of the old demirays (arrows) in a goldfish specimen. Observe that, in addition to the stumps of pre-existing rays, the regenerating demirays are darkly stained by Sirius red. When this staining method is viewed with polarisation microscopy, collagenous structures show up as brightly birefrigent structures that shine against a dark background. Bar, 200 μm.

Fig. 7. Individual ray regenerate (2nd group) in *B. rerio*. Segments are longer than normal, as in the goldfish, and in many instances do not show the first bifurcation. This figure illustrates that segmentation is aberrant, displaying 'fractal' profiles (arrow). Bar, 25 μm.

Fig. 8. The operated individual fin ray may eventually regenerate out of the fin of *B. rerio*. In these cases the isolated rays do not show bifurcations. Bar, 50 μm.

Fig. 9. Segmentation can be aborted during separated fin ray regeneration in the second group of the goldfish. Partial (asterisk) or totally absent segmentations may appear in fin ray regenerates widely separated from the other lepidotrichia. Bifurcation may not occur following this operation. Bar, 100 μm.

Fig. 10. Recorded fin ray blastema after continuous scissor separation may lead to expansions and contractions in the fin structure of the goldfish as observed in this figure. Notice that the lateral isolated fin ray reapproximates to the neighbouring ray and even bifurcates after complete healing because the isolating daily scissor cuts have been interrupted. Bar, 200 μm.

Fig. 11. Blastema of a regenerating tail fin of *Carassius auratus*. The most apical differentiation sign of segmentation (arrowhead) may occur near the apex, 200–300 μm proximal to apical epidermis. Bar, 50 μm.
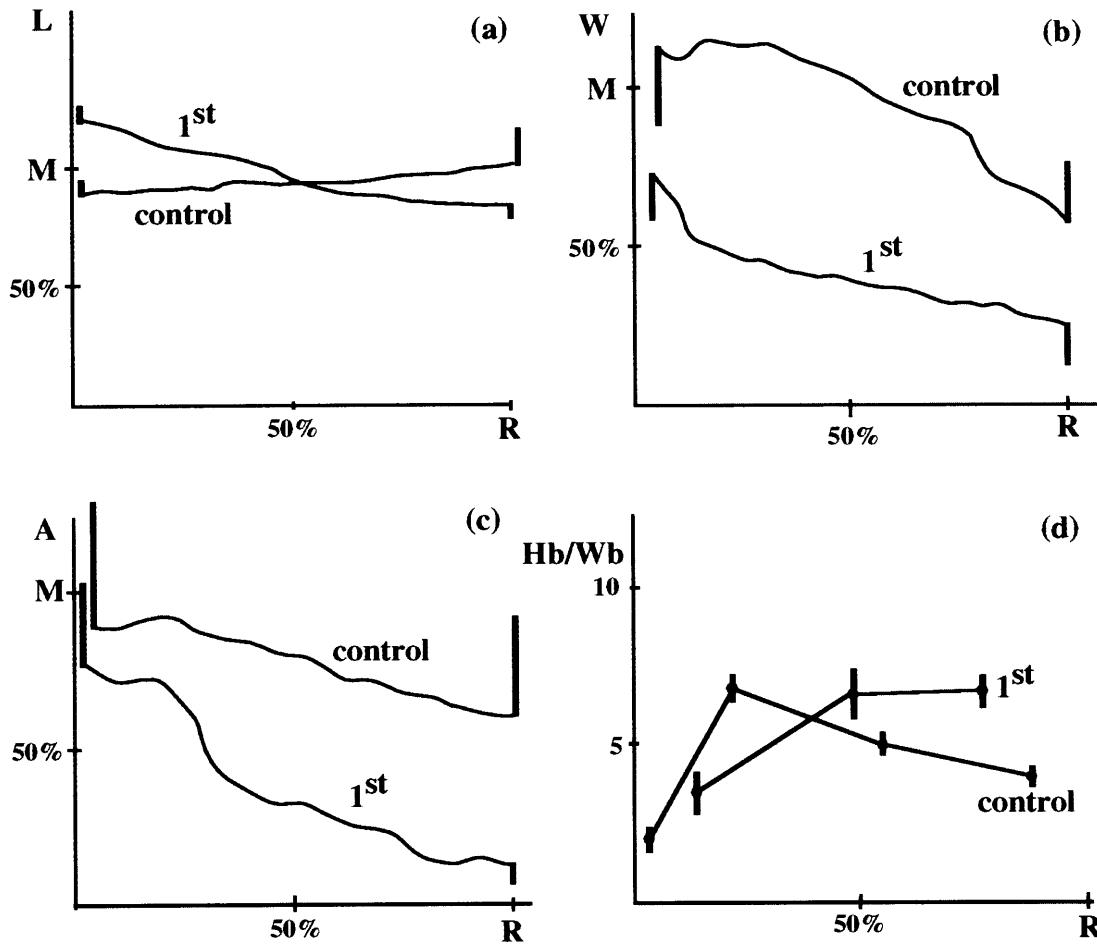
Fig. 12. Regenerative pattern of the goldfish tail fin. *M* corresponds to the control value. 1st and control correspond to regenerates obtained in each group of different operation types. Bars show standard deviation. (*a*) Harmonic means of fin ray segment length (L) dependent on position in the regenerates. R, length of the regenerate, 50 d after amputation. (*b*) Harmonic means of fin ray segment width (W) dependent on position in the regenerates. (*c*) Harmonic means of fin ray segment area (A). (*d*) Blastemal height/width parameter (Hb/Wb) follows a variation with proximodistal position (R) in each operation (1st group) similar to the inverse of the harmonic means of segment length (*a*). Correlations have not been obtained due to technical difficulties.

