

Explanation for Naturally Occurring Supernumerary Limbs in Amphibians

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ABSTRACT The occasional occurrence of high frequencies of limb abnormalities, including extra limbs, in natural populations of amphibians has long been a puzzle. In this paper we report the discovery of a population in which such limb abnormalities appear to be caused by a parasitic flatworm (trematode) that uses amphibians as intermediate hosts. The cercarial larval stage of the trematode attacks amphibians, penetrating the skin to form cysts (metacercariae). The cysts are preferentially localized in the cloacal region, including the developing hind limb regions in larvae of both frogs (*Hyla regilla*) and salamanders (*Ambystoma macrodactylum*). A wide range of limb abnormalities are seen, including duplicated limb structures ranging from extra digits to several extra whole limbs. We hypothesize that these limb abnormalities result from localized regulatory responses of developing and regenerating limb tissues to mechanical disruption caused by the trematode cysts. We have tested this idea by implanting inert resin beads into developing limb buds of frogs and salamanders. Since this treatment can cause supernumerary limb structures, our hypothesis is sufficient to explain the naturally occurring extra limbs.

Reports of amphibians with extra limbs in natural populations have been in the scientific literature for centuries (Van Valen, '74). When such animals occur as single individuals they are usually dismissed as isolated developmental anomalies. More problematic are cases where populations show a high frequency of individuals with extra limbs (Volpe, '81; Reynolds and Stephens, '84), suggesting that a major biological perturbation has spread through the population. Possible explanations for such exceptional events include genetic mutation, viruses, parasites, injury, temperature, radiation, and chemical pollution (Van Valen, '74; Reynolds and Stephens, '84; Meyer-Rochow and Koebke, '86). However, in all previous reports of high frequencies of multilegged amphibians from natural populations neither the cause nor the developmental mechanism has been identified.

In this paper we report a high frequency of limb abnormalities, including extra limbs, in two populations of Pacific tree frogs (*Hyla regilla*), and long-toed salamanders (*Ambystoma macrodactylum*), in which we have identified the causative factor as a parasitic flatworm (trematode) that uses amphibians as an intermediate host. These multilegged amphibians were discovered in a pond in the summer of 1986 during a mark-release-recapture survey of an endangered population of *A. macrodactylum* in Aptos, Santa Cruz

County, northern California (Ruth, unpublished observations). Multilegged *Hyla* were also found at another pond in nearby Rio del Mar. *Ambystoma* with abnormal digits were earlier recorded at the Rio del Mar site in 1974 (Ruth, unpublished observations). We hypothesize that metacercariae, representing the encysted stage of the trematode life cycle, cause the observed limb abnormalities. The cysts may have an effect through the secretion of a teratogenic or morphogen-like substance, or, alternatively, they may simply act as solid obstacles that first injure limb tissues and then passively disrupt morphogenetic processes during subsequent wound healing and continued limb development. The latter possibility was tested experimentally by implanting resin beads into the developing limb buds of captive frog and salamander larvae. Our results suggest that mechanical disruption of developing (or regenerating) tissues by trematode cysts is sufficient to stimulate the outgrowth of supernumerary limb structures.

MATERIALS AND METHODS

Specimens of *Hyla regilla* and *A. macrodactylum* were collected during a study of the aut-

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TABLE 1. Most commonly observed limb abnormalities in 280 *Hyla regilla* tadpoles and froglets from two adjacent ponds in Northern California

Limb abnormality	No. of specimens
Extra limbs	140
Mirror-image duplicates ¹	78
Grossly distorted	55
Ectopic structures	30
Hypomorphic	27
Skin fusions	25
P-D truncation ²	22
Slightly abnormal	21
Shortened limb	13
Bifurcations	10
Extra pelvis	9
D-V duplications ³	5
Reversed D-V axis ⁴	4
Trifurcations	4
Missing limb	3
Missing forelimb	1

¹Symmetrical anterior-posterior structures (see text).

²P-D = proximal-distal axis.

³D-V = dorsal-ventral axis.

⁴Limb is upside down relative to body axis.

ecology of *A. macrodactylum* at a pond near Aptos, Santa Cruz County, California, between February 15, 1986 and May 31, 1987. Juvenile and adult specimens of *Ambystoma* were captured with drift fences; larval specimens were captured with dipnets and seines at 5- to 9-day intervals in conjunction with growth studies between February 12 and September 20, 1986. *Hyla* with abnormal limbs were discovered at the Aptos pond on July 12, 1986, and samples of larval and transforming frogs were collected on July 19 and 22 and August 2, 1986. Recently transformed *Hyla* were collected at a nearby pond in Rio del Mar on July 31, 1986. A sample of adult *Hyla* was collected at the Aptos pond on March 6, 1987. Owing to the endangered-species status of *A. macrodactylum*, virtually all specimens were released within 72 hours of capture. Only 10 animals were collected in addition to those found dead or that died during the field study. Collected frogs and salamanders were fixed in 10% buffered formalin, rinsed in water, and postfixed in 70% ethanol. Specimens were then stained with Alcian blue (for cartilage) and alizarin red (for bone), macerated with trypsin and sodium borate, and cleared with potassium hydroxide and glycerin (Hanken and Wassersug, '81).

