

# Developmental Deformities in Amphibians

Stanley K. Sessions & Brandon Ballengée 2010

This scientific paper posits current explanations for the origins of limb deformities among natural populations of amphibians. Ballengée and Sessions introduced their 'selective predation hypothesis' in 'Explanation for missing limbs in deformed amphibians' published in the *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*. Volume 312B Issue 7, Pages 665 - 788 (15 November 2009).

## Abstract

Amphibian deformities have remained one of the most prominent and controversial environmental issues of the past fifteen years. During this time we have presented strong evidence that suggests the proximate causes for anuran hind limb deformities featuring supernumerary limbs and segments are caused by parasitic infection by the trematode *Ribeiroia ondatrae*. Secondly, those featuring missing limbs and segments are caused by selective predation by aquatic predators. Three key features of amphibian limb development have been important in understanding the etiologies for these limb deformities: 1) the hind limbs undergo all or most of their development after hatching such that they are exposed to the aquatic environment (parasites and predators) from early limb bud stage, 2) the limbs of most amphibian species can regenerate at least to some degree and some point in their lives, and 3) as in other tetrapods, limb development and regeneration involve pattern-forming mechanisms that depend on spatial and temporal patterns of biochemical signaling and other kinds of cell-cell interactions. Two important underlying developmental mechanisms in amphibian limbs fully explain the entire range of limb abnormalities that we have been studying: 1. Intercalation to explain those with extra limb deformities caused by parasitic infection 2. Regenerative decline for those with missing limb deformities induced by either inter- or intraspecific predators practicing selective predation.

## Amphibian Limb Development

In amphibians and other tetrapods, two pairs of limb buds form from mesodermal cells on the lateral body of early embryos. At this stage the limb buds are basically lumps of loose cells (mesenchyme) covered by a single layer of ectoderm. The underlying developmental mechanisms are known mainly from studies of amniote (chick) embryos, and those in amphibians are thought to be broadly similar in most respects.

Early events involve extensive cell signaling to establish the primary limb axes: anterior-posterior, dorsal-ventral, and proximal-distal. In chick embryos, early cell signaling leads to the creation of a thickened band of cells along the distal edge of the bud called the apical ectodermal ridge (AER). The AER in turn acts through signaling to maintain a band of proliferating cells called the Progress Zone (PZ), which is responsible for the proximal-distal outgrowth of the limb bud.

Subsequent signaling sets up specific cellular regions in the AER regulated by homeobox or HOX genes. Hox genes do this by switching on and off other genes causing cascades of gene expression and the establishment of differentiated regions of the developing limb. As part of this process, groups of cells in the posterior region of the bud form a more-or-less distinct signaling region called the Zone of Polarising Activity (ZPA).

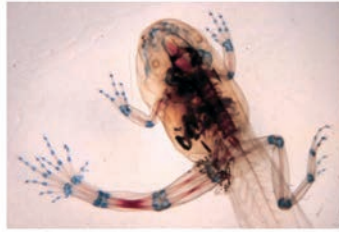


Fig. 1

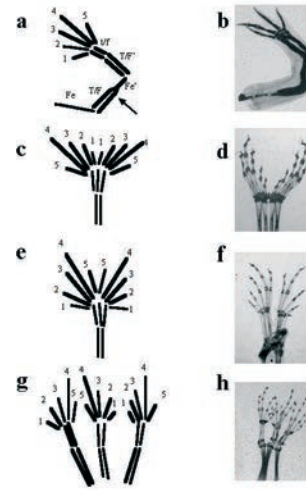


Fig. 2

Cells in the ZPA produce a biochemical signal called sonic hedgehog (shh), which helps establish the anterior-posterior axis (from “pinky” to “thumb”). The sequence of cell interactions leading to the establishment of the ZPA is thought to involve a gradient of retinoic acid (RA), which triggers the initial Hox gene expression leading to shh signaling. In amphibians, a distinct AER is absent or reduced (Richardson et al., 1998), and the ZPA is much less well-defined. The significance of these differences is not yet understood.