'b' and 'c', since values close to 0.6 are obtained for the Pearson correlation. Moreover, unexpected negative correlations of −0.7 have been obtained between the percentage of segment length and the value calculated for 'th' (i.e. the maximal rate of regeneration) in controls. These 2 values are unique in their significance, as all other cases showed much lower absolute values for the correlation coefficient.

Different fin rays from the same and/or different animals also differ in the number of their segments and the values estimated for shape parameters. This precludes comparisons segment by segment, as these may not be intraspecific homologous structures. For this reason we performed Fourier harmonic analysis of the whole set of data coming from the study of single variables in single fin rays, in order to compare different fin positions or among different animals. The logic of this procedure was explained in Material and Methods.

Proximodistal variations of segment length, width, and area, measured in a normal fin ray show a characteristic pattern (Fig. 12*a*). Harmonic amplitudes may be useful for comparing different segments from different animals and treatments. Harmonic analysis provides also a harmonic (trapezoidal) mean in our experiments. This pattern is characterised by an undulating profile, in which any segment parameter can be interpolated. The simulation of the goldfish mean regeneration pattern is shown in Figure 12*a–c*, where it can be observed that, following amputation, regeneration differentiates a smaller segment than that normally found in nonregenerating fins (Fig. 12*a*). Individual fin ray regenerates differentiate longer than normal segments initially. In any case, regeneration restores normal segment length as it proceeds. Segment width and length parameters also show variations, which are dependent on the position of the segment within the regenerate. Segments are narrower

than those of controls in individual fin ray regenerates (Fig. 12*b*). Segmental area is a combination of segment width and length, and although both measurements are proximally similar in each experimental regenerate, they dramatically change through regeneration in individual ray regenerates (Fig. 12*c*).

