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MORPHOLOGICAL ABNORMALITY PATTERNS IN A CALIFORNIA AMPHIBIAN COMMUNITY

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ABSTRACT: Increasing reports of amphibian limb malformations from many parts of North America have prompted investigations into the potential causes of these abnormalities and their implications for amphibian populations. Over a two-year period, we monitored the frequency and composition of morphological abnormalities in four amphibian species (*Hyla regilla, Taricha torosa, Bufo boreas,* and *Rana catesbeiana*) from two California ponds. The frequency of abnormalities differed significantly by species, life-history stage, pond, and season. Generally, the frequency and severity of abnormalities were greater in the amphibians from Frog Pond over those from Hidden Pond, and in larval stages over emerging and adult amphibians. Larvae of *T. torosa* exhibited the highest rate of abnormalities, ranging from 15–50%, followed by larval and metamorphic *H. regilla* (10–25%), and finally by metamorphic *B. boreas* and *R. catesbeiana,* both of which had rates of less than five percent. Within each species, the composition of abnormalities was strongly consistent between years, ponds, and early life-history stages. We recorded the most severe malformations in *H. regilla,* and more than 60% of the abnormalities in treefrogs involved extra hindlimbs, femoral projections, and skin webbings. Similarly severe, the abnormalities of *R. catesbeiana* were dominated by extra and missing hind- and forelimbs. In *B. boreas* and *T. torosa,* the most common morphological abnormalities were missing limbs and digits, which accounted for approximately 75% and 95%, respectively, of their total abnormalities. Potential causes of the observed abnormalities, including infection by the trematode *Ribeiroia,* and the conservation significance of amphibian malformations are discussed.

Key words: Amphibian; Abnormality; Malformation; Deformity; *Hyla regilla*; *Bufo boreas*; *Taricha torosa*; *Rana catesbeiana*; Trematode; *Ribeiroia*

RECENT increases in the number of malformed amphibians reported in North America have prompted considerable concern and investigation (Fort et al., 1999*a*; Hoppe, 2000; Johnson et al., 1999). More than 50 species from 44 states in the United States are now included in those reports (Kaiser, 1999). To date, most accounts detail metamorphic frogs with missing, malformed, or supernumerary limbs from one of several areas: the midwestern U.S. (Burkhart et al., 1998; Gardiner and Hoppe, 1999; Helgen et al., 1998), the western U.S. (Johnson et al., 1999; Sessions et al., 1999), and southern Canada (Bonin et al., 1997; Ouellet et al., 1997).

The high frequency of malformations observed in many of these cases has raised concerns about the implications of these phenomena for environmental health. The few studies that have examined background abnormality rates in amphibians due to mutation, errors in development, and injury suggest a frequency of 0–5% consisting predominantly of digit abnormalities for both anurans (Dubois, 1979; Martof, 1956; Tyler, 1998) and urodeles (Meyer-Rochow and Asashima, 1988; Roberts and Verrell, 1984; Zaffaroni et al., 1992). However, many recent accounts consistently report amphibians with severe limb malformations at rates of 15% or greater (e.g., Hoppe and Mottl, 1997), and some researchers have suggested that in-

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creasing numbers of malformed amphibians may reflect environmental degradation or contamination (Fort et al., 1999*a*; Gardiner and Hoppe, 1999; Ouellet et al., 1997). The role of these malformations in continuing trends of amphibian population decline and extinction remains largely conjectural (Wake, 1998). Malformed frogs are, however, suspected to suffer greater mortality rates (Johnson et al., 1999; Sessions and Ruth, 1990), and an increase in malformations could exacerbate declines in already threatened populations. Ultimately, answers to questions about the environmental significance of the observed abnormalities will depend upon the causative agent(s) responsible. Potential causes include UV-B radiation (Ankley et al., 1998; Blaustein et al., 1997), retinoid exposure (Gardiner and Hoppe, 1999), pesticide contamination (Burkhart et al., 1998; Fort et al., 1999*a,b*; Tyler, 1998), predation (Bohl, 1997; Viertel and Veith, 1992), and trematode infection (Johnson et al., 1999; Sessions and Ruth 1990; Sessions et al., 1999).

One hindrance to the investigation of amphibian malformations has been the lack of baseline data. Frogs and salamanders with limb abnormalities at frequencies ranging from anomalous (<1%) to 30% or more have been recorded from across the world for three centuries (e.g., Bateson, 1894; Vallisneri, 1706; Van Valen, 1974). Unfortunately, these cases are rarely studied intensively and the causes of such abnormalities, while a frequent subject of speculation, are usually left undetermined. Relative to the historical precedent, the current abnormality phenomena are notable in the (1) wide geographic range affected, (2) involvement of numerous amphibian species, and (3) high frequencies of severe malformations reported for many of the sites. Nonetheless, the level of background abnormalities resulting from hereditary, developmental, and traumatic factors—and how that background rate varies among species, life history stages, habitat types, and geographic areas—remains poorly understood, making it difficult to differentiate between "natural" abnormalities and those resulting from human impacts. The likely involvement of multiple causative agents among the current abnormality reports further emphasizes the need for intensive, multi-year data on the patterns of morphological abnormalities to facilitate comparisons among sites and species.

In this paper, we present comparative data on the abnormality patterns of three anurans and one urodele from two ponds in northern California, U.S.A., where large numbers of malformed individuals of *Hyla regilla* have previously been reported (Johnson et al., 1999). The data encompass multiple life history stages for each species and span two seasons. We specifically ask (1) what types of abnormalities are occurring?, (2) at what rate?, and (3) how do abnormality frequency and composition differ among species, seasons, life history stages, and sites? As our null hypotheses, we postulate that the average abnormality frequency for each species will remain within established baseline levels (0–5%) (Dubois, 1979; Johnson and Lunde, 2001; Ouellet et al., 1997), that abnormality composition will not differ within or among species, and that what abnormalities are observed will be "minor" and similar to what has been described in previous, baseline studies (i.e., dominated by ectrodactyly) (Hoppe, 2000; Meyer-Rochow and Asashima, 1988; Tyler, 1998). Finally, we present morphological descriptions of the abnormalities encountered in each species and discuss known and possible causative agents.

Materials and Methods

Site Descriptions and History

Our study focused on the amphibian communities of two permanent ponds in Santa Clara County, California, U.S.A. (T10S, R02E, sec. 03; 250 m). Both ponds are spring-fed and supplemented by rainfall, with surface areas of approximately 1800 m^2 (Frog Pond) and 2200 m^2 (Hidden Pond). The surrounding habitat is pasture-land and consists primarily of oak woodland and chaparral. The two ponds were built in the 1960s as stock ponds and are highly eutrophic. Each supports four amphibian species: Pacific treefrogs (*Hyla regilla*), western toads (*Bufo boreas*), California newts (*Taricha torosa*), and the invasive American bullfrog (*Rana catesbeiana*).

A local resident first observed malformed treefrogs in 1995 and reported the finding to biologists at Stanford University. Two of the authors (J. K. Reaser and A. E. Launer) found a variety of abnormal anurans during three visits in 1995 and 1996. High rates of abnormalities have been recorded in multiple species each subsequent year (1997–1999). It is not known if malformations were present prior to 1995.