As the limb buds develop they form a conical shape in which the interacting cells begin to undergo overt morphogenesis to form the structures of the complete limb. The limb bud next undergoes flattening to form the “palette” stage soon followed by the formation of digits, which, in amniotes, involves extensive cell death (apoptosis) in the interdigital tissues. Here again amphibians differ from amniotes in that apoptosis either plays no role or a very minor role, and instead the digits form mainly through differential cell proliferation (Cameron and Fallan, 1977).

The formation of morphological pattern in amphibian limb development (i.e. the precise size, shape, and arrangement of limb elements) is formally called “pattern formation” (Gilbert, 2000). Many years of research have shown that pattern formation in developing amphibian limb buds depends on intimate interactions among cells in the embryonic limb field (Gilbert, 2000). In this sense,

Pattern Formation refers to the cellular responses to biochemical signaling mechanisms that characterise early limb development.

These early limb cells have information about their positions in the major axes of the limb field (“positional values”), and if their spatial organisation is disturbed they undergo a characteristic cellular growth response called “intercalation” (French et al., 1976; Bryant et al., 1981). This phenomenon is the basis of the well-known Polar Coordinate Model (PCM) of limb pattern formation (French et al., 1976; Bryant et al., 1981). The PCM was developed not only to understand the cellular response to grafting experiments but is also a powerful model for conceptualising normal limb outgrowth, even though it has not yet been fully integrated with the molecular and biochemical aspects of limb development.

According to the PCM, if cells with different positional values are forced to interact, they will undergo intercalation, producing daughter cells (via mitosis) with intermediate positional values and thereby re-establishing pattern continuity at the cellular level. In other words, intercalation fills in gaps between cells with disparate positional information. The astonishing effects of this simple regulatory mechanism on amphibian limb morphology have been noticed for decades (Swett, 1926; Harrison, 1969).

Intercalation is usually manifested at the morphological level by supernumerary limbs in

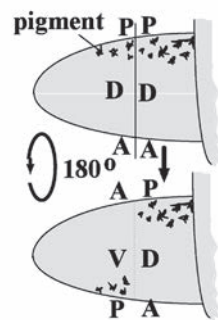


Fig. 3

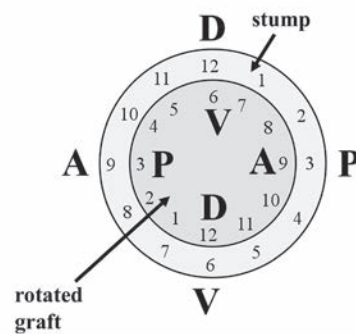


Fig. 4



Fig. 5

mirror-image orientation (Sessions et al., 1999; Fig. 7). These mirror-image limb duplications include double anterior mirror image duplications (AMIDs), double posterior mirror image duplications (PMIDs), mirror image triplications (MITs), and dorsal-ventral mirror image duplications (DVMIDs; Fig. 2; Sessions et al., 1999). Although the precise molecular basis of the PCM has not yet been worked out, many of the signals important in limb patterning are reviewed (Johnson and Tabin, 1997; Pearse and Tabin, 1998; Ng et al., 1999).

This information makes it possible to superimpose molecular expression onto the polar coordinate model. For example, we can consider the posterior values of the PCM (i.e. values 2-4) to correspond to the ZPA, or the expression of *shh*. When limb duplications are developing, producing extra regions of posterior positional values through intercalation, this should be manifested as ectopic ZPAs, marked by ectopic *shh* expression. This will be an area of future investigation.

Intercalation has been demonstrated in the lab with simple grafting experiments in both amphibians and amniotes, which result in the outgrowth of symmetrical supernumerary limb structures and other limb abnormalities (Swett, 1926; Bryant et al., 1987). For example, mechanically splitting a limb bud into anterior and posterior halves often leads to mirror-image duplications along the anterior-posterior axis (Swett, 1926). Likewise, rotating a limb bud around its anterior-posterior axis can induce the outgrowth of two or more limbs from a single limb bud (Hecker and Sessions, 2001, Figs. 3, 4 from Stopper et al. 2002.).