Blastema morphometry. Proximodistal length, width, and thickness in the right–left axis were obtained in blastemas from histological slides of the regenerates at d 3, 6, 11, and 18 in the control group. These variables were plotted on total regenerating length, normalised to positional percentage of blastema. We expected that some of these variations might accompany the general modifications of segmental variables obtained in the anatomical study. Whereas absolute data and length/thickness or width/thickness ratios do not change similarly to the anatomical variables studied, the length/width ratio changes inversely in relation to segment length (Fig. 12*d*), thus supporting the hypothesis that LFCs sheet form is a controlling element of segment length. No other obvious relations were observed.

DISCUSSION

The present observations show that individual fin ray regeneration has proved to be a good model to study the reconstruction of the form and number of segments, as well as the bifurcation process in fin rays. To analyse it, we have developed a harmonic method that allows comparisons between different operations and species. Previous work on this subject classified fish species into 2 classes according to their capacity to regenerate the complete structure: those which show homomorphic (complete) and heteromorphic (partial) regenerates (Wagner & Misof, 1992). We think that through proper experiments, a huge variety of different regeneration forms may appear, as shown by our present results, and that a continuum of variation among them may occur, including fin ray fusions, such as those observed following retinoid injection (Santamaría et al. 1993; Géraudie et al. 1994) or even under natural regeneration processes in other fish species (Wagner & Misof, 1992).

Pattern abnormalities can be understood as dependent on affected blastema cell interactions (Fig. 13). Normal blastemas (the ones with intact interactions with apical epidermis, neighbouring blastemas and within themselves) regenerate bifurcated and segmented fin rays normally (Fig. 13*a*). The absence of such apical, lateral and contralateral interactions leads to abnormal patterns lacking bifurcation (Fig. 13*b*, *c*) or results in irregular segmentation (Fig. 13*c*).

The importance of apical epidermis in blastema formation has been widely recognised. Normal regeneration provides a direct interaction between apical epidermis and the blastema through a very particular basal lamina (Becerra et al. 1996). In the present experiment, individual excision of a fin ray promoted immediate re-epithelisation of the whole wound, filling as a wedge the space of the excised tissue and thus leaving the apical epithelial fold at the tip of the fin, a long way away from the blastema formed over the remaining ray stump, which is in contact with well developed basement membranes of the left and right (lateral) epithelial coverings. In this experiment, abnormal blastemas, initially formed of a smaller number of cells (probably due to the lack of appropriate interaction with the apical epidermal cap), resulted in narrower fin rays along the whole extension of regeneration (Fig. 13*b*).