Sampling Methods

In 1997 and 1998, we monitored Hidden and Frog ponds weekly from April through September and episodically during rainfall events for the remainder of the year. In total, we made 23 site visits in 1997 and 27 in 1998. During weeks in which larval amphibians were present, we performed standardized sweeps with a dipnet (1.4 mm mesh size; 2600 cm^2 opening) every 15 m around each pond's circumference. At each sampling station, we pulled the dipnet rapidly through the water column for 1.5 m in a line perpendicular to the shore (adapted from Heyer et al., 1994). After emptying the contents of the sweep into a sorting tray, we recorded the numbers and identities of all amphibian larvae, fish, and macroinvertebrates. A subset of the amphibian larvae was returned to the laboratory for a thorough inspection.

As *H. regilla* and *B. boreas* metamorphosed and emerged from the ponds, we conducted species-specific, time-constrained transects either (a) around the entire circumference of the pond or (b) in multiple, haphazardly assigned sections of the circumference. The latter method was preferred when frogs and toads were sufficiently abundant (>0.5 individuals/m^2). In all cases, transects followed the shoreline in a band measuring from the water's edge to 2 m inland. At the conclusion of each transect, metamorphic frogs and toads were inspected and scored for abnormalities in the field. Abnormal specimens and a subset of the normal individuals were collected for further examination while the remaining individuals were released within the transect area. We surveyed metamorphosing bullfrogs and adults of all species during timed searches. Bullfrog inspections were conducted during eight nocturnal surveys in 1997 and 1998 while adults of *H. regilla* and *T. torosa* were inspected on the nights of 17 and 23 January 1998.

For each species and life-history stage, we calculated the frequency of morphological abnormalities for the entire season (seasonal frequency) and for the 2-wk period with the highest rate (peak frequency). We then compared these values (a) to our null-hypothesized frequency of 5% or less using goodness of fit tests (*G*-statistic) and (b) between species, life-history stages, ponds, and years using *G*-tests of independence (Sokal and Rohlf, 1995).

Morphological Analysis and Terminology

The teratological terminology in this paper is largely adapted from Landau et al. (1986), Tyler (1998), and Wise et al. (1997). We use the general term "abnormality" to describe any gross deviation from the normal range of morphological variation, be it traumatic or developmental. "Malformations" are more specific, encompassing the class of permanent and often lethal, structural defects resulting from abnormal development (see review by Beltrame and Giavini, 1990). The detection of abnormalities depends on the scale of examination, and we report only on substantial deviations of body plan (e.g., the number of limbs or digits). Skeletal variations or abnormalities which require body measurements or detection via radiography or clearing and staining are not included, and our estimates of abnormality frequency are therefore conservative. Table 1 presents the more specific abnormality classifications used in this study and their respective definitions. While many of these terms imply a developmental origin, it should be emphasized that the distinction between traumatic and developmental abnormalities is not always immediately evident. As anurans' regenerative capacity weakens to-

TABLE 1.—Descriptions of abnormality categories. Terms adapted from Landau et al. (1986), Tyler (1998), and Wise et al. (1997).

Condition	Description
Anophthalmy	Absence of one or both eyes.
Apody	Absence of one or more feet.
Brachydactyly	Abnormal shortness of one or more digits.
Brachymelia	Abnormal shortness of one or more limbs.
Cutaneous fusion	Constriction or "fusion" of limb longbones by a superficial skin layer; skin webbing.
Ectrodactyly	Complete absence of one or more digits.
Ectromelia	Complete absence of one or more limbs.
Edema	Fluid-filled swelling; either localized or general (distension).
Femoral projection	Presence of one or more non-articulating microappendages from the dorsal skin of a hindlimb; may contain ossified elements.†
Hemimelia	Partial or complete absence of distal portions of one or more limbs.
Limb hyperextension	Excessive or rigid flexure of a limb joint.
Mandibular dysplasia	Underdevelopment of mandible.
Micromelia	Abnormal smallness of one or more limbs.
Mirror-image duplication	Digit series duplication in the anterior-posterior axis; may be either a posterior mirror-image duplication (PMID) of the digit pattern 5-4-3-2-1-2-3-4-5 or an anterior mirror-image duplication (AMID) of the pattern 1-2-3-4-5-4-3-2-1.‡
Mirror-image triplication	Digit series triplication in the anterior-posterior axis, usually in the digit pattern 1-2-3-4-5-4-3-2-1-2-3-4-5.‡
Polydactyly	Presence of one or more extra digits or parts of digits.
Polymelia	Presence of one or more extra limbs or parts of limbs; supernumerary limbs.
Polypody	Presence of one or more extra feet or parts of feet.
Syndactyly	Partial or complete fusion of one or more digits.
Taumelia	One or more sharp folds in a limb longbone; bone with limb elements at right angles, externally appear truncated; bony triangle.

† From Johnson et al. (1999).
‡ From Sessions et al. (1999).

ward metamorphosis, injury to the limbs of larvae may produce "malformations" not obviously distinguishable from those of developmental origins (Bohl, 1997; Brunst, 1961; Tyler, 1998). Similarly, errors in urodele limb regeneration can result in malformed limbs and digits (Dearlove and Dresden, 1976; Scadding, 1981). Thus, until the exact cause(s) are known, we use the terms in Table 1 inclusively and do not differentiate between trauma and "true" malformations.

Amphibians collected in the field were subsequently measured and examined using a stereodissecting microscope. We described abnormalities in detail and dissected or cleared and stained subsets of the specimens for further analysis. In larval and metamorphosing amphibians, we frequently encountered specimens with more than one abnormality (e.g., an extra right leg and a missing left leg). Thus, in calculating abnormality composition, individual abnormalities—rather than individual abnormal frogs—were evaluated (as in Tyler, 1998). However, only independent abnormalities were included; an extra digit on an extra limb constituted only a case of polymelia, not both polymelia and polydactyly. As virtually every observed extra leg was itself malformed, this avoided an artificial bias in the estimated number of abnormalities. The severity of the abnormalities in a species or life-history stage were determined by calculating the average number of independent abnormalities per abnormal animal. As this index examines only the abnormal individuals, it is independent of abnormality frequency.

RESULTS

Hyla regilla: *Abnormality Frequency*

At Hidden Pond during 1997, we conducted 34 transects, capturing and examining a total of 2423 treefrogs. In 1998, 63

