

# ONE

## WHAT IS AN AMPHIBIAN MALFORMATION?

Symmetry is an unmistakable sign that there's relevant information in a place. That's because symmetry is a property shared by a relatively small number of things in the landscape, all of them of keen interest to us. . . . Symmetry is also a sign of health in a creature, since mutations and environmental stresses can easily disturb it.

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The word “malformation” literally means “bad form.” Bad form in most animals means an unintended lack of symmetry, or an imbalance in structure, color, or other quality. A lack of symmetry can arise through one of three mechanisms:<sup>2</sup>

*Genetic* Genes are flawed, or the expression of genes during development is flawed. Albinism (in which animals have white bodies and pink eyes) is a familiar genetically determined malformation. Albinism is often caused by a mutation that results in the failure of embryonic neural crest cells to migrate. Similar genetic mutations in northern leopard frogs produce blue or partially blue axanthic individuals (Color Plate 1),<sup>3</sup> the kandiyohi pigment morph (which has tight blotches rather than spots; Color Plate 2), and the burnsi pigment morph (which lacks dorsal body spots; Color Plate 3).<sup>4</sup> These mutations are not fatal and in the cases of the kandiyohi and burnsi morphs are caused by an autosomal dominant allele and need only be passed on by one parent. Roughly 50% of all offspring between one of these morphs and a normally pigmented leopard frog inherit the pattern; from 75–100% of offspring between two of these pigment morphs inherit the pattern. Pieter Johnson and his colleagues<sup>5</sup> have suggested using the term “deformity” for malformations such as these that arise from genetic mechanisms. Not everyone has followed this suggestion, although such precision in thought guides understanding.

*Epigenetic* Genes and gene expression are normal but some deviation from expected environmental circumstances occurs where and at the time when genes are being expressed (due perhaps to a lack of nutrition, to the presence of toxins that disturb cell-to-cell interactions, to the presence of other chemicals that disrupt the endocrine system or cause chromosomal aberrations, or to mechanical disruptions). Both genetic and epigenetic problems are apparent before development is complete, and are called congenital abnormalities when present at birth in humans. Epigenetic mechanisms, as we shall see below, are the most likely cause of the current malformed frog phenomenon.

*Trauma (or other post-developmental mechanisms)* Injuries or disease. Injuries can occur during failed predation attempts, territory or mate defense, amplexus (mating), or environmental exposure (desiccation or frostbite). Infections secondary to injury can also occur and have permanent effects.<sup>6</sup>

It can be difficult to determine the cause of any given malformation, but a sound knowledge of natural history offers perspective. For example, in Minnesota and other areas of the Upper Midwest, and in New England, most malformed frog hotspots are isolated wetlands; separated from other known hotspots by tens, if not hundreds, of miles. But frogs are mobile, they travel from wetland to wetland, and genes get spread among populations. If genetic mechanisms are the cause of malformations we would expect—in much the same way that burnsi and kandiyohi northern leopard frog color morphs are distributed—frogs in wetland clusters to exhibit malformations. Further, within any wetland hotspot, more than one species can be affected and many of the malformations found in these species are of the same type.<sup>7</sup> The chances of the same genetic mutation arising in several species at a single site without any sign of mutations in populations from nearby wetlands (and these nearby wetlands are frequently close, well within the home range of individual frogs) must be astonishingly low. These observations suggest a site-based (epigenetic or trauma-based) cause. In fact, Dave Hoppe has performed the definitive experiment.<sup>8</sup> He collected a newly laid cluster of mink frog (*Rana septentrionalis*) eggs and raised half the eggs in his laboratory, in water known to be suitable for normal development, and half in a pond known to produce malformed frogs. In these genetically similar animals, the lab-reared tadpoles developed normally, the pond-reared tadpoles developed extra legs as well as



PLATE I. (A) Left and (B) right side views of an axanthic northern leopard frog collected along the shore of Sunken Lake in Dickinson County, Iowa, in 2003. Photo by Daniel Fogell; used with permission.