Experimental implantations of resin beads were performed using larvae of laboratory bred *Xenopus laevis* and *Ambystoma mexicanum*. Beads were selected that were approximately the

TABLE 2. Number and location of hind limbs in *Hyla regilla* tadpoles and frogs from ponds in Northern California.¹

Location and No. of hind limbs: left/right	No. of specimens	Frequency
1/1	133	.504
1/2	33	.125
2/1	31	.117
2/2	13	.049
1/3	11	.042
3/1	10	.038
3/2	8	.030
4/1	6	.023
1/0	3	.011
1/4	3	.011
2/3	3	.011
3/4	2	.008
1/5	1	.004
2/4	1	.004
4/2	1	.004
4/3	1	.004
5/1	1	.004
5/3	1	.004
6/2	1	.004
9/3	1	.004

¹Hind limb combinations are listed in order of frequency.

same size as the metacercarial cysts (150–200 μm) and surgically implanted into early (pre-digit stage) hind limb buds. Three to five beads were implanted into each limb bud. Control limb buds were damaged in a similar way to the experimental limb buds but without implantation of beads. All specimens of *Hyla regilla* and *Ambystoma macrodactylum* analysed in this study will be deposited in the Museum of Vertebrate Zoology, University of California, Berkeley.

RESULTS

The specimens of *Hyla regilla* from our July 19 and 22, 1986 sample ranged from tadpoles at pre-limb bud stages to fully metamorphosed froglets. Approximately 72% of a total of 280 tadpoles and young froglets from the Aptos pond presented a wide range of hind limb abnormalities, from missing limb structures to variable numbers of extra whole hind limbs with various kinds of limb deformities (Tables 1, 2; Figs. 1, 2). Duplicated whole limbs or limb structures (long bones and/or digits) sprout from anywhere along the length of a normal limb, or from their own extra pelvic structures (Fig. 1). The total number of hind limbs per individual in this sample of frogs ranged from 1 to 12 (Fig. 2), with a mean number of 2.9. One-half of the specimens had at least one extra hind limb