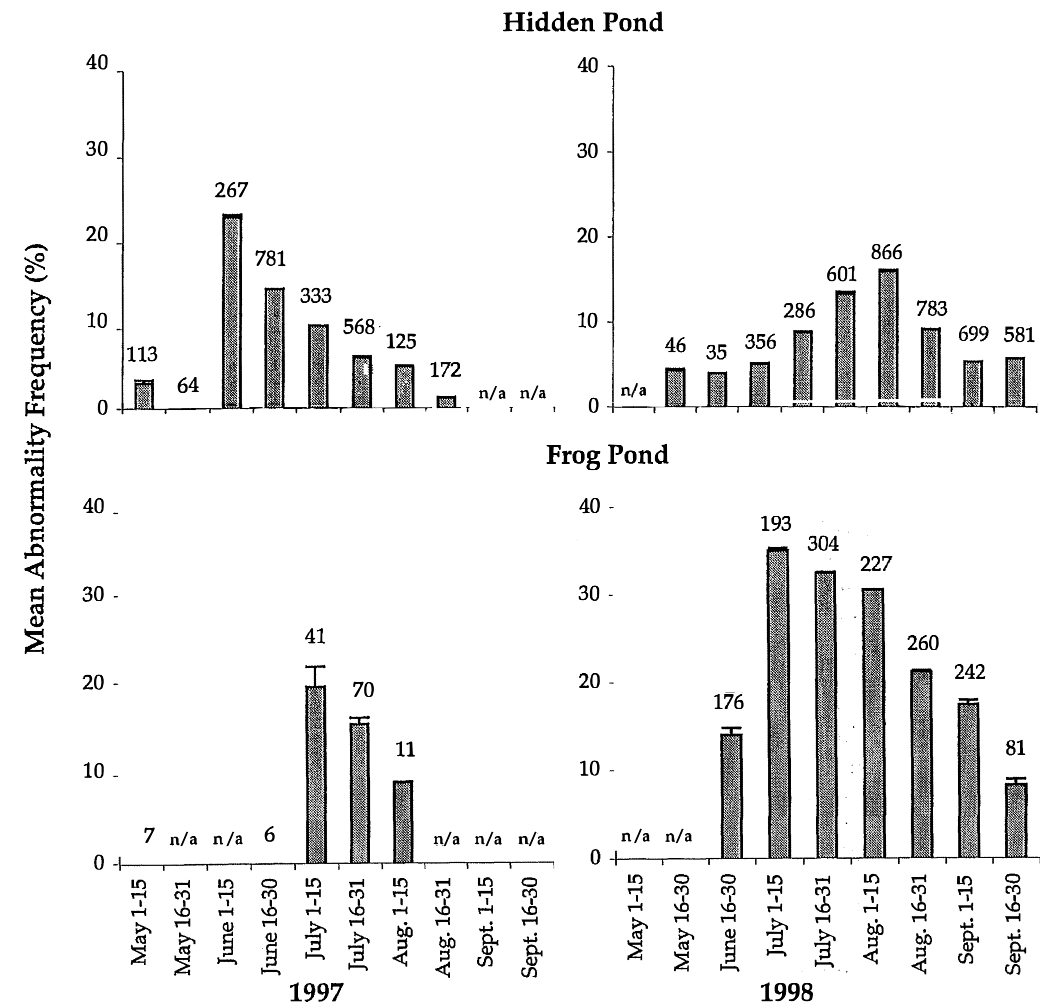

FIG. 1.—Frequency of morphological abnormalities in metamorphic Pacific treefrogs (*Hyla regilla*) from Hidden and Frog ponds (1997–1998). Bars represent the mean abnormality rate ± SE for a 2-wk period. Total sample sizes (number of frogs examined) for the 2-wk periods are displayed above the bars. All standard error values are ≤2.4. For periods in which no transects were conducted or insufficient animals were available, an "n/a" is indicated.

transects yielded a total of 4253 inspected treefrogs. The abnormality rates were high both years but varied across the season (Fig. 1). For each year, the seasonal frequency significantly exceeded the hypothesized baseline range of 0–5% (*G*-test, df = 1, $P < 0.0001$; Table 2), and peak values for the two seasons approached or exceeded 20% (Fig. 1). In 1997, both the peak and seasonal frequencies were greater than those recorded in 1998, but these differences were not significant (*G*-test, df = 1, $P > 0.05$).

Frog Pond supports substantially fewer Pacific treefrogs than Hidden Pond. In 1997, we scored 138 metamorphic treefrogs for abnormalities during 15 timed transects. Treefrogs were more abundant in 1998, and 67 transects yielded nearly 1500 frogs. As found for Hidden Pond, the peak and seasonal abnormality frequencies at Frog Pond in 1997 and 1998 significantly exceeded the expected baseline (*G*-test, df = 1, $P < 0.005$; Table 2), and the 1998 values were greater than those from 1997 (*G*-test, df = 1, $P < 0.05$; Table 2).

TABLE 2.—Seasonal abnormality frequencies for the amphibians at Hidden and Frog ponds (1997–1998). Frequencies calculated as the number of abnormal individuals divided by the total number of examined individuals for a given species and year. Sample sizes are listed in parentheses. For entries in which insufficient data were available, an "n/a" is indicated. Within a row, differences in superscripts denote significant differences (G-test, df = 1, $P < 0.05$) in the abnormality frequency.

	Hidden Pond		Frog Pond	
	1997	1998	1997	1998
Hyla regilla: metamorphs	9.9%^{a,b} (2423)	9.3%^{a} (4253)	14.5%^{b} (138)	25.1%^{c} (1483)
Hyla regilla: larvae	16.6%^{a} (361)	9.7%^{b} (516)	n/a	17.2%^{a} (151)
Taricha torosa: larvae	18.9%^{a} (482)	14.7%^{a} (382)	48.9%^{b} (303)	34.6%^{c} (344)
Bufo boreas: metamorphs	3.5%^{a} (145)	0.9%^{a} (320)	3.7%^{b} (572)	2.2%^{a,b} (641)
Rana catesbeiana: metamorphs	4.4%^{a} (23)	20.0%^{a} (10)	3.5%^{a} (259)	5.1%^{a} (254)

With respect to differences between the ponds, the peak and seasonal abnormality rates at Frog Pond in 1998 were approximately twice the values from Hidden Pond for the same year (G-test, df = 1, $P < 0.0001$; Table 2). However, no significant differences in abnormality rate were observed between the ponds in 1997 (G-test, df = 1, $P > 0.05$; Table 2).

Overall, larvae of *Hyla regilla* generally exhibited higher abnormality rates and a different temporal pattern than those observed in metamorphic frogs (G-test, df = 1, $P < 0.01$). Unlike the bell-shaped trend associated with abnormalities in metamorphs, the frequency of abnormalities in larvae rose steadily throughout the season (Fig. 2). The peak frequencies for all larval series of *H. regilla* occurred on the last sampling date during which larvae were available. At Hidden Pond, the seasonal frequency in larvae for 1997 was significantly greater than that for 1998 (G-test, df = 1, $P < 0.005$; Table 2). At Frog Pond, abnormality data for treefrog larvae were only collected in 1998, for which the seasonal frequency exceeded the rate for Hidden Pond in 1998 (G-test, df = 1, $P < 0.05$) but not in 1997 (G-test, df = 1, $P > 0.05$; Table 2). Collectively, the larvae and metamorphs of *H. regilla* exhibited significantly higher abnormality rates than the

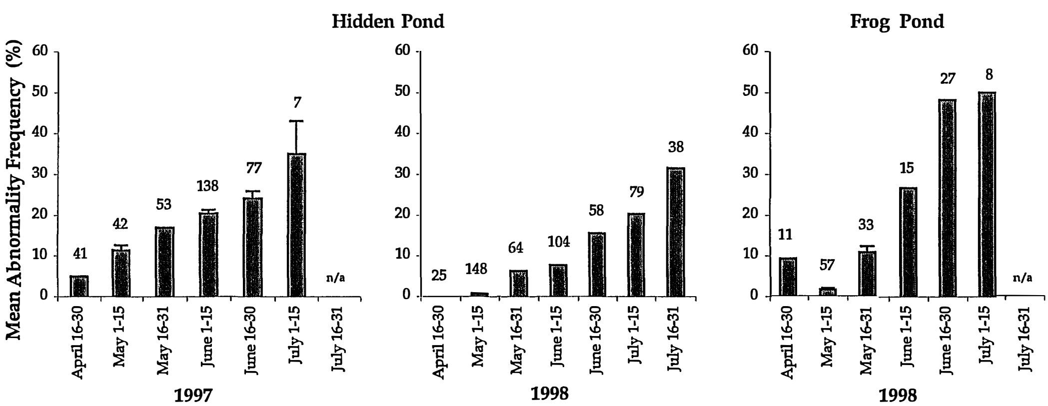

FIG. 2.—Frequency of morphological abnormalities in larval Pacific treefrogs (*Hyla regilla*) from Hidden and Frog ponds (1997–1998). Bars represent the mean abnormality rate ± SE for a 2-wk period. Total sample sizes (number of frogs examined) for the 2-wk periods are displayed above the bars. All standard error values are ≤8.0. For periods in which no surveys were conducted or insufficient animals were available, an "n/a" is indicated. Too few larvae were present at Frog Pond in 1997 to obtain meaningful data.