In most tetrapods the formation of digits, and of the whole limb, occurs very early in embryonic development while in amphibians (except a few direct developing species), this occurs relatively late, usually well after hatching (Alford & McDiarmid 1999, Galis et al. 2003). Differences in the details of limb development between anurans and urodeles are also important. In urodeles (or at least the ones with aquatic larvae), the forelimbs undergo most of their development before hatching, well in advance of the hind limbs, and so are protected by the egg jelly coats, and all limbs (in most species) can completely regenerate throughout life. In anurans, on the other hand, all four limbs develop more-or-less simultaneously, but the forelimbs undergo most of their development (up to metamorphosis) within the protection of an enclosed gill chamber.

Also extremely important is the fact anurans lose the ability to regenerate their limbs as they develop; young tadpoles can completely regenerate their limbs but this ability is gradually lost as the tadpoles approach metamorphosis and their limbs complete development (Muneoka et al., 1986). This process is called regenerative decline, and the maximum regeneration response in the limb of a metamorphosed frog or toad is a cartilaginous outgrowth (e.g. "spike") of some kind (Fig. 5).

#### Missing Appendages

The vast majority of deformed amphibians in wild populations are those with missing limbs and limb segments (Lannoo, 2008; Ballengée and Sessions, 2009). Hypotheses for such abnormalities have included chemical pollution and genetic damage caused by UV radiation believed to be effecting the normal development of embryonic amphibians.



Fig. 6

These hypotheses are based on the belief that what we are seeing among wild frogs are actually malformations similar to chemical induced human birth defects. Although certain chemicals such as retinoids and high levels of UV have been shown in laboratory simulations to induce malformed amphibian embryos, they do not match what we are finding in nature (please see review below). Our experimentally demonstrated hypothesis is that what we are finding among frogs are natural regenerative responses to injury (*selective predation*) from specific predators and even tadpoles to one another. Our evidence does not exclude the potential secondary role that environmental degradation may play in complex ecosystems and food webs but it does supply a concrete explanation for the origin of such injuries and for the full range of missing limb deformities found in the wild.

Selective predation, either inter- or intraspecific, can cause missing appendages identical to those seen in field-caught amphibians (Sessions, 1997; Ballengée and Sessions, 2009). Selective predation refers to predation by predators that are too small, or who have mouthparts that are too small, to consume an entire prey animal and instead remove only a portion of the prey, such as an exposed hind limb (Ballengée and Sessions, 2009). Enclosure/exclosure experiments in the field and in laboratory led to the conclusion that missing hind limbs in European Common Toads (*Bufo bufo*) were caused by selective predation by dragonfly nymphs (Ballengée and Sessions, 2009).

Although Licht reported observing dragonfly nymphs removing hind limbs in tadpoles in 1974, our study was the first to specifically link nymphs to the most

common types of limb deformities found in the wild. Other researchers have recently reported dragonfly nymph-induced hind limb deformities in populations of Cascades frogs (*Rana cascadae*; Bowerman et al., 2009). In our continued European and North American experiments we have identified nymphs of five different Dragonfly species that practice selective predation on anuran tadpoles (Fig. 6). Three of these, the Shadow darner (*Aeshna umbrosa*), Green darner (*Anax junius*), and Darter species (probably either *S. striolatum* or *S. sanguineum*), were able to produce permanent missing limb deformities among metamorphic anurans in our experimental enclosures (Ballengée & Sessions 2009, Ballengée et al., in prep., 2010).

In addition to dragonfly nymphs, certain crustaceans and other invertebrate predators, especially leeches, are also known to attack anuran limbs, sometimes causing limb loss or injury (Licht, 1974; Formanowicz and Brodie, 1982; Duellman and Trueb, 1986; Viertel and Veith, 1993; Johnson et al., 2001a). Limb deformities in Western toads were caused by stickleback fishes, an introduced predator (Johnson et al. 2001; Bowerman et al., 2009). Our own experimental simulations show that aggressive attacks by Three-spined sticklebacks (*Gasterosteus aculeatus*) injure and/or remove limb segments in *B. boreas* and Common frog tadpoles (*Rana temporaria*). We also now know that attacks by Brook sticklebacks (*Culaea inconstans*) cause injuries to toes and eyes in late-stage tadpoles of Woodfrogs (*Rana sylvatica*).