According to our results and other recently published studies on gene expression (Laforest et al. 1998), we propose the following controlling mechanisms operating in fish fin regeneration: (1) apical epidermis-blastema bidirectional cross-interaction acting during blastema formation, and later in regeneration, (2) local cell-to-cell interactions at LFC precursor sheet, and (3) long distance interactions controlling bifurcation and segmentation.
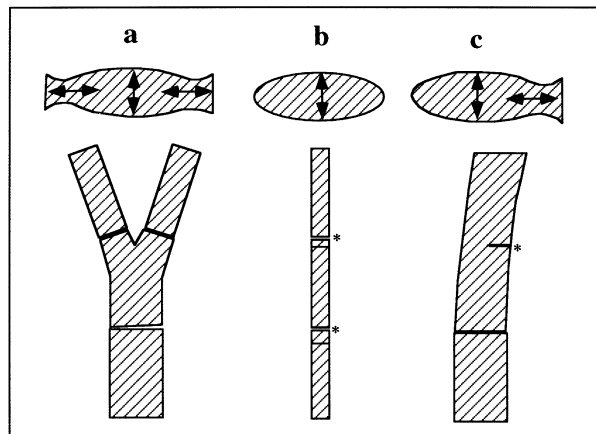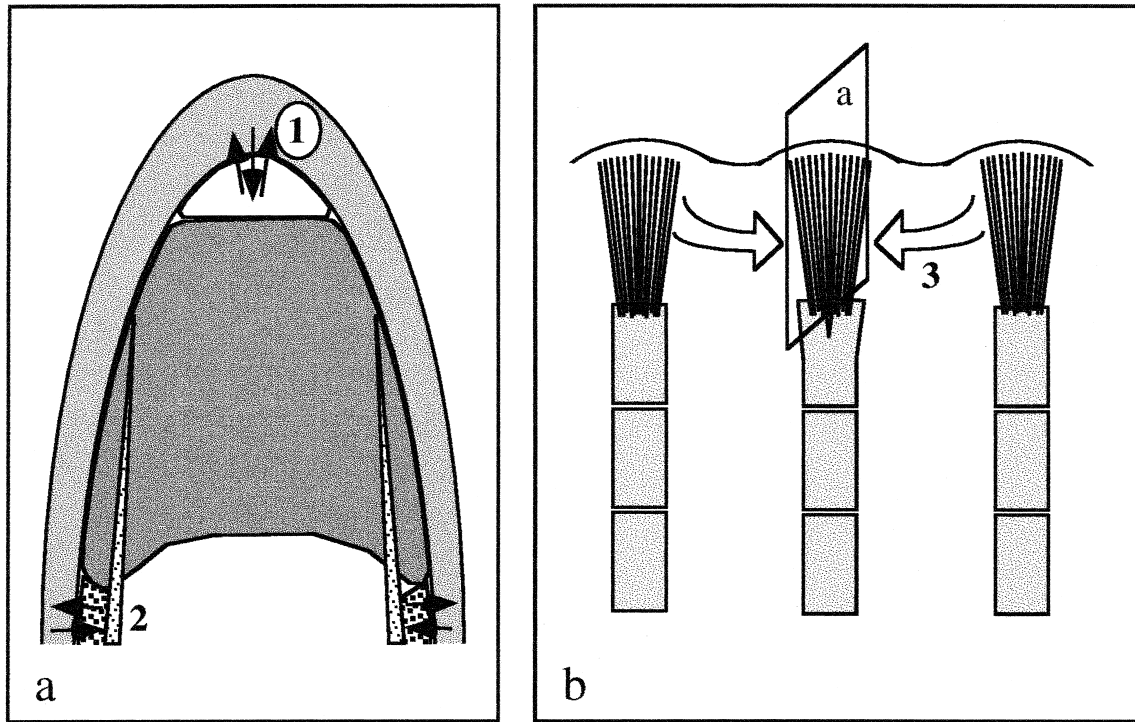

Fig. 13. Schemes of regenerates following each operation. A transverse section of the regenerated blastema is also shown. Arrows display probable interactions in intact tissues. (*a*) Normal regeneration independent of cutting level produces normal bifurcations and segmentation in dermal bones of each fin ray. (*b*) Lack of lateral interblastema interaction during individual fin ray regeneration leads to a reduction in bifurcations or a delay to more distal portions, and an extreme reduction of segment width probably as a reduction in the number of blastemal cells. The segmentation process (asterisks) is not significantly affected except for initial segmentation in which coincidence of hemisegmentation may be absent and long unsegmented fin hemirays differentiated. (*c*) Lack of lateral interblastema interaction during isolated fin ray regeneration also leads to a reduction in bifurcations or a delay to more distal portions. Segmentation process (asterisk) may be absent or partially differentiated during the isolation.

Fig. 14. Cell to cell and intertissular interactions modulating pattern of the fin ray of teleosteans. (*a*) (1) Apical epidermis induces and polarises distalisation. Early determination and cell recruitment during blastema formation (positional information (P.I.)) is probably dependent on epidermal induction (down pointing distal arrow) whereas local *msx D* over-expression in the covering epidermis (slightly shaded profile) (unpublished results) may be dependent on blastema induction (up pointing arrows). (2) LFC patterning might depend on a hierarchical local determination process initiated in the distal leading blastemal cell population (white distal profile) interacting with the epidermis whereas right-left or dorsal-ventral interactions may occur. At LFC determination connective-epidermis interaction are also active (transverse arrows). Basal epidermis may also induce LFCs differentiation (grossly pointed profile) (according to Laforest et al. 1998) (transverse arrow). (*b*) (3) Long-range blastemal interactions may also operate between apical or proximal blastema (strongly shaded profile) modulating segmentation and bifurcation (oblique arrows) determination. a indicates the longitudinal section of scheme (*a*).