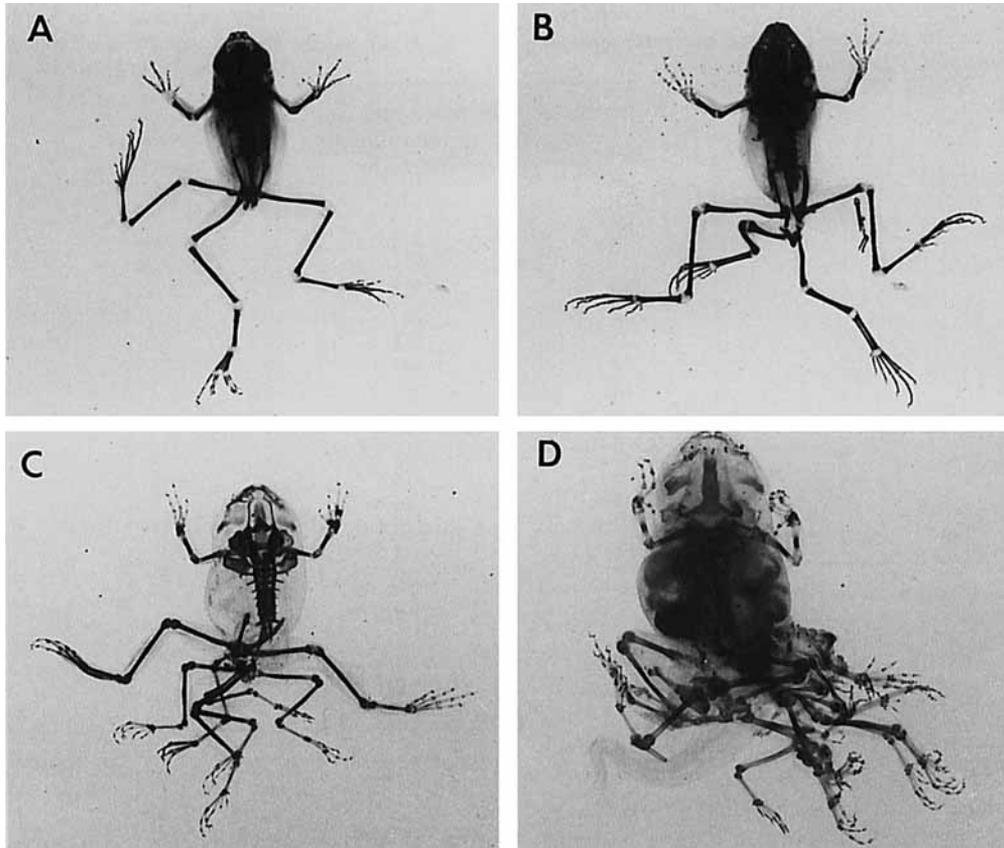


Fig. 1. Specimens of *Hyla regilla* with (A) three hind limbs, (B) four hind limbs, an extra pelvis, and small ectopic limb elements, (C) nine hind limbs, and (D) 12 hind limbs. All specimens in this and other figures were cleared and stained with Alcian blue/alizarin red, unless otherwise indicated.

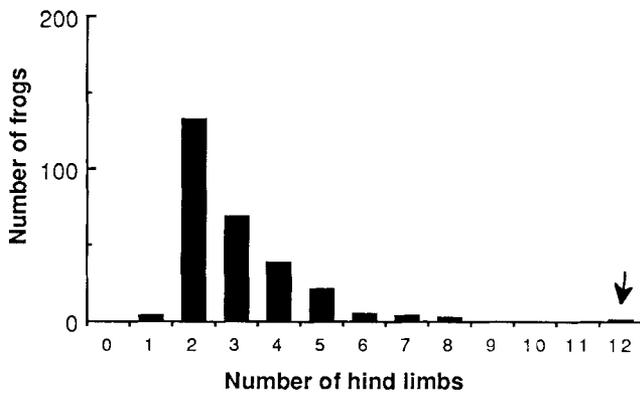


Fig. 2. Frequency of hind limbs in *Hyla regilla* from two adjacent ponds in Northern California.

(Table 1; Fig. 2), and extra limbs occur with approximately equal frequency on the right (48%) and left (52%) sides of the body (Table 2). The most common morphological deformity seen among the extra limbs was mirror image duplica-

tion of the anterior-posterior axis (Table 1; Fig. 3), fitting "Bateson's Rule" of supernumerary limb symmetry (Bateson, 1894, cited in Bryant et al., '80). Only five hind limb duplications in the dorsal-ventral axis were found. The only forelimb abnormality was a single specimen in which one forelimb was missing.

Specimens of *Ambystoma macrodactylum* from the same pond showed a much lower frequency of limb abnormalities than the frogs (Tables 3–5). Because of their endangered-species status, the majority of captured *Ambystoma* were released, and only a few specimens with obvious limb abnormalities were retained for whole-mount examination. None of the 25 samples of salamander larvae taken prior to 2 August showed any sign of abnormal limb development. In the eight samples taken from August 2 to September 20, between 9% and 25% of the larvae had obvious limb abnormalities. These samples were pooled to facilitate comparisons with juveniles and adults (Table 3).

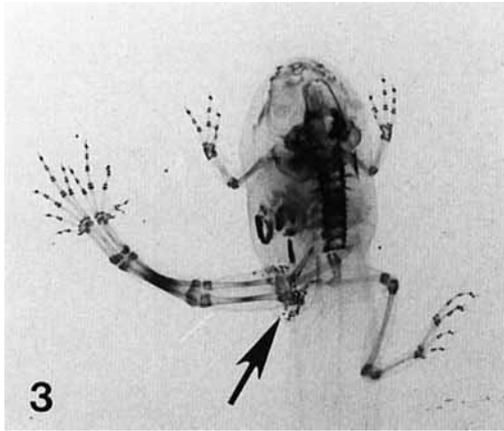


Fig. 3. Specimen of *H. regilla* with a mirror-image duplication of the left hind limb. Metacercaria cysts can be seen as small black dots around the base of the abnormal limb (arrow).

TABLE 3. Numbers of specimens showing limb abnormalities in a sample of 915 larval salamanders (*Ambystoma macrodactylum*) from the pond in Aptos, California

Abnormality	Forelimb		Hind limb	
	Left	Right	Left	Right
Extra digits	2	3	51	41
Forked digits	0	0	2	4
Fused digits	0	0	3	0
Stubby digits ¹	1	0	12	7
Missing digits ¹	0	1	3	4
Ectopic digits	0	0	8	6
Extra limbs	0	0	6	5
Ectopic hand/foot	0	0	29	40
Short limb ¹	3	0	62	55
Missing limb ¹	0	0	3	2
Other	1	1	1	3

¹Could easily be explained by attempted predation.