Even tadpole to tadpole predation maybe a factor. Historic accounts of cannibalism and 'larva against larva' behavior among ranid tadpoles are reported by several authors (Dickerson, 1906; Noble, 1931; Borland and Rugh, 1943). Excessive cannibalistic behavior may have even led to the economic demise of the Bullfrog leg market at the turn of the last century (Wright, 1920). At sites in New York State that produced high frequencies of metamorphs with missing appendages, captured Bullfrog (*R. catesbeiana*) and Green frog (*R. clamitans*) tadpoles showed obvious evidence of recent trauma to their hind limbs, including bloody stumps and protruding limb bones (Sessions, 1997). Bullfrog tadpoles raised under crowded conditions in the lab were observed to attack each other, causing missing limbs, missing feet, and even missing eyes in the metamorphs, the same range and kinds of abnormalities observed in field caught tadpoles and newly metamorphosed frogs (Sessions, 1997). Likewise, field-collected



Fig. 7

Southern Quebec Northern Leopard frog (*R. pipiens*) tadpoles were observed nibbling off the hind toes of smaller Green frog tadpoles when raised in our 2009 laboratory enclosures.

The key to understanding these kinds of deformities, at least in anuran tadpoles, is regenerative decline combined with extent of injury. When an anuran limb is amputated, epidermal cells quickly cover the resulting wound (Rose, 1944). If this occurs at an early-stage of limb bud development the epidermal cells are transformed into a layer of signaling cells called the Apical Epithelial Cap (AEC; Sturdee and Connock, 1975; Christensen and Tassava, 2000). Simultaneously, fibroblast cells migrate across the wound surface concentrating in the center. As these multiply they become a blastema or mesenchymal growth zone (MGZ).

Inside the blastema, undifferentiated cells are programmed to undergo the normal cell-cycle leading to regenerated structures (Brockes, 2003). This natural regenerative response is temporary and undergoes a steady decline as the tadpole develops and fully disappears by complete metamorphosis (“regenerative decline”; Muneoka et al., 1986). So if a predator consumes a section or entire limb in an early-stage tadpole the resulting regenerative structure would be very different than if it occurred late in development (Ballengée and Sessions, 2009).

In addition to regeneration, amphibians have other remarkable wound repair abilities. Their skin is made entirely of living cells, whereas most reptiles, birds or mammals have layers of dead keratin-filled cells at the surface. When an amphibian is injured these living cells group together to repair damage rapidly (Brunst, 1961) whereas in human adults for instance, wound repair often results in a

non-functioning mass of fibroblastic tissue known as a scar. Because amphibian skin is made from living cells that constantly undergo mitosis and create functioning fibroblasts, prior limb injuries can sometimes be masked (without obvious scars, inflammation, even fully-exposed bone can sometimes be covered gradually by epidermal tissue) (Rose, 1944; Goode, 1967).

We observed this wound repair phenomenon in our recent amputation studies, whereby epidermal tissues at healed wound site and resulting regenerative structures lacked obvious scars and showed little previous signs of trauma except for abnormal pigmentations (Ballengée & Sessions 2009). Additionally, in our 2009 Southern Quebec studies, some Ranid tadpoles with limbs removed by dragonfly nymphs were documented healing without obvious scars Fig. 7 (Ballengée et al., 2010, in prep.).

Several authors have sought to distinguish between injury-induced deformities and development malformations by examining field-collected metamorphosed and adult frogs (Meteyer, 2000; Taylor et al., 2005; Skelly et al., 2007; Lannoo, 2008). Our evidence suggests it is probably impossible to determine the cause of missing appendages without also examining injury rate in limbs among a range of age classes including tadpole stages.