TABLE 3.—Composition of abnormalities in Pacific treefrogs (*Hyla regilla*) from Hidden and Frog ponds (1997–1998). Listed are the numbers of each abnormality type and its proportion (%) relative to the total number of abnormalities observed. The total number of abnormalities may or may not equal the number of abnormal animals, as many specimens had more than one abnormality. The mean numbers of abnormalities per abnormal larva or metamorph, an index of abnormality severity, are offered in the final row.

	Hidden Pond		Frog Pond	
Abnormality type	Larvae	Metamorphs	Larvae	Metamorphs
Cephalic and axial				
Anophthalmy	4 (2.8%)	1 (0.1%)	1 (2.4%)	6 (0.9%)
Mandibular dysplasia	1 (0.7%)	5 (0.6%)	0	4 (0.6%)
Open wound	3 (2.1%)	15 (1.7%)	1 (2.4%)	1 (0.2%)
Edema	6 (4.3%)	1 (0.1%)	4 (9.5%)	1 (0.2%)
Other	0	0	0	0
Forelimb				
Ectrodactyly	0	10 (1.2%)	0	0
Polydactyly	0	1 (0.1%)	0	0
Apody	0	2 (0.2%)	0	1 (0.2%)
Hemimelia	0	5 (0.6%)	0	0
Ectromelia	1 (0.7%)	7 (0.8%)	0	1 (0.2%)
Polymelia	0	0	0	0
Cutaneous fusion	0	2 (0.2%)	0	1 (0.2%)
Other malformed*	0	5 (0.6%)	0	0
Hindlimb				
Ectrodactyly	5 (3.5%)	47 (5.4%)	2 (4.8%)	16 (2.5%)
Polydactyly	5 (3.5%)	25 (2.9%)	1 (2.4%)	13 (2.0%)
Apody	4 (2.8%)	18 (2.1%)	1 (2.4%)	5 (0.8%)
Polypody	1 (0.7%)	23 (2.6%)	1 (2.4%)	11 (1.7%)
Hemimelia	0	35 (4.0%)	0	6 (0.9%)
Ectromelia	5 (3.5%)	26 (3.0%)	0	8 (1.3%)
Polymelia	78 (55.3%)	419 (48.3%)	23 (54.8%)	345 (54.1%)
Femoral projection	12 (8.5%)	85 (9.8%)	3 (7.1%)	99 (15.5%)
Cutaneous fusion	0	26 (3.0%)	2 (4.8%)	64 (10.0%)
Taumelia	6 (4.3%)	35 (4.0%)	0	16 (2.5%)
Micromelia	1 (0.7%)	7 (0.8%)	1 (2.4%)	5 (0.8%)
Limb hyperextension	1 (0.7%)	5 (0.6%)	0	2 (0.3%)
Other malformed*	8 (5.7%)	63 (7.3%)	2 (4.8%)	33 (5.2%)
No. abnormal	106	673	31	413
No. inspected	877	7175	239	1621
No. abnormalities per abnormal animal	1.33	1.29	1.36	1.55

* Other malformed includes brachymelia, brachydactyly, and syndactyly.

remaining anurans (*G*-test, df = 3, $P <$ 0.005; Table 2).

Abnormality severity, as indexed by the average number of independent abnormalities per abnormal frog, follows the pattern noted for abnormality frequency among the two ponds. The treefrogs at Frog Pond showed higher peak and seasonal abnormality frequencies than those at Hidden Pond, whether for larval or emerging individuals. Correspondingly, Frog Pond treefrogs were also more severely malformed than those at Hidden Pond (Table 3).

Hyla regilla: *Abnormality Composition*

We described the abnormalities of 673 metamorphic Pacific treefrogs from Hidden Pond and 413 from Frog Pond. Interannual variation in the types of abnormalities within a pond was slight so we pooled the data for the two years. Likewise, the types of abnormalities and their relative frequencies were similar between the two ponds.

In general, abnormalities in both frogs and larvae of *H. regilla* were unilateral and when bilateral, asymmetric. Approximately 98% of the morphological abnormalities

TABLE 4.—Total number and relative frequencies of mirror-image duplications and triplications from Frog and Hidden ponds. Three types of digit patterns presented: anterior mirror-image duplications (AMID's), posterior mirror-image duplications (PMID's), and mirror-image triplications (MIT's). Sample sizes include larval and metamorphic Pacific treefrogs (*Hyla regilla*).

Site	Sample size	AMID	PMID	MIT
Frog Pond (1997–1998)	84	7 (8.3%)	74 (88.1%)	3 (3.6%)
Hidden Pond (1997–1998)	230	23 (10.0%)	200 (87.0%)	7 (3.0%)
Total (%)	314	30 (9.6%)	274 (87.3%)	10 (3.2%)

observed at both ponds between 1997 and 1998 involved the limbs. Of those, 95% from Hidden and 98% from Frog Pond affected the hindlimbs in particular. We noted fewer than 40 forelimb abnormalities in the 9912 frogs examined. The dominant hindlimb abnormality at both ponds was polymelia (Table 3). The number of extra limbs ranged from 1–6 complete limbs independent of the two "primary limbs" (Gardiner and Hoppe, 1999). Supernumerary limbs typically emerged from the dorsal plane of the pelvic girdle; extra limbs or segments thereof extending from the ventral side of the primary limbs occurred in <4% of the observed cases of polymelia (n = 764). As extra structures were often cutaneously fused to one of the primary limbs, we classified an extra limb as "a supernumerary appendage independent of any other limb for at least one-half the length of the tibiofibula and the entire tibiotarsus." For example, an extra appendage fused along the entire tibiofibula to a primary limb would only constitute an extra foot, not an extra leg.

Polymelous frogs rarely demonstrated motor control of their added limb(s). The extra limbs themselves were invariably malformed, frequently exhibiting mirror-image duplications in the feet (Table 1). Posterior mirror-image duplications (PMID's; Sessions et al., 1999) of the digit pattern 5-4-3-2-1-2-3-4-5, were significantly more common than anterior mirror-image duplications (AMID's; Sessions et al., 1999) of the pattern 1-2-3-4-5-4-3-2-1 (G-test, df = 1, $P < 0.001$). Mirror-image triplications (1-2-3-4-5-4-3-2-1-2-3-4-5) comprised <4% of the total cases of mirror-image polydactyly (Table 4).