1. The apical *epidermis-blastema interactions*. This bidirectional interaction may include at least 3 different processes. (*a*) *Apical epidermis-blastema interactions* modulate blastema fin ray dimension during its early formation (Fig. 14*a*). This process might be time-dependent, as quick re-epithelisation affects fin ray thickness with a correlation of 0.6 ($P < 0.001$). Pectoral fin heteromorphic regeneration and slow epidermis regeneration following single fin ray ablations (processes which are correlated; data not shown) may also explain this process in other species (Misof & Wagner, 1992) as a deployment of epidermal regenerative capacity affecting pseudapical ridge differentiation (Geraudie, 1980). Fin ray polarity may also depend on apical epidermis-blastema interaction. This is suggested by the proximal distalisation, which occurs following large holes in the goldfish tail fin (Nabrit, 1929), probably by epidermis covering the distal cut face of the hole which induces a change of polarity in the regenerate. Distal interactions between apical epidermis and blastema may also control final dimensions by repressing blastema potency during regeneration (Fig. 14*a*). In *long fin* mutants this process may result in the absence or reduction of this interaction, since individual fin ray ablation, performed in the zebrafish, permitted acquisition of total length of the regenerate, whereas this is not achieved during normal regeneration in this mutant (Géraudie et al. 1995). (*b*) In situ hybridisation in individual fin regenerates is also indicative of *msxD* expression, being dependent on later acting *connective-epidermis interaction*, as its expression is only observed in epidermis lateral to distal blastema (Marí-Beffa et al. unpublished observations). Fig. 14*a*. A similar interaction has been observed in the chick limb bud (Carrington & Fallon, 1986). This interaction probably acts during the whole regeneration process, but in the opposite sense, as was discussed previously (see above). (*c*) *Epidermis-blastema interaction* may also control bifurcations: individual pectoral fin ray regenerates, which show continuous connection between epidermis and blas-

tema during the whole process, differentiate normal bifurcations, whereas individual tail fin ray regenerates which do not show this type of connection do not bifurcate until 60–70 % of the regenerate is achieved (Table).

2. *Local cell-to-cell interactions.* Cells differentiating at LFCs determination site are probably controlled by at least 2 different signals: (*a*) signals differentiating basal cells of the epidermis and (*b*) those inducing differentiation of blastemal cells resulting in scleroblasts (LFCs). (*a*) At LFCs formation level, *connective tissue to epidermis interactions* may also be active, as the basal epidermal layer changes the morphology of its cells from cuboidal to columnar as the distalising blastema reaches the level during single fin ray regeneration (Marí-Beffa et al. 1996). (*b*) *Shh* and *ptc-1* expression at LFC precursors level suggests an *epidermis-to-connective-tissue induction*. As *Shh* is expressed at the epidermis and *ptc-1* in both epidermis and LFC sheet (Laforest et al. 1998), *Shh* probably provides polarity and positional information to inner nerve bundles, blood vessels and LFCs precursors (Fig. 14*b*), although it has recently been reported that *Shh/ptc-1* is also probably involved in bifurcation and segmentation patterning (Laforest et al. 1998).