Examination of the external morphology of a total of 915 larval (Table 3), 3,233 juvenile (Table 4), and 1,778 adult (Table 5) captured and released specimens indicated that at least 39.2% of the late larvae, 38.5% of the juveniles, and 4.6% of the adults showed obvious limb abnormalities ranging from missing limb structures to extra digits and extra whole limbs. Limb abnormalities include Bateson mirror-image duplicates (Fig. 4). In contrast to the frogs, salamanders showed abnormalities in both forelimbs and hind limbs; nevertheless, in larval and juvenile salamanders the hind limbs showed more extensive abnormalities than the forelimbs (Table 3, 4).

The abnormal limb structures in both amphibian species are associated with massive infesta-

TABLE 4. Numbers of specimens showing limb abnormalities in a sample of 3,233 juvenile salamanders (*Ambystoma macrodactylum*) from the pond in Aptos, California

Abnormality	Forelimb		Hind limb	
	Left	Right	Left	Right
Extra digits	165	140	171	166
Forked digits	5	0	8	7
Fused digits	1	1	4	2
Stubby digits ¹	4	2	6	1
Missing digits ¹	6	9	12	19
Ectopic digits	0	0	11	13
Extra limbs	1	0	16	23
Ectopic hand/foot	0	0	131	138
Short limb ¹	0	0	79	86
Missing limb ¹	0	0	0	1
Other	0	0	8	10

¹Could easily be explained by attempted predation.

TABLE 5. Numbers of specimens showing limb abnormalities in a sample of 1,778 adult salamanders (*Ambystoma macrodactylum*) from the pond in Aptos, California

Abnormality	Forelimb		Hind limb	
	Left	Right	Left	Right
Extra digits	13	8	8	10
Forked digits	3	0	1	1
Fused digits	1	1	1	1
Stubby digits ¹	2	1	2	3
Missing digits ¹	6	4	1	2
Ectopic digits	0	0	0	0
Extra limbs	0	0	0	1
Ectopic hand/foot	0	0	4	6
Short limb ¹	0	0	1	1
Missing limb ¹	0	0	0	0
Other	0	0	0	0

¹Could be easily explained by attempted predation.

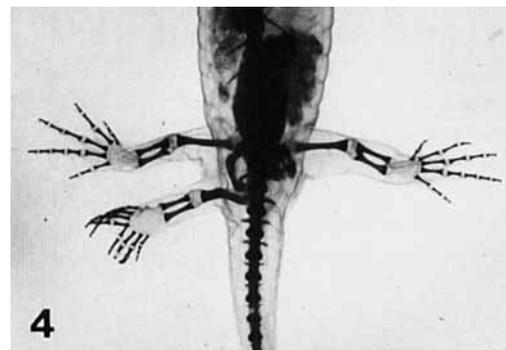


Fig. 4. Salamander (*A. macrodactylum*) with a mirror-image supernumerary limb on the left side.

tion of an encysted stage of at least one species of digenetic trematode (Figs. 3, 5–7). The cysts were identified as trematode metacercariae by the presence of ventral and oral adhesive organs (Fig. 5). A tentative identification of the metacercariae found in the frogs is *Manodistomum syntomentera* of the family Plagiorchiidae (J. N. Caira, pers. comm.), a digenetic trematode that uses garter snakes (*Thamnophis*) as its primary host (Ingles, '33). The metacercaria cysts, each approximately 200 μm in diameter, were embedded primarily within the tissues at the base of the hind limbs (Figs. 3, 5, 6), but were also found elsewhere in the bodies of the two species of amphibian. In some parts of the body, especially around the cloacal region, the infestation is closely associated with irregular outgrowths (Figs. 3, 6). Cyst-infested outgrowths sometimes appear to completely disrupt the hind limb-forming regions in young tadpoles and to obviously affect the pattern of chondrogenesis of skeletal elements of the developing limb (Fig. 6). Cysts were found in close physical association with duplicated limb structures in both frogs (Fig. 3) and salamanders (Fig. 7). Only salamanders with abnormal limbs were collected and examined for the presence of cysts in this study, and all of these were infected. All frog specimens were infected with cysts, regardless of whether limb abnormalities were present. A sample of 103 adult *Hyla* that were captured and released at the Aptos location on March 6, 1987 included three with small accessory limb structures.

Resin beads surgically implanted into developing limb buds of larval *Xenopus* and axolotls induced substantial limb abnormalities including duplicated limb structures in nine (20%) of a total of 44 experimental animals (Figs. 8, 9), compared to only one of 16 controls (a minor digit bifurcation in an axolotl larva). In addition, a close physical association between beads and duplicated limb structures was seen in experimental salamander limbs (Fig. 9) similar to the association between cysts and duplicated structures observed in wild-caught specimens (Fig. 7).

Chemical analysis of a water sample taken from the Aptos pond on July 17, 1986, testing for heavy metals, chlorinated hydrocarbons, and petroleum hydrocarbons, gave negative results.