Given the available evidence, predation/cannibalism appears to be a much more plausible explanation for missing appendages than either chemical pollution or UV-B. It is also consistent with the observation that missing forelimbs are much less frequent than missing hind limbs in field-caught anurans.

Additional research is needed to determine if there is a correlation between recent outbreaks of frogs with missing appendages and introduced predators, especially fishes, in amphibian breeding habitats. Other potential anthropogenic factors also need to be examined such as alteration of frog breeding habitats favoring dragonfly populations or other potential aquatic predators. If predation is occurring at preternatural rates, it could represent changes in amphibian larval behavior or ontological defenses against predators (Ballengée and Sessions, 2009). Our own results indicate that in some cases, increased abnormalities may indicate high population densities and cannibalistic interactions between tadpoles, at least in American bullfrogs (Sessions, 1997).

#### Extra limbs

From a developmental perspective, extra limbs (or supernumerary limbs) are easier to explain than missing limbs. A wide variety of factors could cause a missing limb, depending on whether it

is a developmental defect or a traumatic injury (Ouellet, 2000). On the other hand, there are only two alternative confirmed causes of high frequencies of duplicated limbs in amphibians: exogenous retinoids, and mechanical perturbation (Sessions et al., 1999; Sessions, 2003; Stopper et al., 2002).

The key to understanding deformities featuring extra limbs is intercalation. The results of grafting experiments described earlier support the idea that trematode cyst-induced abnormalities in amphibians result from mechanical disruption of early limb buds followed by intercalation (Sessions and Ruth, 1990; Sessions et al., 1999; Hecker and Sessions, 2001; Sessions et al., 2001). The mechanical effects of trematode cyst infestation have been shown to be *sufficient* to cause the outgrowth of supernumerary limbs in amphibians (Sessions and Ruth, 1990), and this has been recently confirmed by additional work (Hecker and Sessions, 2001; Stopper et al., 2002).

The trematode has since been identified as *Ribeiroia ondatrae* (Sessions et al., 1999; Johnson et al., 2001), a digenetic trematode with a complex life cycle in which amphibian larvae serve as an intermediate host (Schell, 1985). It has now been possible to observe the process of *Ribeiroia* cyst infection of tadpoles in the lab, including histological analysis of the effects of the cysts on developing amphibian limb buds (Stopper et al., 2002). The cercariae cause extensive damage when they encyst in or around the developing limb bud, triggering extensive localised cellular growth and clearly disrupting the normal limb pattern forming mechanisms.

Previous reports showed that laboratory-induced infection of early stage tadpole limb buds by cysts of the digenetic trematode *Ribeiroia ondatrae* can produce limb deformities in Pacific Treefrogs (*Hyla regilla*) and Western toads (*Bufo boreas*) that are strikingly similar to those found in the wild (Johnson et al., 1999; Johnson et al., 2001). This trematode has been implicated as the cause of similar limb abnormalities in several other species of amphibians (Sessions and Ruth, 1990; Sessions et al., 1999). Our results show that this trematode can also cause limb deformities in species of the genus *Rana*, which includes the most commonly reported deformed amphibian species (Stopper et al., 2002).

The developmental mechanism by which the cysts cause limb deformities in frogs is easily understood in terms of what is currently known about the effects of perturbation of pattern formation in the developing limbs of amphibians. We have found that *Ribeiroia* cyst infection in the lab produces the same range of limb deformities as that induced by surgical rotation of early limbs. The deformities closely resemble those seen in wild-caught

deformed amphibians (Sessions and Ruth, 1990; Johnson et al., 1999; Sessions et al., 1999; Johnson et al., 2001). These results suggest that cysts induce limb deformities in frogs by perturbing the spatial organisation of cells and triggering intercalation in the limb buds during early stages of development. Our histological analysis supports this conclusion, indicating that the cysts cause massive perturbation of the spatial organisation and mitotic activities of cells in the limb buds and surrounding tissues.

The abnormal limb outgrowths include the kinds of mirror-image duplications and triplications predicted from intercalation following mechanical perturbation. These results indicate that the probable mechanism by which trematode cyst infestation causes limb deformities in frogs is perturbation of the spatial organisation of cells in the developing limb buds followed by intercalation. Several researchers have offered an alternative view, based on the possible role of environmental chemical pollutants or UV-B radiation.