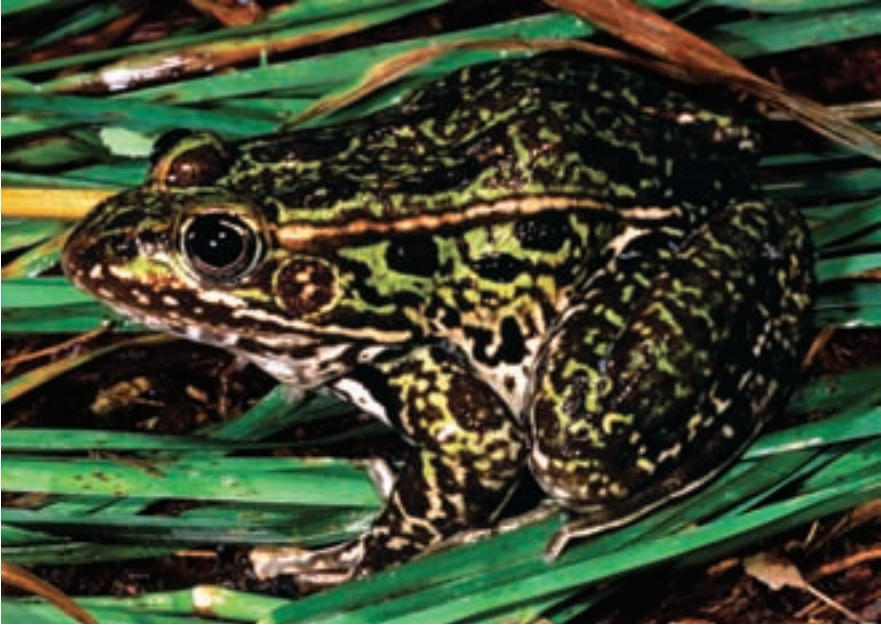


PLATE 2. A green kandyohi northern leopard frog morph from western Minnesota. Photo by Michael Redmer©/www.mikeredmer.com.



PLATE 3. (A) A brown burnsi morph northern leopard frog from northwestern Iowa. Note the lack of dorsal spotting. Northern leopard frogs may be either brown or green, and in the Upper Midwest, brown and green animals occur in about a 50:50 ratio. (B) A green burnsi morph northern leopard frog (or is it?—note the two elongate dorsal spots) from northwestern Iowa. This animal teaches a lesson: when working with amphibians we must be willing to consider variants that defy convenient characterization. Photos by the author.



PLATE 4. *Rana pipiens*. 50 mm SUL. Collected on 24 September 1997 at the SUN site in Ottertail County by Minnesota Pollution Control Agency field biologists. Note on the left thigh, proximal to the site where the limb segments are missing, the normal barred pattern along the dorsal hind limb (see right side of animal) is disrupted. Spotting is smaller and oriented along the long axis of the limb. Failed predation cannot produce this pigment pattern. Animals from this site are also shown in Figures 2.16 and 2.54.

other malformations. Hoppe's conclusion was unavoidable—these malformations were not genetically based, the cause was in the water.

In deciding between epigenetic and trauma-based causes, trauma often-times seems the least likely. Trauma can and does happen, and perhaps most trauma in amphibian larvae comes from failed predation attempts. However, we expect trauma to produce scarring, and most malformed animals show no signs of scarring or any other type of wound-healing process. This is true despite the fact that limb development, and therefore the opportunity for limb trauma, immediately precedes metamorphosis (remember that most malformed frogs are collected at or just after metamorphosis, when scarring should be obvious). Further, many frogs have malformations that simply cannot be due to trauma (e.g., eyes not in their proper location, multiple bent bones, multiple fluid-filled sacs, bloating, abnormal pigment patterns, small heads), or if they were due to trauma the injuries sustained (missing pelvic and spinal components) would likely be inconsistent with life. Forelimb malformations in newly metamorphosed animals are also unlikely to arise from trauma. For most of a tadpole's life forelimbs are tucked under enclosed gill coverings and therefore hidden from visually oriented predators, who are unlikely to selectively eat what they cannot see.