DISCUSSION

Digenetic trematodes have a complex life cycle involving a primary host (usually a vertebrate carnivore) in which the adult flatworm lives, and

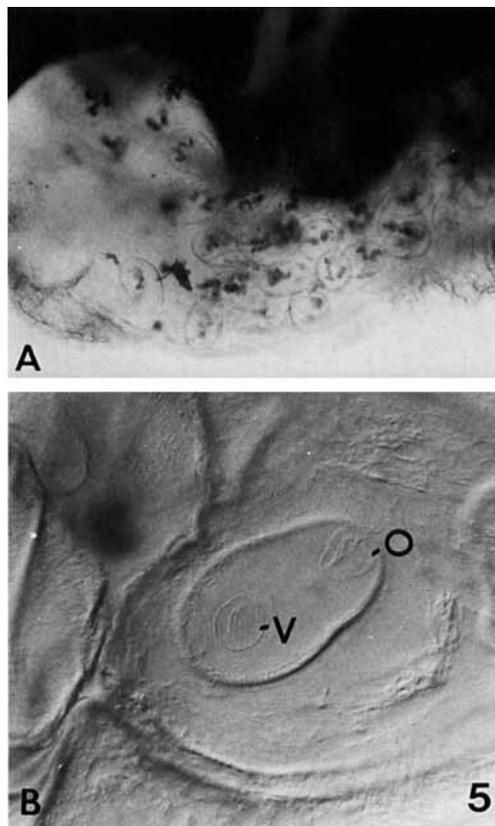


Fig. 5. A: Cloacal/hind limb-base region of a froglet showing massive infestation of metacercariae. B: High magnification of metacercaria cyst embedded in the hind limb-bud region of a tadpole (Nomarski optics); oral (O) and ventral (V) adhesive organs are visible.

one or two intermediate hosts in which larval development and/or encystation of the trematode occurs (Barnes, '68). Details of the life cycle vary widely among species of trematodes. In a generalized life cycle of a digenetic trematode, the eggs are released into pond water with the faeces of the primary host (e.g., garter snakes). The eggs then hatch as a first larval stage (miracidium), which infects pond snails. After passing through several developmental stages within the snail, the trematode is released as a free-swimming larval stage, (cercaria) which seeks out a secondary intermediate host (in this case, amphibians). The cercariae penetrate the skin of the secondary intermediate host and form cysts (metacercariae). The trematode's life cycle is completed when the secondary intermediate host is eaten by the primary host. The amphibians we discovered are therefore serving as secondary intermediate hosts in the trematode's life cycle. Possibly more than one

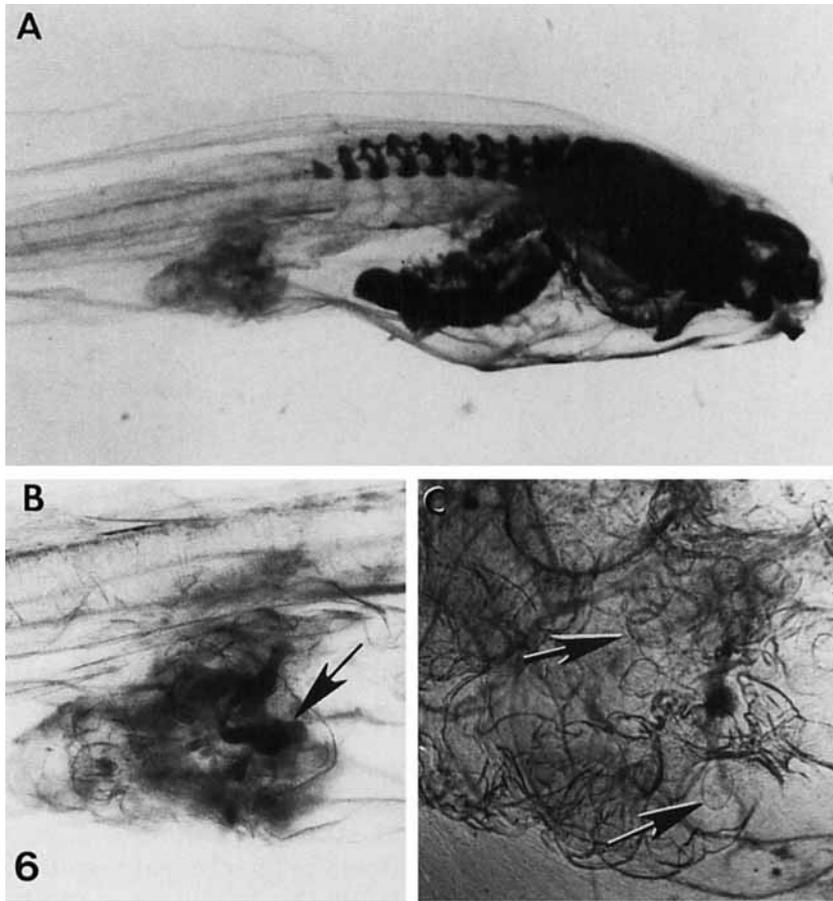


Fig. 6. **A:** *Hyla* tadpole with disorganized, cyst-infected hindlimb region. **B:** High magnification of hind limb region of same tadpole showing distorted limb cartilages (arrow). **C:** High magnification of same hind limb region showing numerous cysts (arrows) embedded in tissue.