The second most common abnormality in treefrogs from both ponds was a femoral projection (Fig. 2H in Johnson et al., 1999). "Skin webbings," or cutaneous fusion ranked as the third most prevalent abnormality in metamorphic frogs from Frog Pond (Table 3). More severe cases of this condition precluded the limb's extension and positioned the tibiotarsus immediately adjacent to the pelvic girdle. This malformation could affect one or both hindlimbs, and in two instances involved a forelimb. The third most frequently encountered abnormality at Hidden Pond involved missing and partially missing hindlimbs (ectro- and hemimelia).

A final abnormality commonly observed in frogs from both ponds was taumelia. In many of the frogs affected with this condition, the tibiofibula was folded over on itself creating the appearance of a severely truncated tibiofibula with several spikes extending from the knee. Upon clearing and staining, we found that this condition was typically associated with bony triangles (Gardiner and Hoppe, 1999; Johnson et al., 1999).

Abnormalities in the larvae of *H. regilla* (1997–1998) totaled 141 for Hidden Pond and 42 for Frog Pond and were similar to those in emerging frogs (Table 3). The trend in abnormality composition described for metamorphic frogs from each of the two ponds remained consistent in their respective larval stages. Supernumerary limbs again accounted for >50% of the observed abnormalities in each of the two ponds (Table 3). The extra limbs were typically smaller and delayed in development relative to the primary limbs, making detection in early stage individuals difficult. With the aid of a stereodissecting scope, however, we observed limb duplications as early as stage 29 (Gosner, 1960). Again, femoral projections constituted the second

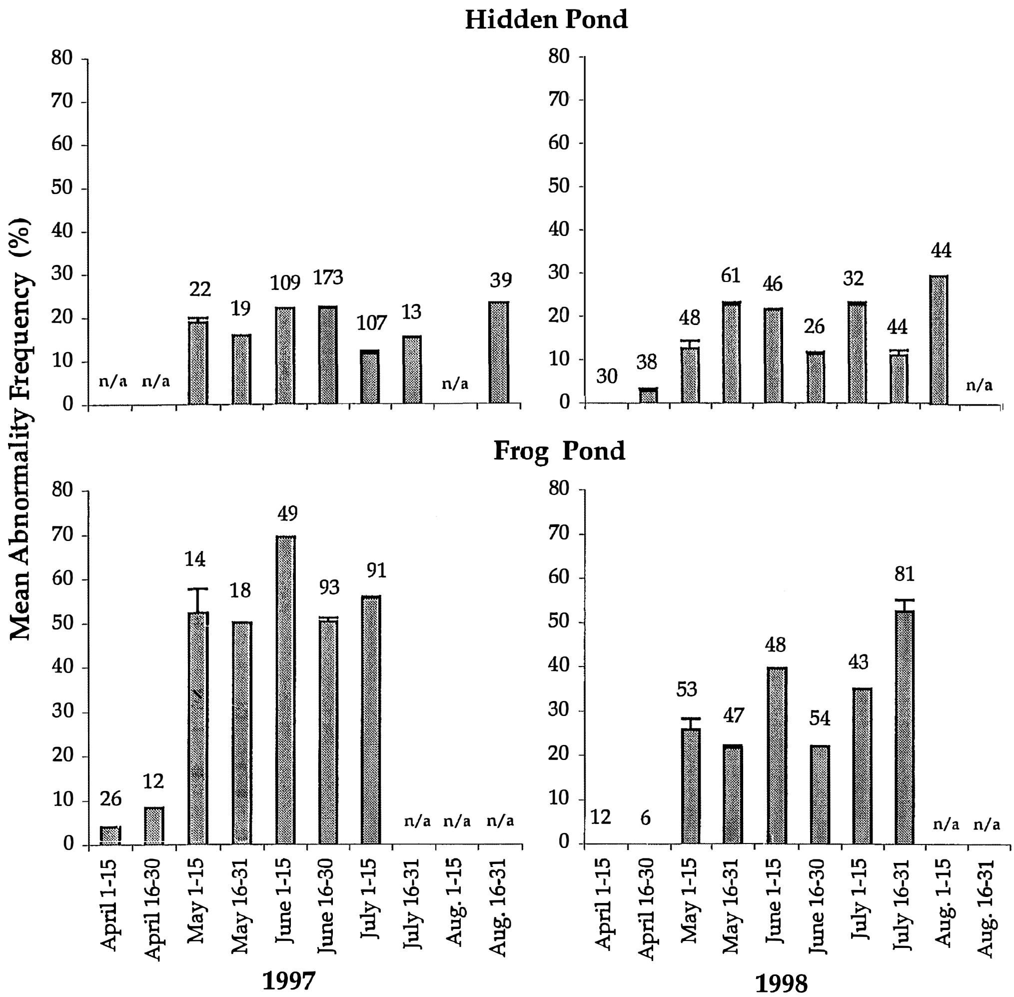

FIG. 3.—Frequency of morphological abnormalities in larval California newts (*Taricha torosa*) from Hidden and Frog newts (1997–1998). Bars represent the mean abnormality rate ± SE for a 2-wk period. Total sample sizes (number of newts examined) for the 2-wk periods are displayed above the bars. All standard error values are ≤5.5. For periods in which no surveys were conducted or insufficient animals were available, an "n/a" is indicated.

most prevalent malformation for the two ponds while cutaneous fusion ranked third at Frog Pond and ectromelia ranked third at Hidden Pond (Table 3).

Of the 77 inspected adults of *H. regilla,* one individual with a small, dorsal extra leg was collected. No other abnormal adults were found.

Taricha torosa: *Abnormality Frequency*

At Hidden and Frog ponds, the seasonal and peak abnormality frequencies in larval *T. torosa* were significantly greater than the rates recorded for each of the other species (*G*-test, df = 3, *P* < 0.0001). In 1997 and 1998, the abnormality rate in larvae of *T. torosa* from both ponds remained high and stable between May and July (Fig. 3). The newt larvae from Frog Pond suffered substantially greater abnormality rates than those from Hidden Pond (Fig. 3). In both 1997 and 1998, the peak and seasonal abnormality frequencies at Frog Pond were approximately twice those recorded at Hidden Pond (*G*-test, df = 1, *P* < 0.001; Table 2). And, while the seasonal frequency in larvae from Hidden Pond showed no significant change between the two seasons, the peak and seasonal frequency in newt larvae from Frog Pond declined significantly between 1997 and 1998 (G-test, df = 1, $P < 0.001$; Table 2).

Taricha torosa: *Abnormality Composition*

In 1997 and 1998, we collected 92 and 63 abnormal California newt larvae, respectively, from Hidden Pond. Unlike treefrogs, *T. torosa* showed less of a disparity between fore- and hindlimb abnormalities (60% hind and 40% fore; G-test, df = 1, $P < 0.001$, $n = 186$). Ninety-seven percent of the observed abnormalities involved missing limbs and missing sections of limbs, with apody in the hindlimbs and ectrodactyly in both the fore- and hindlimbs predominating (Table 5). We found only one polymelous and two polydactylous larvae. The single abnormal adult found at Hidden Pond was also polydactylous ($n = 100$).

The 267 abnormal newt larvae from Frog Pond exhibited a very similar pattern of abnormality composition. Fifty-four percent of the observed abnormalities affected the hindlimbs (G-test, df = 1, $P < 0.001$, $n = 427$) and 99% of the total abnormalities involved missing parts of limbs, feet, or digits, many of which appeared to be regenerating. Hindlimb apody was again the most common abnormality followed by ectrodactyly in the hind feet and apody in the forelimbs (Table 5). One larva from Frog Pond had a supernumerary hindlimb.