Given this evidence, the amphibian malformations that most concern us probably arise from epigenetic mechanisms—consequences of the environment in which genetic expression is occurring. As Michael Pollan has observed:<sup>1</sup> “. . . the ecological effects of changes to the environment often show up where we least expect to find them (p. 211).”

#### MOST AMPHIBIAN MALFORMATIONS ARE FROG MALFORMATIONS

Martin Ouellet assembled a comprehensive review of the literature on amphibian malformations<sup>9</sup> and from his study we can conclude that most amphibian malformations are frog and toad malformations. Ouellet's search found data on malformations occurring in more frog and toad species, sites, and specimens (Table 1.1).

The data my colleagues and I have collected from the United States support this conclusion. In the past decade we have radiographed 2,377 malformed, or suspected-to-be malformed, amphibians; 2,363 were frogs, 14 were salamanders. There were, however, sampling biases. In field protocols

TABLE 1.1. *Summary of Martin Ouellet's data showing most amphibian malformations are frog and toad malformations*

	<i>Frog and Toad vs. Salamander Numbers</i>	<i>Ratio</i>
Species affected	67:26	2.6:1
Sites where found	159:43	3.7:1
Numbers collected	11,687:2,512	4.5:1

followed by both the Minnesota Pollution Control Agency (~ 702 specimens) and U.S. Fish and Wildlife Service (666 specimens through the 2005 field season), there was a stated emphasis on collecting malformed frogs rather than malformed salamanders. Therefore the species and sites sampled, the timing of sampling, and other practices favored the collection of frogs.

#### FROG SKELETONS

Because the form, or basic size and shape, of vertebrates is generated from their skeletons, and because skeletal tissue is the first to form in developing limbs, any serious description of malformations must include an examination of skeletons. There are several ways to examine skeletons, but we rely on radiographic (or roentgenographic in the old eponymous and cumbersome terminology<sup>10</sup>) analyses—we take “x-rays” (although, strictly speaking, x-rays are the type of radiation that produces the image on film; the actual image itself is termed a “radiograph”). To know what is abnormal, we must first know what is normal.

Frog skeletons are simple but variable (there are, after all, over 5,200 recognized species of frogs; in fact, the late Dr. Alan Holman used the shape of the hip joint [acetabulum; see below] to identify fossilized frog bones).<sup>11</sup> Figures 1.1 and 1.2 show labeled illustrations of frog and toad skeletons.<sup>12, 13, 14</sup> From these images, and following the recent review of Handrigan and Wassersug<sup>15</sup> we can see that frogs exhibit:

- a large skull with a broad jaw and prominent eye sockets;
- a rigid, shortened vertebral column with five to eight vertebrae (humans have 25);