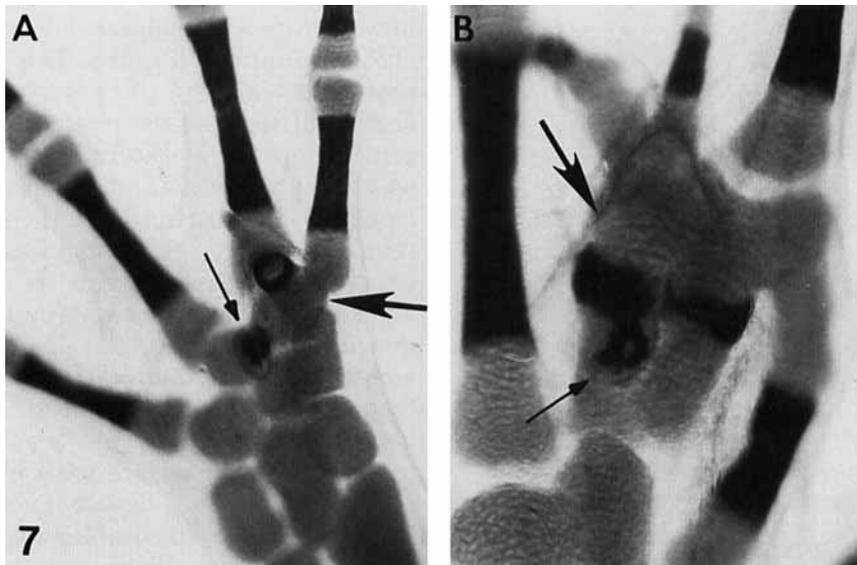


Fig. 7. Hand (**A**) and foot (**B**) of a salamander larva (*A. macrodactylum*) showing a close physical association between cysts (small arrows) and extra digits (large arrows).

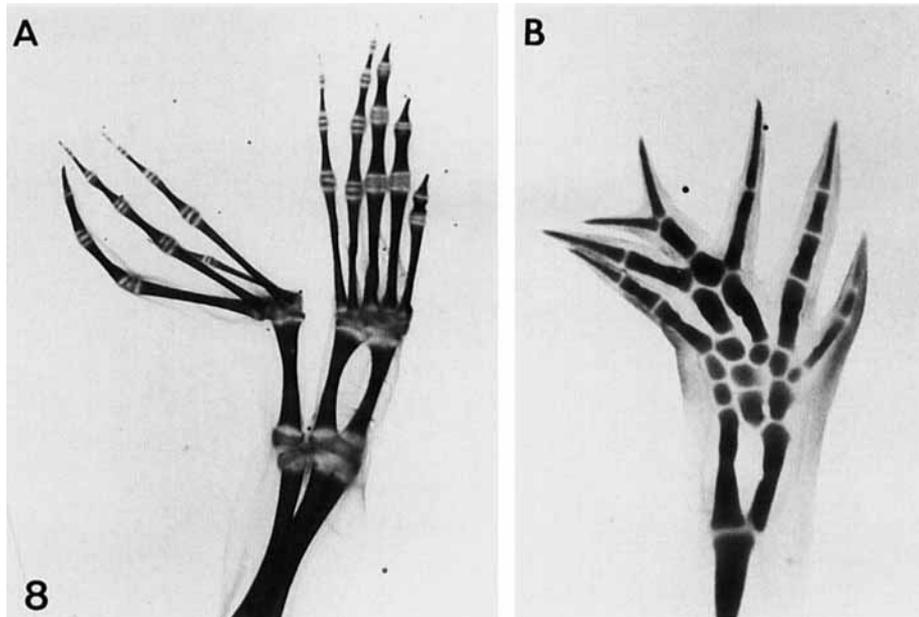


Fig. 8. Duplicated hind limb structures experimentally induced by bead implantation in the hindlimb buds of (A) *Xenopus* and (B) axolotl (*A. mexicanum*).