3. *Long distance interactions.* Each fin ray blastema may also receive a morphogenetic control acting over long distances, modulating the direction of regeneration (probably by cell traction), as well as the determination of segmentation and bifurcation (since those processes are also affected in its absence) (Fig. 14*b*). Lateral interactions with adult tissue may not exist, since individual fin ray regenerates and isolated fin ray regenerates have led to similar results in the absence of bifurcations and abnormalities in segmentation. As fin ray polarity may also depend on these interactions, we speculate about a probable source of these signals at the apical epidermis-distal blastema interface, either in the interray or ray blastema (Fig. 14*b*). Recent results obtained in our laboratory support this hypothesis. Proximal fragments of the first, unbifurcated long ray were grafted to the interray between the shortest long rays (i.e. between each lobe in the tail fin) and then amputated with the whole fin. The regenerated grafted rays bifurcated in some instances as neighbour blastemas were also present (Marí-Beffa et al. unpublished results). This long-range interaction may also occur in normal fins from neighbour tissues, and controls the proper action of this signal in each fin ray blastema. The bifurcation in single fin ray regenerates probably occurs due to sufficient long-range bifurcation signal

coming from self ray. Segmentation may also be absent in isolated fin rays, thus leading to double length segments.

Local modulation of cell growth and segment pattern may account for the observed correlation between maximal regeneration rate and segment length, which is probably related to the classic observations of correlation of regeneration rate with amputation level (Tassava & Goss, 1966). We have also found a lack of correlation between blastema form and segment form, except for the general form of the basal lamina adjoining LFCs. A segment-controlling mechanism may be exerted at LFC determination in a single cell sheet. Recently, Laforest et al. (1998) suggested that the gene *Shh*, which is expressed in the basal epidermal layer near the scleroblasts adjoining the basal lamina, could control by *ptc-1* and *bmp-2* the patterning of the fin rays.

Final morphogenesis is partially dependent on cell properties, which result in the segmentation and bifurcation of fin rays. Properties such as cell adhesivity and cell tractions (abnormal lepidotrichia pattern of partial condensations; Marí-Beffa et al. 1996) or niches of proteoglycans around the cells (Santamaría & Becerra, 1991), cell migration (initial re-epithelisation) or external LFCs migration and differentiation (Marí-Beffa, 1987; Santamaría & Becerra, 1991), blastema cell proliferation (Santamaría et al. 1996) and extracellular matrix (ECM) deposition and degradation (actinotrichia growth control; Marí-Beffa et al. 1989), may modulate the regeneration outcome. Morphogenetic models (Oster et al. 1983, 1985) have also been proposed to act during fin development (Oster et al. 1990). The appearance of trifurcations (Nabrit, 1929; Oster et al. 1983) (unexpected in many theoretical models of development), fin ray polarity changes dependent on lateral fin ray interactions (Morgan, 1902; separated fin regenerates) and overall segment length changing in parallel to LFCs surface form, are all data supporting the notion of cell interactions acting within sheets of cells adjoining the basal lamina, which modulate their cell behaviour (Oster et al. 1983).

Fin rays constitute a classical determination character widely used in fish taxonomy. Systematic comparisons of fin regeneration capability (Wagner & Misof, 1992), or population analysis of regeneration processes, as pollution detection indexes (Weis & Weiss, 1980), may be morphometrically characterised using the harmonic method developed here. The usefulness of experimental embryology in proposing evolutionary processes is exemplified by the case of the lateral, nonbifurcated fin rays in most fish orders,

or by the nonarticulated isolated rigid spines and fin rays which are characteristic of a variety of fish families, i.e. *Notacanthidae* (*Notacanthus*; Bloch, 1788), *Triglidae* (*Trigla*: Linnaeus, 1758), *Peristediidae* (*Peristedion cataphractum*; Linnaeus, 1758) (Whitehead et al. 1984); or the unsegmented pelvic rays of *M. falcifer* subadult females (Parenti, 1986). These phenotypes have also been observed experimentally, following fin ray separation or individual fin ray regenerates. This suggests that lateral blastemal interactions may also control these processes, and that the proposal of their reduction in lateral or isolated fin rays in species of the latter groups is a plausible evolutionary explanation. Experiments in these fishes could help to test this hypothesis.

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