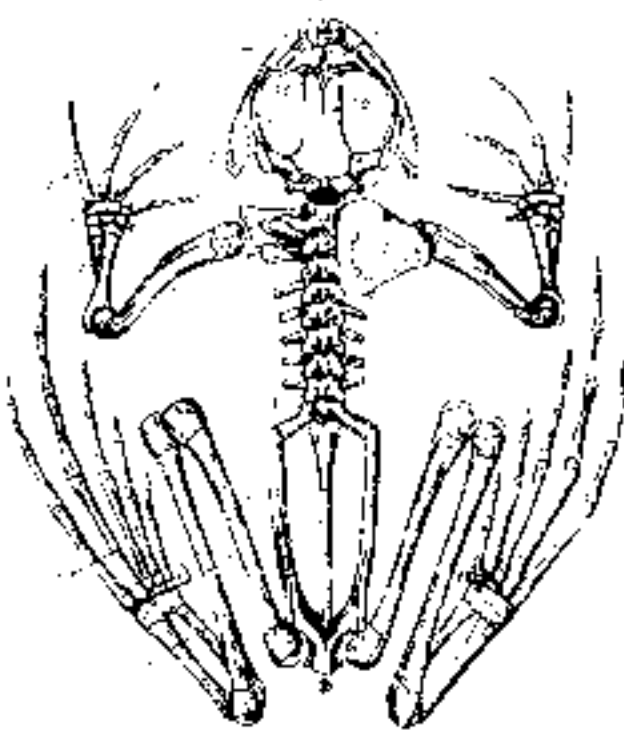


FIGURE 1.1 Frog skeleton (presumably *Rana* sp.). Left suprascapular and scapular removed. From J. Z. Young.<sup>13</sup> Letters code to bones as follows: *a*, tibiale (astralagus); *c*, fibulare (calcaneum); *d*, suprascapular; *e*, exoccipital; *f*, femur; *fp*, frontoparietal; *g*, metacarpals; *h*, humerus; *i*, ilium; *k*, metatarsals; *l*, carpus; *m*, maxilla; *n*, nasal; *o*, prootic; *p*, pterygoid; *pm*, premaxilla; *q*, quadratojugal; *r*, radioulna; *s*, squamosal; *se*, sphenethmoid; *t*, tibiofibula; *u*, urostyle; *sv*, sacral vertebra. Used with permission of Oxford Press.

no ribs (this generalization holds for most frogs, including all species found in North America)

relatively simple shoulder and pelvic girdles; and  
strong but simple limbs.

Shoulder girdles consist of sternal (breast) bones along the midline of the belly, scapular bones (shoulder blades) along the back, and coracoids and

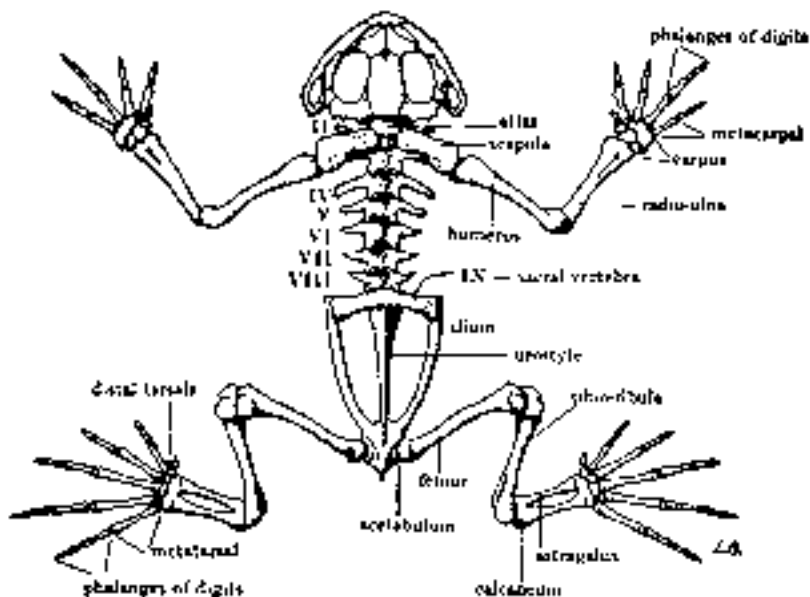


FIGURE 1.2 Skeleton of “common” toad (presumably *Bufo* sp.). From A.S. Romer.<sup>14</sup> In his caption, Romer notes: “Except in minor details, this is identical with the frog skeleton. (After Jammes.)” Used with permission of The University of Chicago Press.

clavicles (collar bones) connecting the sternal bones to the scapulars. The coracoid and scapular bones form the socket for the forelimb humerus.

Pelvic girdles consist of sacral, coccygeal, ischial, and pelvic bones aligned with the backbone, and paired iliums to either side (laterally). The acetabulum, or articulation between the pelvis and the femur of the leg, occurs at the junction of the ilium, ischium, and pelvic bones.