#### **Possible Roles of Chemical Pollution and UV- radiation**

A number of recent reports have investigated or proposed the possibility of environmental pollutants as malforming agents (chemical teratogens). Water, sediment, and sediment extracts from several ponds where high-incidences of limb abnormalities have been reported were shown to cause developmental abnormalities in *Xenopus laevis* in a FETAX assay (frog embryo teratogenesis assay – *Xenopus*; Burkhart et al., 1998; Fort et al. 1999a). In some of the experiments, water was treated in such a way as to rule out the presence of trematodes and predators, and developmental abnormalities were observed. However, one experiment reported in Fort et al., (1999a) exposed tadpoles to untreated pond water for 30 days. This was the only treatment from these studies that was reported to have induced limb abnormalities, which are the predominant deformities observed at the ponds from which water was taken.

Fort et al., (1999b) showed that sediment extracts from one of the same affected sites are capable of causing limb abnormalities in *Xenopus*. It was previously shown that sediment extracts from this site were appreciably more toxic to *Xenopus laevis* than pond water from the same site (Burkhart et al., 1998; Burkhart et al., 1999). Therefore, it is clear that the developmental toxicity of the sediment extracts does not reflect that of the pond water to which the natural populations of frogs were exposed. Unfortunately, the morphologies of the induced limb abnormalities are not presented, and their similarity to the deformities found in nature is not discussed (Fort et al., 1999b). These studies suggest the presence of teratogenic compounds at these sites;

however their role in causing limb deformities in natural populations remains speculative and the role of trematodes and predators was not ruled out.

La Clair and colleagues (1998) reported that *S*-methoprene, a retinoid mimic used for flea and mosquito control, and the products of its exposure to sunlight, can cause malformation in a FETAX assay. This study allowed a portion of the individuals to develop past metamorphosis for characterisation of deformities and found no effect on limb development. Ankley et al. (1998) also found no effect on limb development in methoprene treated individuals. Furthermore, *S*-methoprene and its photoproducts and metabolites have not been shown to exist at teratogenic levels in connection with amphibian deformities in the wild (La Clair 1998).

The insecticide carbaryl was shown to induce deformity and mortality in the Southern Leopard Frog (*Rana sphenocephala*), and limb deformities were found in two individuals: a missing limb and an extra forelimb (Bridges, 2000). Despite the low numbers of limb deformities produced (these were well within background levels for natural populations of frogs), the fact that they were produced at all is notable. However, the specific morphologies of the limb deformities were not reported and their relationship to morphologies of limb deformities observed in the field was not discussed. No report is made of carbaryl found at natural sites of amphibian deformities at any level (Bridges, 2000).

Gardiner and Hoppe (1999) examined 27 deformed mink frogs (*Rana septentrionalis*) from a site in Minnesota. All of the examined individuals had deformed hind limbs and none had deformed forelimbs. They suggest environmental retinoids as the probable cause of the reported deformities. The possibility that the deformities in their investigation were caused by trematodes cysts was discussed very little because of a lack of “massive” parasite infections, and the lack of observation of cysts in association with *distal* structures, hypomorphic limbs, or bony triangles. However, it has been shown that even light exposure to *Ribeiroia* cercariae can induce a range of limb deformities in Pacific Treefrogs (*Hyla regilla*; Johnson et al., 1999) and Western toads (*Bufo boreas*; Johnson et al., 2001), encompassing all of the deformities reported by Gardiner and Hoppe (1999). Furthermore, a lack of cyst association is only reported for a subset of their deformities, and our current results show limb deformities are often not associated with cysts, even in controlled lab infection with trematodes. We find that cysts are rarely present distal to the femur. This probably reflects the fact that the developmental effects of the cysts occur in the early limb bud, and

the cysts do not “ride out” with the growing limb, so distal deformities are often present in adults that do not appear to arise in close association with one or more cysts.