Bufo boreas: *Abnormality Frequency*

We conducted 25 time-constrained transects at Hidden Pond and 32 at Frog Pond to assess temporal changes in abnormality frequency of metamorphic toads. The frequency of abnormalities in emerging western toads from both ponds remained low throughout the study (Fig. 4). At Frog Pond, where *B. boreas* was considerably more abundant, the peak frequencies for metamorphosing toads in 1997 and 1998 ranged between 4–9% (Fig. 4). Hidden Pond's toads exhibited a comparable range of frequencies (Fig. 4). At no time, however, did the abnormality frequency in the emerging toads of either pond significantly exceed the hypothesized baseline rate of 5% (G-test, df = 1, $P > 0.05$). Correspondingly, the seasonal frequencies at both ponds were similarly low, ranging between 1–4% (Table 2), and no differences were observed either between years or overall between ponds.

The rapid rate of limb development and metamorphosis in larval *B. boreas* precluded the collection of meaningful data regarding temporal changes in their abnormality frequency. In both ponds across both years, the overall frequency of abnormalities in toad larvae was low. Out of 470 inspected larvae from Frog Pond, 13 (2.8%) suffered some abnormality. At Hidden Pond, only one of the 97 larvae examined exhibited improper development.

Bufo boreas: *Abnormality Composition*

Abnormalities in metamorphic *B. boreas* were more common in the hindlimbs for both years and ponds but not to the degree recorded in *H. regilla.* The division approximated 70% hindlimb to 30% forelimb within the combined metamorph samples (G-test, df = 3, $P < 0.001$, $n = 56$).

As with the previously discussed species, patterns in the composition of morphological abnormalities were consistent within ponds and across years. The majority of the observed abnormalities at both Frog and Hidden ponds involved hemimelia of a hindlimb or ectrodactyly of a fore- or hindlimb (Table 5). We collected one polymelous toad from each pond in 1997, but none were observed in 1998. Both individuals had a single, dorsally articulating supernumerary hindlimb, one of which exhibited a PMID.

Rana catesbeiana: *Abnormality Frequency*

We examined bullfrog metamorphs during nocturnal transects on 16 April, 25 July, 23 August, and 22 September of 1997 and 17 April, 10 and 30 August, and 6 September of 1998. At Frog Pond, where bullfrogs were more abundant than at Hidden Pond, the frequency of abnormalities was

TABLE 5.—Composition of abnormalities in western toad (*Bufo boreas*) metamorphs, California newt (*Taricha torosa*) larvae, and American bullfrog (*Rana catesbeiana*) metamorphs from Hidden and Frog ponds (1997–1998). Listed are the numbers of each abnormality type and its proportion (%) relative to the total number of abnormalities observed. The total number of abnormalities may or may not equal the number of abnormal animals, as many specimens had more than one abnormality. For each species, the mean number of abnormalities per abnormal larva or metamorph are offered in the final row.

	Frog Pond		Hidden Pond		
Abnormality type	*Bufo boreas*	*Taricha torosa*	*Bufo boreas*	*Taricha torosa*	*Rana catesbeiana*
Cephalic and Axial					
Anophthalmy	0	1 (0.5%)	0	0	1 (4.3%)
Mandibular dysplasia	0	1 (0.5%)	0	0	0
Open wound	0	0	3 (7.5%)	0	0
Edema	0	0	0	0	3 (13.0%)
Other	0	0	0	0	0
Forelimb					
Ectrodactyly	3 (15.8%)	25 (13.4%)	6 (15.0%)	58 (13.6%)	0
Polydactyly	0	0	0	0	0
Apody	1 (5.3%)	19 (10.2%)	1 (2.5%)	81 (19.0%)	0
Hemimelia	0	7 (3.8%)	1 (2.5%)	13 (3.1%)	0
Ectromelia	0	20 (10.8%)	1 (2.5%)	47 (11.0%)	5 (21.7%)
Polymelia	0	0	0	0	4 (17.4%)
Cutaneous fusion	0	0	0	0	0
Other malformed*	0	0	1 (2.5%)	0	1 (4.3%)
Hindlimb					
Ectrodactyly	4 (21.%)	37 (19.9%)	8 (20.0%)	74 (17.3%)	0
Polydactyly	0	2 (1.1%)	0	0	0
Apody	2 (10.5%)	36 (19.4%)	4 (10.0%)	81 (19.0%)	0
Polypody	0	0	0	0	0
Hemimelia	5 (26.3%)	12 (6.5%)	6 (15.0%)	13 (3.0%)	0
Ectromelia	0	25 (13.4%)	2 (5.0%)	59 (13.8%)	1 (4.3%)
Polymelia	1 (5.3%)	1 (0.5%)	1 (2.5%)	1 (0.2%)	3 (13.0%)
Femoral projection	0	0	0	0	0
Cutaneous fusion	0	0	0	0	0
Taumelia	0	0	0	0	0
Micromelia	1 (5.3%)	0	1 (2.5%)	0	1 (4.3%)
Limb hyperextension	0	0	0	0	0
Other malformed*	2 (10.5%)	0	5 (12.5%)	0	4 (17.4%)
No. abnormal	17	147	38	267	22
No. inspected	453	861	1168	647	513
No. abnormalities per abnormal animal	1.12	1.26	1.05	1.60	1.05

* Other malformed includes brachymelia, brachydactyly, and syndactyly.

low on each sampling trip and averaged 3.5% for 1997 (n = 259). In 1998, the seasonal frequency was 5.1% (n = 254). Neither of these values differed significantly from each other or from the null-hypothesized baseline rate (G-test, df = 1, P > 0.05; Table 2). In 1997, we surveyed larval *R. catesbeiana* during several seine hauls and inspected them in the field. Of the 333 examined, six (1.8%) had abnormalities. We also captured 17 adults at Frog Pond, four of which were abnormal. Hidden Pond, which supports few bullfrogs, yielded 23 metamorphs between 1997 and 1998, three of which showed signs of injury or malformation.

Rana catesbeiana: *Abnormality Composition*

Limb malformations accounted for >90% of the observed abnormalities in larval, metamorphic, and mature *R. catesbeiana* from Frog Pond. The predominance of hindlimb abnormalities was slight (55% hind- to 45% forelimb) among the 22 abnormal metamorphs. Missing and su-