In frogs that jump, such as northern leopard frogs (see Fig. 1.1 and compare to Fig. 1.2), the pelvis and hindlimbs are modified to generate thrust; the shoulders and forelimbs are modified for landing. Male frogs may have larger forelimbs than females, to better grasp females during mating (amplexus).

Frog limbs are similar to the limbs of all vertebrates, including humans, in having the closest segment to the body supported by a single long bone (humerus in the arm, femur in the leg), and the next closest segment sup-

ported by two long bones (radius and ulna in the arm, tibia and fibula in the leg), which in frogs are fused to form the radioulna and tibiofibula, respectively. Wrist (carpal) and ankle (tarsal) bones are numerous but may be reduced or fused, depending on the species. Ankle bones include the elongated tibiale and fibulare. Hands are composed of metacarpal bones (the palm) and phalanges (fingers). Feet are composed of metatarsal bones (the foot in a restricted sense) and phalanges (toes). Four digits are expected on the hands, five digits are expected on the feet.

#### THE NATURE OF BONE

When we think of skeletons we think of bones. Bones, particularly long bones, consist of two parts: an outer, hard compact surface (periosteal bone), and an inner, chambered, cancellous core (marrow cavity).<sup>16</sup> Bones are vascularized (have a blood supply). Bone tissue consists of calcium supported by a protein matrix called hydroxyapatite. With life experiences, bones are remodeled and reshaped. This complicated process is accomplished by differential addition to or subtraction from the bone surface. Bone addition is achieved through the actions of cells called osteoblasts; bone subtraction is accomplished by osteoclasts.

Most bones, including the bones of the spine, shoulder and pelvic girdles, and limbs, develop from cartilage. Cartilage is unvascularized—it has no capillary network of its own—and is made of protein (collagen and proteoglycan complexes) and water. During the metamorphosis from an aquatic tadpole to a terrestrial juvenile, most portions of frog skeletons undergo a conversion from cartilage to bone. This conversion is triggered in part by the growth of arteries into the cartilaginous skeletal elements. Cartilage is much less dense than bone, so radiographs of frog tadpoles do not show the contrast between skeletal and non-skeletal elements seen in metamorphosed frogs (Figure 1.3).

#### HOW DO MALFORMATIONS ARISE DURING DEVELOPMENT?

All vertebrate tissues develop from one of three embryonic germ layers: ectoderm (giving rise to epidermis, pigment cells, and nervous tissue), mesoderm (which creates the dermis of the skin, skeleton and muscle tissue), and