Gardiner and Hoppe (1999) further report that the production of extra hind limbs and bony triangles are particularly significant, and suggest induction by retinoids, as they know of no other chemical that can induce these malformations. However, retinoids actually inhibit limb development in anuran hind limbs, producing reduced limbs (Scadding and Maden, 1986; Stocum, 2000). During regeneration, limb duplications can be induced by retinoids in amphibians, but these duplications are of the proximodistal axis (e.g. a limb with a complete extra limb growing from its distal end: PD duplication; Sessions et al., 1999). Duplications of this type are strikingly absent from natural populations, where the duplications are of the anteroposterior axis or the dorsoventral axis (e.g. two limbs, both with the proximal femoral articulation near the pelvis; Sessions and Ruth, '90; Sessions et al., '99; Meteyer et al. '00), just as those produced in the lab by trematode cysts (Johnson et al., 1999; Sessions et al. 1999; Stopper et al., 2002). Although retinoids are the only chemical known to produce bony triangles, our results show that bony triangles are also produced by both mechanical rotation, cyst-infection and was observed in one of our predator-induced deformed metamorphic frogs (Ballengée et al., in prep. 2010), suggesting a purely mechanical origin for those found in wild frogs

Degitz et al., (2000) investigated toxicity of retinoic acid in *Xenopus laevis* and four species of *Rana* and report that their results contradicted the idea that retinoids are causing deformities in the wild, as they found that retinoic acid was lethal at much lower concentrations than those necessary to cause reductions and deletions of hind limbs. The results of current research are not consistent with the interpretations of results of Gardiner and Hoppe (1999), and there is currently no evidence that directly links any of the limb abnormalities in wild-caught amphibians to environmental retinoids (Stocum, 2000; Stopper et al., 2002).

Ouellet et al., (1997) found a correlation between proximity of sites to agricultural habitats and rates of anuran deformities featuring missing limbs and segments. However, they found that the hind limb abnormality rate in “control sites” (n = 12) was actually not significantly smaller than the deformity rate in those sites “subjected to pesticides” (n = 14). Furthermore, the correlation found relies on a single “subjected” sample site in a single year (Ouellet et al., 1997) in which deformity rates were more than six-fold higher than rates at all other sites, and at

that same site in the following year. In addition they sampled over three times the number of frogs at “pesticide” sites in comparison to “control” sites. Results from our 2009 Southern Quebec field-studies indicate that it is important to examine as many specimens as possible at both known-polluted and sites without excess levels of pollution for these also produce deformed frogs and may be reflective of regional background levels (Ballengée et al., 2010, in prep.).

Ouellet et al. (1997) did not report any attempt to quantify or confirm the existence of pesticides at any site, and though they noted fish at some sites, species were not published nor any other data on potential predators that could have induced these deformities. In summary the team was unable to identify the presence of any possible causative agents besides parasite cysts.

Helgen et al. (1998) investigated populations of frogs with high-incidence of deformities, mostly confined to the hind limbs, captured from field sites in Minnesota, USA. They detected parasitic cysts in most of the frogs from their largest population sample, and in all frogs of one species (Treefrogs) from all sites. Although they did not find parasites in one population, it is difficult to rule them out as a causative agent, since our results show that species of *Rana* are apparently able to rid themselves of cysts as they approach metamorphosis (Stopper et al., 2002). In addition, potential tadpole predators were not studied at sites. They reported no other direct evidence of the presence of possible causative agents, at any level, in the frogs themselves, or in the ponds from which the frogs were collected (Helgen et al., 1998).

Meteyer et al. (2000) found the ranges of deformities in Northern leopard frogs (*Rana pipiens*) to differ depending on site, and suggests that this implies multiple etiologies. However, as in other field studies, they were unable to find direct evidence of the presence of any proposed causative agent besides metacercarial cysts. In addition deformities resulting from predation injury were dismissed based on the lack of obvious signs of trauma such as scarring or exposed skeletal protrusions. Our results, along with those of Johnson et al. (1999, 2001) show that a wide range of limb abnormalities may result from just two etiologies: trematode cysts and selective predation.