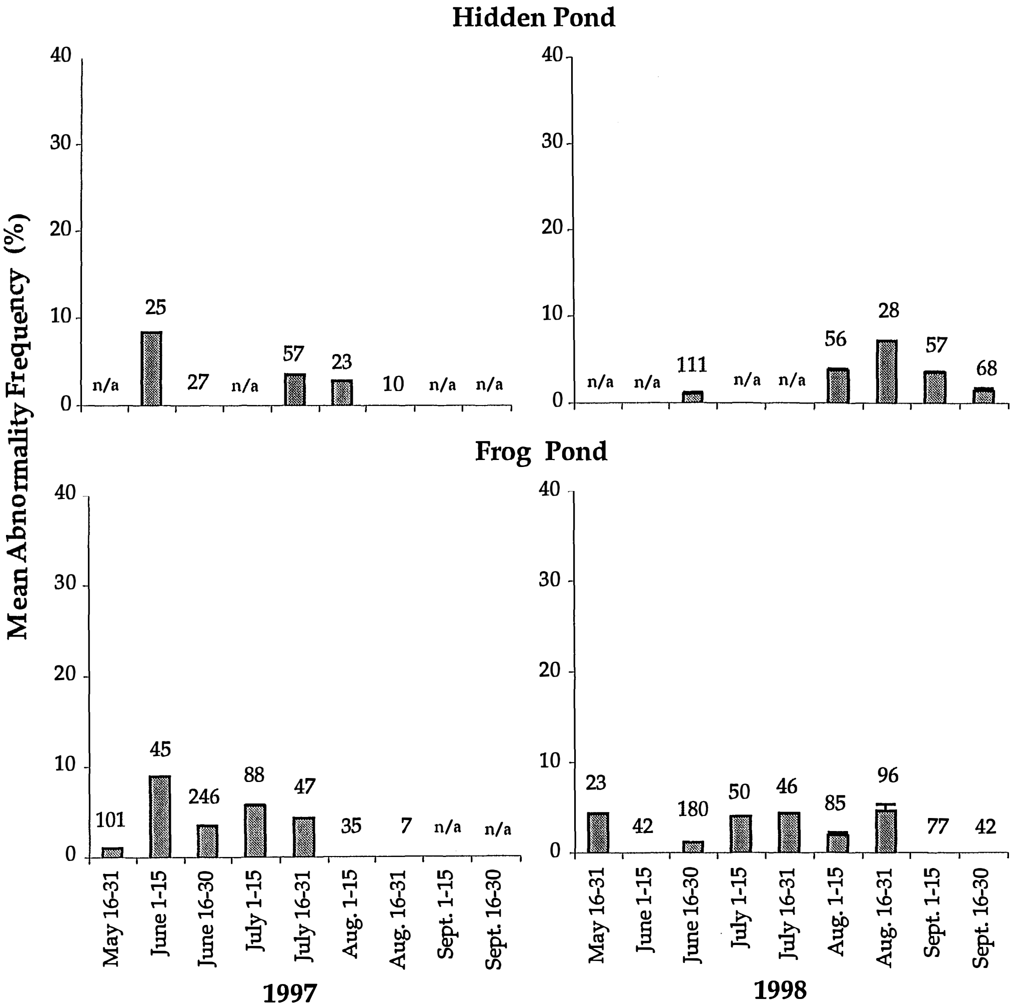

FIG. 4.—Frequency of morphological abnormalities in metamorphic western toads (*Bufo boreas*) from Hidden and Frog ponds (1997–1998). Bars represent the mean abnormality rate ± SE for a 2-wk period. Total sample sizes (number of toads examined) for the 2-wk periods are displayed above the bars. All standard error values are ≤0.7. For periods in which no transects were conducted or insufficient animals were available, an "n/a" is indicated.

pernumerary forelimbs were the most common abnormalities followed by malformed (e.g., limb hyperextension, reduced musculature) and supernumerary hindlimbs (Table 5). Two individuals, each with two extra forelimbs, and three individuals with extra hindlimbs were collected. Two of the three supernumerary hindlimbs projected ventrally from the primary limb and were poorly developed. The third was dorsal to the primary limb and well-developed, with a PMID digit pattern in the foot. Within the larval samples from Frog Pond, hindlimb polymelia accounted for most of the observed malformations. No polymelous adult bullfrogs were observed, but one suffered polydactyly on a hind foot while another two had abnormalities on a hindlimb. The three abnormal metamorphs from Hidden Pond included one obvious trauma case with a fractured mandible and a crushed eye and two individuals with malformed feet or digits.

DISCUSSION

Our results reveal dramatic differences in the patterns of morphological abnormalities in amphibians between seasons, ponds, and among species and life-history stages. With respect to our null-hypotheses, we found that the larvae of *T. torosa* and the larvae and metamorphs of *H. regilla* from both ponds consistently exhibited an abnormality frequency significantly and substantially above the hypothesized baseline range of 0–5%. The hierarchy of abnormality frequency among species was the same for Hidden and Frog ponds and for 1997 and 1998: the larvae of *T. torosa* had the highest abnormality rates, with peak frequencies in excess of 50%, followed by the larvae and metamorphs of *H. regilla,* and finally by *R. catesbeiana* and *B. boreas,* both of which suffered low rates of abnormalities within the expected baseline range. Within each species, however, we observed significant differences in the magnitude of abnormality frequency between seasons and between ponds, with amphibians from Frog Pond generally exhibiting higher peak and seasonal frequencies. Life-history stage was also a significant determinant of abnormality frequency, and the proportion of abnormal animals typically decreased with progressive stages. Abnormal adults were rarely recorded, and, when present, had only slight abnormalities, suggesting that abnormal larvae and metamorphs may suffer higher mortality than normal conspecifics.

The majority of the abnormalities in all species affected the limbs, particularly the hindlimbs. Within a species, the specific types of limb abnormalities observed and their relative frequencies were strongly consistent between seasons, ponds, and early life-history stages. Among species, however, we documented substantial variation in abnormality composition. For Pacific treefrogs from both ponds, severe malformations such as polymelia, femoral projections, and cutaneous fusion comprised approximately 60% of the observed abnormalities in larvae and metamorphs. Several treefrogs with as many as six complete extra limbs were collected. In the larvae of *T. torosa,* the abnormalities observed at Hidden and Frog ponds consisted of 97% ectrodactyly, ectromelia, and hemimelia. These same abnormalities accounted for between 70 and 80% of the abnormalities documented in western toads from the two ponds. In *R. catesbeiana,* we encountered approximately equal numbers of animals with ectromelia and polymelia, which together comprised 60% of the total abnormalities. No bullfrogs with ectrodactyly were collected.

The differences in abnormality frequency and composition observed within and among these species are most likely a consequence of one (or both) of two possibilities: (1) exposures to different causative agents or (2) different exposures or responses to a single agent. Even while inhabiting the same ponds, these species have different phenologies, relative sizes, larval development periods, and habitat preferences, all of which may affect the likelihood that they are influenced by a teratogenic factor. For example, *Bufo boreas,* the species with the lowest abnormality rate, also has the shortest larval development period, possibly reducing its exposure to teratogens. However, unlike the pattern described by Hoppe and Mottl (1997), we did not observe a positive correlation between larval development period and abnormality rate among our species. *Rana catesbeiana,* the only overwintering species in the study, also exhibited a low rate of abnormalities. Although water tests have failed to detect any pesticides, heavy metals, or polychlorinated-biphenyls (PCBs) in Hidden and Frog ponds, several other agents warrant discussion in explaining the observed abnormalities.