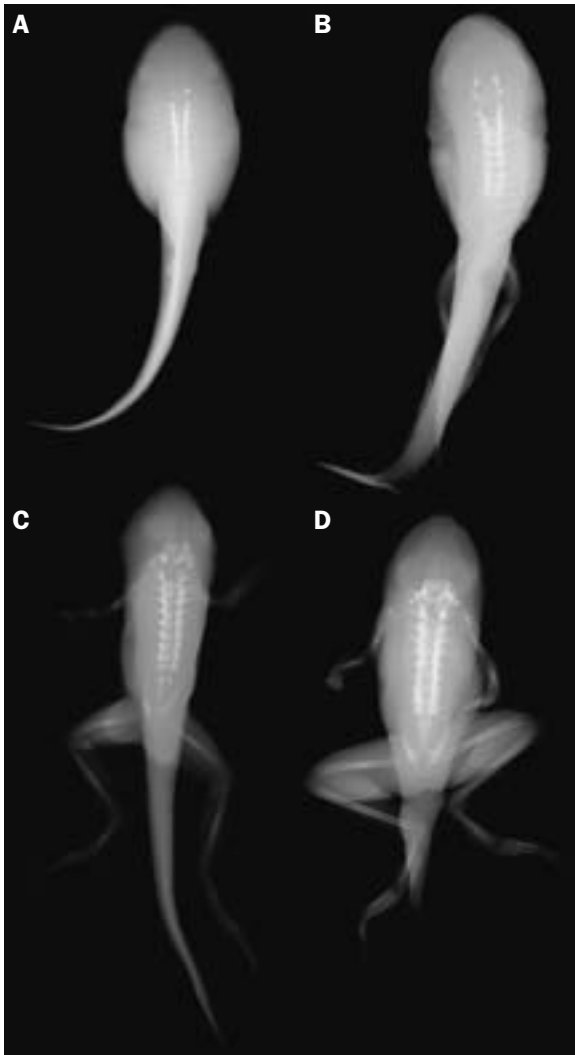


FIGURE 1.3 *Rana pipiens* tadpoles and mid-metamorphic animals collected in Minnesota during the 1996 and 1997 field seasons, illustrating the partial sequence of bone ossification: (A) collected August 1997, ~39 mm SUL; (B) collected June 26 1996, ~31 mm SUL; (C) collected August 1997, ~31 mm SUL; (D) collected June 26 1996, ~33 mm SUL.

endoderm (responsible for forming the lining of the gut and digestive glands). In amphibians, the three most common sites for malformations are the limbs, the jaws, and the eyes. In each of these regions, malformations are the result of mesodermal and/or ectodermal problems (i.e., legs, jaws, and eyes have no gut tissue and thus no endodermal contribution to their development). There can be no doubt that endodermal malformations arise, but because they are internal, these malformations are less likely to be observed. Additionally, because gut function is essential, animals with endodermal malformations may not survive their tadpole stage to reach metamorphosis, the age where malformations are detected, using current field collection protocols.

### LIMB DEVELOPMENT

Perhaps no aspect of amphibian developmental biology is better known than the process of limb formation.<sup>17</sup> Not only does the study of amphibian limb development have a long history, but several high profile labs continue to study this important topic. To understand the malformed limbs of amphibians, it is useful to have in mind seven concepts underlying the development of normal limbs:

1. limbs develop from limb buds;
2. limbs have three axes: proximal–distal (from the body out to the fingers or toes); dorsal–ventral (back to belly); and anterior–posterior (head to tail);
3. limb buds give rise to limbs from proximal to distal, which means the farther a structure is from the body, the later it will develop;
4. limb components develop in the following order: cartilage, blood vessels, nerves, and muscles;
5. the shape of limbs varies by species (compare the long limbs of frogs to the short limbs of toads), and within an individual by position (compare forelimbs to hindlimbs) and side (limbs on opposite sides are mirror–image oriented);
6. limb growth after the shape pattern has been established is critical for proper limb function;

7. amphibian limbs, especially the developing limbs of anurans and larval and adult limbs of some salamanders, have impressive abilities to regenerate lost structures.

*Some Details* As mentioned above, mesoderm and ectoderm combine to form limbs. To begin, aggregations of mesoderm from an area known as the lateral plate form beneath the surface ectoderm to create limb buds. At the distal end of each limb bud, the surface ectoderm thickens to form a discrete growth zone known as the apical ectodermal ridge (AER). Normal limb development is dependent on the interaction between limb bud mesoderm and the AER.

As the limb bud elongates, it contains an embryonic tissue termed mesenchyme. Mesenchyme consists of embryonic cells found as individuals or in groups, rather than in sheets. Mesenchymal cells can be derived from mesoderm or ectoderm (for example neural crest cells, which are mesenchymal and produce pigment, derive from ectoderm). Mesenchymal cells form precursors to cartilage (the formation of cartilage is called chondrogenesis) that will develop into the bones of the shoulder and pelvic girdles, and later into the bones of the limbs (the formation of bone is termed osteogenesis).

During early stages of outgrowth, the limb bud is invaded by structures called angiogenic cords (the primordia of blood vessels), which establish a blood supply (the formation of blood vessels is termed angiogenesis). Nerves follow and make motor and sensory connections (a process called neurogenesis). Finally, cells for the precursors to muscle (myogenic primordia) enter, condense, and form muscle masses, which subsequently segregate to form individual muscles (the process of developing muscle tissue is termed myogenesis).