Sower et al., (2000) reported decreased levels of the gonadotropin-releasing hormones (GnRH) and androgens among frogs with missing limb deformities collected in New Hampshire. GnRH and other hormones are synthesized and released by neurons in the hypothalamus portion of the brain. These and other hormones are vital towards the

development of the normal reproduction systems and considerable attention has been given to endocrine-disrupting pollutants recently (Hayes et al., 2002). Sower et al. (2000) reported that in normal metamorphic Bullfrogs GnHR levels were nearly three times higher than those with abnormal limbs leading the team to suggest the involvement of endocrine-disrupting pollutants. Yet, other studies suggest that this is a normal response to trauma. Chu et al., (2008) found that GnHR levels decrease in laboratory rats subjected to some forms of injury. Likewise, research into human patients also shows natural decreases in hypothalamic secretions following injuries to the head (Clark et al., 1992). More research is needed but studying GnHR levels in missing limb deformed frogs may help to further establish the role that injury may play in their induction.

Limb deformities have been produced by exposure to UV-B in the lab (Ankley et al., 1998). However, these deformities are generally bilateral truncations, which are rarely seen among deformed amphibians from natural populations (Sessions and Ruth, 1990; Sessions et al., 1999; Meteyer et al., 2000). UV-B radiation has been shown to induce mortality in outdoor lab experiments on three species of *Rana* (Tietge et al., 2001) and mortality and non-limb deformities in populations of amphibians the field (Blaustein et al., 1994; Blaustein et al. 1997). Mortality effects appear to be species-specific based on location of egg deposition and differential ability to repair UV-induced genetic damage (Blaustein et al., 1998). Although UV radiation may be playing a role in amphibian population declines, it has been recently rejected as a substantial cause for limb deformities among wild populations (Ankley et al., 2004)

### Conclusion

To our knowledge, there is no direct evidence that limb deformities are induced by chemical teratogens or UV-B radiation in natural populations of amphibians. While we agree that chemical pollution and UV radiation are threats to amphibians and other organisms, current evidence suggests that chemical pollutants are at best only indirectly involved in deformed amphibians of the kind we have been studying and UV may not be a factor at all (Degitz et al., 2003; Ankley et al., 2004; Johnson and Chase, 2004; Rohr et al., 2008). It seems far more likely that a complete understanding of the deformed amphibian phenomenon will depend on additional information concerning the ecology and evolution of anuran parasites and their predators.



Figure 1. Top: Mirror image duplication (AMID) Bottom: Mirror image triplication (photograph by Stanley K. Sessions)

Figure 2. Different kinds of mirror-image duplications in amphibians limbs: a & b, proximal-distal duplication (PDD) in a salamander limb that was treated with retinoic acid; c & d, double posterior mirror-image duplication (PMID); e & f, double anterior mirror-image duplication (AMID); g & h, mirror-image triplication (MIT). Retinoic acid is known to induce PDDs and PMIDs, but not AMIDs or MITs. Mechanical perturbation, including trematode cysts, can produce AMIDs, PMIDs, and MITs, but not PDDs. (photographs by Stanley K. Sessions, from Sessions et al. 1999)

Figure 3. Surgical rotation meant to confront cells in the developing limb bud of a frog tadpole (from Stopper et al., 2002)

Figure 4. Creation of confrontations between cells with different positional information via surgical rotation (shown in previous Figure).

Figure 5. Cartilagenous spike in peri-metamorphic Common toad/ *Bufo bufo*, collected summer 2006 at Havercroft Village Green Pond, West Yorkshire, England (photograph by Brandon Ballengée)

Figure 6. Green frog/ *Rana clamitans* tadpole with a developing hind limb fully removed by Green darner/ *Anax junius* dragonfly nymph in experimental enclosures, 2009 Southern Quebec studies (photograph by Brandon Ballengée)

Figure 7. Dragonfly nymph induced deformed metamorphic Green frog/ *Rana clamitans* without obvious signs of scarring from 2009 Southern Quebec studies experimental enclosures (photograph by Brandon Ballengée)

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