Previous research at these same study sites has demonstrated that the majority of the malformations in treefrogs from Hidden and Frog ponds can be caused by the cathaemasiid trematode, *Ribeiroia* sp., an abundant parasite at both ponds (Johnson et al., 1999). Using field-derived levels of *Ribeiroia* infection, Johnson et al. (1999) induced high frequencies of polymelia, femoral projections, cutaneous fusion, and a wide array of other limb malformations in similar proportions to the observed field patterns. Furthermore, Sessions et al. (1999) have suggested that PMID's—-the most frequently encountered form of polydactyly in *H. regilla* from our study sites-are indicative of a mechanical disturbance in limb development such as trematode in-fection. *Ribeiroia* is a digenetic trematode which uses planorbid snails as its first in-termediate host, amphibians or fish as its second intermediate host, and birds or mammals as the definitive host (Beaver, 1939; Yamaguti, 1975). The mobile cercar-ia stage of the parasite emerges from an infected snail and infects the hindlimb re-gion of amphibian larvae, wherein it en-cysts as a metacercaria (Johnson and Lun-de, personal observation). If an infected frog is eaten by a suitable definitive host, the parasite excysts, completes its development, and sexually reproduces with another mature worm. Indeed, it is suspected that inducing limb malformations may benefit *Ribeiroia* by increasing the likelihood that an infected frog is predated and the parasite transferred to its final host (Johnson and Lunde, 2001; Johnson et al., 1999; Sessions and Ruth, 1990).

Host-parasite interactions between *Ri-beiroia* and Pacific treefrogs may exp lain several of the trends in the abnormality pattern recorded for *H. regilla*. First, *Ri-beiroia* cercariae preferentially infect and encyst in the hindlimb region of anurans, potentially accounting for the large bias (> 95%) in favor of hindlimb malformations in larval and metamorphic *H. regilla*. Sec-ond, Frog Pond supports a greater density of the aquatic snail *Planorbella tenuis*, a first intermediate host for *Ribeiroia*. Cor-respondingly, treefrogs from Frog Pond frequently exhibited higher intensities of *Ribeiroia* infection, possibly explaining their generally higher rates of more severe limb malformations. In addition, higher in-fection intensities of *Ribeiroia* may also ex-plain the increasing rate of malformed lar-vae of *H. regilla* at both ponds toward the end of the emergence period. Both the population density of snails and the per-cent parasitized by *Ribeiroia* increase dur-ing the late spring and summer (Johnson and Lunde, unpublished data), exposing later cohorts of *H. regilla* to heavier infection intensities. Greater dosages of *Ribeiroia* cercariae, in turn, directly increase not only the likelihood that an infected larva will be malformed but also the severity of the resulting malformation (Johnson et al., 1999).

The cause(s) of the abnormalities in the three remaining amphibian species are not yet known. While all three were regularly found infected with *Ribeiroia* metacercariae, aspects of water quality, traumatic events, and a host of other factors might additionally or alternatively be involved. The polymelous western toad and bullfrog metamorphs are most likely the result of *Ribeiroia* infection, given the rarity of mutational events, the high frequency of polymelia caused by *Ribeiroia* in the laboratory (Johnson et al., 1999), and the scarcity of other agents shown to cause anuran supernumerary limbs in the field. However, the missing limbs in these species and in the California newt larvae are less easily explained. If these abnormalities are not the result of parasite infection, a suite of other agents are known to affect amphibian limb growth. Injury to the developing limbs of amphibian larvae followed by partial regeneration can create the appearance of developmental malformations. Bohl (1997) in Germany and Bowerman (personal communication) in Oregon found high frequencies of toads with missing or malformed limbs which, after experimental trials, they concluded were due to predatory attacks on the larval stages by leeches (*Erpobdella octoculata*) and sticklebacks (*Gasterosteus aculeatus*), respectively. Both Hidden and Frog ponds support dense populations of introduced mosquitofish (*Gambusia affinis*), which voraciously attack amphibian larvae in the laboratory (Gamradt and Kats, 1996; Goodsell and Kats, 1999). Gamradt and Kats (1996) found that invasive individuals of *G. affinis* were undeterred by the potent skin toxins of *T. torosa*, and they preyed heavily on larvae in field and laboratory experiments. Predatory attacks by the non-native *G. affinis* could explain the high rates of limb and digit truncations in our larval samples of *T. torosa*. The density of mosquitofish and California newts are both greater at Frog Pond than at Hidden Pond, coinciding with the consistently greater frequencies of abnormalities from this pond. Moreover, the number of mosquitofish at Frog Pond declined significantly between 1997 and 1998 (Johnson and Lunde, unpublished data), corresponding to the significant decrease in the abnormality frequency of larval *T. torosa* during this period. Alternatively, conspecific predation, which is a well-documented phenomenon in a variety of salamander species, might be involved in the abnormalities of these newt populations (Polis and Myers, 1985). Ultimately, however, these hypotheses, along with *Ribeiroia* infection and contaminant exposure, will require experimental testing.

A final consideration is the degree to which such abnormalities at the observed rates pose a threat to affected populations. Malformed amphibians are not new to California (e.g., Banta, 1966; Crosswhite and Wyman, 1920; Cunningham, 1955), but they may be occurring with greater severity or frequency. Of the four species that we examined, only *B. boreas* exhibited a low enough abnormality frequency composed of "minor" abnormalities to conform to our null-hypothesized baseline pattern. While also occurring at a low frequency, abnormal individuals of *R. catesbeiana* were predominantly afflicted with polymelia and ectromelia, two severe abnormalities uncommonly reported in studies on background variation in amphibian morphology. Larvae of *T. torosa* had the highest overall frequency of abnormalities but are also endowed with greater regenerative capabilities than their anuran counterparts. Most studies of morphological abnormalities in urodeles examine only adults (e.g., Meyer-Rochow and Asashima, 1988; Roberts and Verrell, 1984; Shubin et al., 1995), and, as many of the missing limbs and digits observed in our newt larvae may have subsequently regenerated, it is not clear if these high rates represent a threat to the population or are even unusual in this species. Nonetheless, even if these missing appendages do not directly lead to elevated mortality, they may be indicative of it. Amputations caused by the attacks of mosquitofish or other predators represent only the failed attempts; actual predation rates could be much greater.

Larvae and metamorphs of *H. regilla* consistently exhibited high frequencies of permanent, severely debilitating malformations. Although not considered a declining species, Pacific treefrogs are expected to suffer substantially reduced survivorship as a consequence of these malformations. After a summer in which >300 abnormal larval and metamorphic treefrogs were observed at Hidden Pond, only one abnormal adult was found the subsequent winter. Moreover, larvae of *H. regilla* infected with cercariae of *Ribeiroia* in the laboratory exhibited more than twice the mortality of uninfected control animals (Johnson et al., 1999). At this point, however, we need more information on the long-term effects of *Ribeiroia* infection and other causes of abnormalities on amphibian populations.

These findings offer some important considerations for the continued investigation of malformed amphibians. First, as observed in *H. regilla,* abnormality rate can vary substantially across a given season, emphasizing the importance of frequent monitoring efforts which include multiple life history stages. Samples from a single site visit could result in misleading data. Abnormality rate can also vary spatially, and we noted differences as great as 10% from one side of a pond to the other on a single day. Second, individual agents may affect species in different ways or to differing degrees. While the abnormality patterns were largely consistent within each species, we observed dramatic differences in the composition and frequency of morphological abnormalities among the four species. Studies focusing on one amphibian species may fail to notice severe malformations in a second species. Finally, a single agent, namely *Ribeiroia* infection, appears to have caused similar frequencies and types of malformations within *H. regilla* across two ponds and two years (Johnson et al., 1999). If this constancy in the abnormality signature for a species holds for other sites and potentially other causative agents, it may be possible to develop a field diagnostic system which would differentiate between abnormality causes by their respective signatures. Broad-based regional surveys, currently hindered by the influence of multiple causative agents, would be greatly strengthened by such information.

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