Limbs develop not only by the assembly of structures but also by their disassembly. Areas of programmed cell death (necrotic zones) appear late in development, for example in the mesenchyme between the digits, wrists and ankles, elbows and knees, and armpits and groin. This process of cell death (called apoptosis) plays an important role in shaping limbs.

After the limb pattern is established, a period of rapid growth follows, during which muscles and skeletal tissues mature, tendons and ligaments are formed, and joints develop. The limb must be functionally innervated and moving for this to occur properly. As Lanyon and Rubin point out (p. 1):<sup>16</sup>

The development of an organism from an embryo into a normal adult is a complex process requiring both appropriate genetic instructions and adequate nutrition. However, this input alone is not sufficient to form a functional skeleton, since development occurring under these conditions (as in a paralyzed limb) produces skeletal structures that lack the detailed shape, mass, and arrangement of tissue necessary for load bearing. ‘Normalcy’ of architecture, and the structural competence that it reflects, is achieved and maintained only as a result of an adaptive response of the cells to load bearing. This response is functional adaptation.

During development, motor neurons supplying limb muscles are over-produced by anywhere from a factor of two to a factor of eight.<sup>18</sup> Motor neurons that “compete” successfully, live (i.e., make correct synaptic connections on myotubes during the development of limb reflex movements); those that do not “compete” successfully, die. While the absence of a limb does not affect motor neuron proliferation, migration, and initial differentiation, limb absence results in the death of nearly all motor neurons responsible for its innervation. Somatosensory neurons that would normally provide touch, pressure, pain, and temperature information from the limbs are similarly affected. It has long been known that the effects of developing limb amputation depend on developmental stage. In bullfrogs (*Rana catesbeiana*), amputation of a late-stage tadpole limb (when reflexes have become established) results in a degeneration of motor neurons at metamorphosis.<sup>19</sup> After metamorphosis, however, motor neurons do not degenerate following amputation.<sup>18</sup> This developmental process can assist in interpreting causes of missing limbs; under certain circumstances the absence of motor neurons distinguishes limbs never present from limbs once present and now absent.

In vertebrates, limb abnormalities and dysfunctions are among the most commonly encountered developmental problems. In part this is because developing limbs are more or less self-contained: limb buds either have what it takes to make an intact functioning limb or they do not, and when they do not, adjacent or interacting tissues or organs cannot assist by taking corrective action. Further, while limbs are critical for proper behavior and for ecological viability, they are not necessary for basic body functions. Therefore, unlike developmental problems in vital organs or organ systems, glitches in limb development tend not to be fatal. For frogs, this point is particularly

important, because aquatic, swimming tadpoles do not need functioning limbs to survive, or even to thrive. Forelimbs are so unimportant to tadpoles that they are tucked under gill covers. Therefore, as tadpoles, frogs with malformed limbs may be behaviorally and ecologically unaffected. At metamorphosis, of course, this situation changes abruptly from being simply curious to us to being vitally important to the frog.

During metamorphosis, limb assembly and coordination occur quickly, because the requirement to have four functioning limbs wired correctly and working together is behaviorally and ecologically essential. Metamorphosis is the most vulnerable time in a pond-breeding amphibian's life history.<sup>20</sup> Metamorphosing amphibians are concentrated along wetland edges and relatively immobile; they are easy prey for garter snakes, raccoons, opossums, and various bird species. And whereas having limbs that work is not a requirement for amphibian life, having working limbs at metamorphosis enables amphibians to escape from predators, and is therefore crucial for amphibian survival.

#### JAW DEVELOPMENT

Vertebrate jaws develop from an embryonic structure called the first visceral arch.<sup>21</sup> Visceral arches (there are six total) form in the region of the pharynx (neck) and are produced from all three germ layers (ectoderm, mesoderm, and endoderm). Visceral arches form the structures responsible for swallowing and breathing (including the formation of gills in fishes and aquatic amphibians). The major vessels of the circulatory system, including the heart, aorta, and big vessels to and from the arms and brains, develop in visceral arches and later migrate into the thorax.