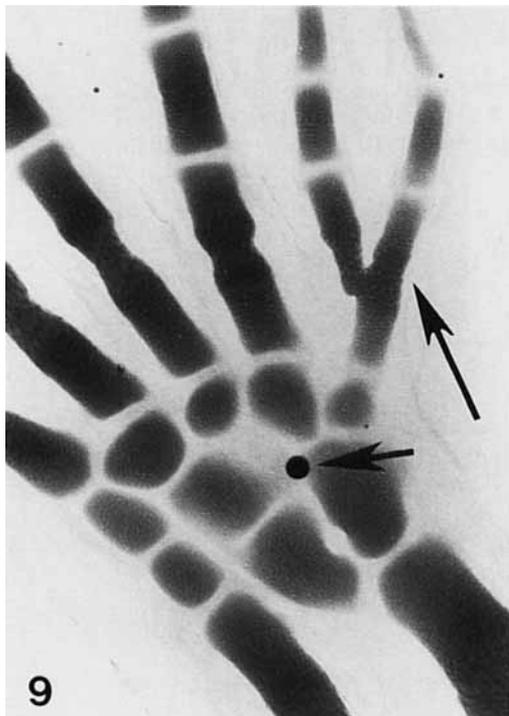


Fig. 9. Hand of an axolotl larva (*A. mexicanum*) in which beads were surgically implanted, showing close physical association between a bead (short arrow) and abnormal, bifurcated digit (long arrow).

species of trematode parasitizes these populations of amphibians, since adult flukes (tentatively identified as *Clinostomum sp.*) have been isolated from the pharyngeal region of adult *A. macrodactylum* (M. Dailey, pers. comm.). Subnormal rainfall resulting in early pond drying for the past two years has delayed efforts to study the trematode life cycle.

We hypothesize that the trematode cysts interfere with normal limb development in amphibian larvae to produce abnormal limb structures, including duplicated limbs. This possibility is supported not only by the association between limb abnormalities and the presence and localized concentrations of metacercariae in two unrelated species of amphibians in the same pond, but also by the visible disruption of the hind limb-forming region by cysts in young tadpoles. Further evidence comes from specimens that showed close physical association between cysts and supernumerary limb structures. An important question is whether the trematode cysts disrupt pattern through the secretion of a teratogenic or morphogen-like substance or simply act as solid obstacles that passively interfere with morphogenetic processes during limb development. We tested this latter possibility by implanting inert beads into the developing limb buds of larvae of frogs (*X. laevis*) and salamanders (*A. mexicanum*). We were able to insert only a relatively few beads

compared to the number of cysts commonly found in our wild-caught specimens. Nevertheless, this treatment induced duplicated limb structures in a significant number of cases. In addition, a close physical association between beads and duplicated limb structures was seen in experimental limbs, similar to the association between cysts and duplications in wild-caught specimens. The results of the bead implantation experiment indicate that mechanical disruption of developing limb tissues by inert objects similar in size and shape to trematode cysts is sufficient to stimulate the outgrowth of supernumerary limb structures. However, these results do not preclude the possibility that the metacercaria cysts also secrete a growth-stimulating substance, but they do show that such a scenario is not necessary to explain the observed abnormalities.

Our conclusion is that the limb abnormalities in these amphibians are probably caused by the mechanical disruption of developing and regenerating tissues by trematode cysts. First, by burrowing into the tissues of developing limb buds or regenerating limbs the parasites probably cause extensive, localized tissue damage. These localized injuries would trigger a wound-healing and/or regenerative response, depending on the species and the developmental state of the limb tissue. This response would be complicated by the presence of the cysts as relatively large, solid objects embedded in the tissues of the limb field or developing limb bud. Disruption of limb-forming tissues by cell death or experimental tissue rearrangements commonly results in regulatory responses leading to pattern duplications in a variety of organisms, including insects and vertebrates (Bryant et al., '80). These observations have been interpreted to indicate that such cellular perturbations can result in the confrontation of cells with disparate positional information. A characteristic regulatory response to such positional confrontations leads to the reestablishment of pattern continuity through intercalary mitotic growth (Bryant et al., '80). This response results in the acquisition by cells of normal nearest neighbors, whether or not the resulting overall limb pattern is normal. Mirror-image limb duplications are a classic example of global morphological abnormalities produced by processes that restore pattern continuity (Bryant et al., '80). The observed abnormal limb patterns reported here, including mirror-image duplications, suggest that extensive tissue rearrangements, probably including cell displacement and death,

have occurred during limb development in these amphibians.

The variable morphological effects of the parasitic infestation are probably explained by ontogenetic differences between the two species as well as the stage of limb development or regeneration at which exposure to the cercariae occurs in different individuals. Frogs gradually lose the ability to regenerate limb structures during ontogeny as tadpoles approach metamorphosis (Muneoka et al., '86); therefore, duplicated limb structures could be induced by metacercaria infestation only during early tadpole stages when limb bud regeneration is possible. Cercaria attack on the intact or amputated limbs of older tadpoles and post-metamorphic frogs would not induce limb duplications, and this probably explains why our sample included infested frogs with normal limbs. Salamanders, on the other hand, can regenerate their limbs throughout life, and cercaria attack could result in duplicated limb structures at any time during ontogeny. Because of differences in the life cycle between the two amphibians and because of the high frequency of attempted cannibalism among the salamander larvae sampled in August and September of 1986 (Ruth, unpublished observations), we think that many of the limb abnormalities seen in *Ambystoma* are due to cercaria attack on regenerating limbs (amputated via predation by other *Ambystoma*) rather than developing limb buds. At very early stages before limb outgrowth in either species, the cysts could mechanically partition the entire embryonic (or regenerative) limb field, and regulatory responses of the affected tissues within the subdivisions could result in major morphological abnormalities, including extra whole limbs. The morphological consequences of cercaria attack at later limb bud/regenerative stages would be correspondingly diminished.