To form jaws, the neural crest-derived mesenchyme of each side of the first visceral arch splits in two to form the upper jaw (mandibular process) and the lower jaw (maxillary process). These processes grow towards the front of the face, meet, and fuse. At metamorphosis, frog jaws undergo further change as they reconfigure from the small scraping, sucking cartilaginous mouthparts of tadpoles, to the large grabbing, biting, bony mouthparts of adults.

#### EYE DEVELOPMENT

In vertebrate animals, proper eye development is the result of a series of complicated ectodermal and mesodermal tissue interactions.<sup>21</sup> In amphib-

ians, these interactions also include gut (endodermal) tissue—because during swallowing, amphibian eyes close, retract, and push food down their throats—making these processes even more complex.

Eye development in vertebrates begins with the brain, specifically the forebrain (which also produces the cerebral cortex). Optic vesicles form on either side of the forebrain and grow out to where the eyes will eventually be positioned. When an optic vesicle meets the surface ectoderm, the ectoderm becomes transformed to create a lens placode, which invaginates to form a lens vesicle. This lens vesicle breaks away from the surface ectoderm, and this ectoderm becomes a portion of the cornea. At the same time, the lateral surface of the optic vesicle (remember, this is the extension of the forebrain) invaginates to form a nearly complete, bilayered optic cup. The inner surface of the optic cup gives rise to the neural portion of the retina; the outer layer of the optic cup gives rise to the pigmented epithelium of the retina. The optic stalk (now connecting the optic cup to the forebrain) persists, and guides axons from retinal ganglion cells back to the developing brain. When your family physician or ophthalmologist examines your retina, he or she is peeking at a derivative of the same brain structure that created your mind.

In the eye, the iris develops from cells that originated both from the pigmented epithelium and neural crest (ectoderm). Connective tissues in the eye and outside of the eyeball derive from neural crest and mesoderm. Blood supply comes from vessels associated with the first aortic arch (the same source as the blood supply to the jaws). Muscles that move the eyes, including in amphibians the levator and retractor bulbi muscles that assist food manipulation and swallowing, develop from head mesodermal tissue.

#### TIMEFRAMES AND THE APPEARANCE OF MALFORMATIONS DURING DEVELOPMENT

Tadpoles typically hatch from eggs and metamorphose into terrestrial juveniles within a single season, although the tadpoles of larger aquatic frogs such as bullfrogs, green frogs (*Rana clamitans*), and mink frogs may overwinter at least once.<sup>22</sup> In amphibians, rates of development are temperature dependent, so within a species, animals in northern populations take longer to complete life cycles than animals in southern populations. Similarly, animals in spring-fed wetlands grow more slowly than animals in pothole wetlands.

Rates of development vary across species, from as little as four weeks among treefrogs in the family Hylidae, (such as cricket frogs [*Acris crepitans*], eastern gray treefrogs [*Hyla versicolor*], and chorus frogs [*Pseudacris triseriata*]) and true toads in the family Bufonidae (such as American toads [*Bufo americanus*]), to over seven weeks (true frogs in the family Ranidae, such as northern leopard frogs). Some tadpole stages last much longer, including those of the bullfrogs, green frogs, and mink frogs mentioned above.

Malformed structures can only be observed when, or after, structures have (or would have) formed, so different types of malformations arise at different times during development. Eyes form embryonically, so gross eye malformations appear quickly and will be visible in both tadpoles and in adults. Jaws also form early in development, but unlike eyes, get completely reworked at metamorphosis. Gross jaw malformations can be seen in tadpoles or in adults, although they are often most conspicuous in adults. Limbs form in late-stage tadpoles, and limb malformations are often not apparent until tadpoles reach pre-metamorphic developmental stages. In species with a short tadpole stage, such as American toads, limb formation may precede metamorphosis by as little as one or two weeks.