There are at least two possible explanations for why the hind limbs are preferentially affected in these frogs and salamanders. First, cercariae are known to be attracted to orifices in intermediate hosts (M. Dailey, pers. comm.) and the developing hind limb buds are located immediately adjacent to the cloacal opening in both frogs and salamanders. Furthermore, in *Hyla* (as in most other frogs) the forelimbs develop protected within an atrium, whereas the hind limbs develop exposed to the external medium (Nieuwkoop and Faber, '75). Similarly, in *Ambystoma* (as in most other salamanders with aquatic larvae) the forelimbs undergo much of their development before hatch-

ing while the embryo is still enclosed within the egg capsules (Harrison, '69). On the other hand, the hind limbs of the salamanders develop much later (after hatching) and, like the hind limbs of frogs, are exposed to the external medium at very early developmental stages. Apparently, these characteristics are sufficient to protect the early developing forelimb-forming regions from predation or cercaria attack in both species.

The case reported here of duplicated limbs in wild-caught amphibians is important as an example in which morphological perturbation of an entire population is apparently caused by an identified environmental factor: parasitic flatworms. Van Valen ('74) has emphasized the possible importance of such cases in the evolution of morphological novelties through "genetic assimilation." His argument is that if extra legs were advantageous in a certain environment, then selection would favor genetic mutations that impart increased sensitivity of developing limb tissues to the causative agent (e.g., parasites). In other words, it would require fewer and fewer parasites to trigger the development of extra limbs until finally extra limbs would form even in the absence of the parasite. This scenario seems highly unlikely in light of the fact that extra limbs are so easily induced in amphibians and yet apparently no tetrapod lineage has ever evolved extra limbs as a heritable adaptive character (although several groups—including amphibians, reptiles, and mammals—have lost one or both pairs of limbs).

In the amphibians reported in this paper as well as in others (Van Valen, '74; Volpe, '81; Reynolds and Stephens, '84), extra limbs seem to constitute a survival disadvantage. Frogs in particular require functional hind limbs for predator avoidance and swimming, and metamorphosed *A. macrodactylum* may travel a mile or more to and from breeding sites. The extra legs observed in our specimens are apparently poorly innervated since they move little or not at all, are dragged passively and appear to impede function of the animals' "normal" limbs (if present). This is especially true of mirror-image duplicates, which combine limbs of opposite handedness on the same side of the body. Furthermore, most of the captive multilegged frogs in our study died soon after metamorphosis, and many juvenile and larval salamanders were obviously weakened by inflamed, cyst-infested outgrowths. Nevertheless, we have evidence that frogs and salamanders with abnormal limbs including extra limb structures can survive to reproductive maturity. Ap-

proximately 5% of the adult salamanders had abnormal limb structures. Also, approximately 3% of the adult frogs collected in 1987 at the same pond that yielded multilegged froglets in 1986 had limb abnormalities. If there is a selective advantage in this system it belongs to the trematode, since the handicapped amphibians, acting as intermediate hosts in the trematode's complex life cycle, make easy prey for the primary host (probably a small vertebrate carnivore such as a garter snake). Increased levels of trematode infestation, however, would run the danger of causing the local extinction of the amphibian hosts on which the parasite depends for its own propagation.

In conclusion, the observed phenotypic effects reported here, including extra limbs, probably reflect intrinsic regulatory properties of the cells of developing tissues reacting to an extrinsic perturbation (infestation of trematode cysts). Since similar effects on limb morphology can be obtained experimentally by disrupting positional relationships between cells in the developing or regenerating limbs of amphibians (Bryant et al., '81), any of a wide variety of environmental factors could have similar effects. The characteristics of trematode life cycles combined with the regulative properties of amphibian limb tissues make cercaria attack particularly effective in inducing duplicated limbs. Ironically, these phenotypically plastic effects underline the resilience of normal epigenetic patterning mechanisms in the vertebrate limb. We think that the occurrence of multilegged frogs and salamanders reported in this paper reflects a sporadic and localized population explosion of trematodes and/or one of its hosts. This possibility should be explored in previously reported, but unexplained occurrences of multilegged amphibians.

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