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Morphological Abnormalities in Amphibians in Agricultural Habitats: A Case Study of the Common Frog *Rana temporaria*

HENNA PIHA, MINNA PEKKONEN, AND JUHA MERILÄ

Recent studies suggest that the incidence of morphological abnormalities has increased in many amphibian populations, often exceeding the estimated background deformity frequency of 0–5%. Many chemical contaminants, including agrochemicals, can cause abnormalities in amphibians, but data on the occurrence of morphological abnormalities in wild amphibian populations in Europe is anecdotal at best. In a large-scale study covering 264 ha and 26 farmland breeding populations of the Common frog (*Rana temporaria*) in southern Finland, we investigated whether the incidence of morphological abnormalities in metamorphs differed from the background level of 0–5% and among populations along an agrochemical gradient. Abnormalities occurred in a low frequency (1% of the studied individuals; 40/4115), the highest population-specific frequency being 4%. We found no evidence for increased abnormality frequencies in the habitats most likely exposed to agrochemicals. Hence, the data suggest that current Finnish agrochemical practices are not causing increased incidences of morphological abnormalities in Common frog populations breeding in farmland areas.

THE occurrence of exceptionally high frequencies of morphological abnormalities in natural amphibian populations has received considerable attention during recent years (Sessions et al., 1999; Ouellet, 2000; Johnson et al., 2002). Trauma related and developmental abnormalities are typically found in amphibian populations at frequencies between 0–3% (Meyer-Rochow and Asashima, 1998; Gillilland et al., 2001), and it is when abnormality frequencies exceed 5% that their occurrence is considered abnormally high (e.g., Ouellet, 2000). In North America, populations with abnormality frequencies exceeding 15% have been found in at least eight amphibian species (e.g., Sessions and Ruth, 1990; Ouellet et al., 1997; Johnson et al., 1999; Johnson et al., 2001b). Isolated findings of high abnormality frequencies in anurans have also been made in Europe (e.g., Henle, 1981), but in general, significantly fewer observations of amphibian populations with high abnormality frequencies have been made there than in the U.S. (Ouellet, 2000). In Europe, the observations have most often been of single abnormally developed adult amphibians, which have been made by chance during other field investigations (e.g., Koskela, 1974; Meyer-Rochow and Koebke, 1986).

Although abnormally developed amphibians have been found globally (Ouellet, 2000), comprehensive population level studies are lacking from most parts of the world. Insufficient knowledge of the status of the majority of amphibian populations makes it impossible to estimate whether the incidence of abnormalities has increased at a global level (Ouellet, 2000; Carey et al., 2003). The lack of large-scale population censuses and historic data also prevents inference as to whether incidence of abnormalities in amphibian populations has actually increased with time.

A number of abiotic and biotic factors including UV radiation (Ankley et al., 1998; Pahkala et al., 2001; Ankley et al., 2002), trematode parasites (Sessions et al., 1999; Johnson et al., 2001a, 2002; Schotthoefer et al., 2003), retinoids (Gardiner and Hoppe, 1999; Sessions et al., 1999), pesticides (Alvarez et al., 1995; Britson and Threlkeld, 1998; Harris et al., 1998a; Hayes et al., 2002), other chemical contaminants (Burkhart et al., 1998; Rowe et al., 1998; Hopkins et al., 2000), and predation (Meyer-Rochow and Koebke, 1986; Sessions, 2003) can cause morphological abnormalities in amphibians. Hind limb abnormalities, which are the most common type of abnormality reported in wild-caught amphibians, can be caused by each of these factors (Ouellet et al., 1997; Gardiner and Hoppe, 1999; Johnson et al., 2002). Often the causal factors behind morphological abnormalities occurring in the wild have not been identified (Johnson et al., 2003). Despite this, recent findings suggest that infection by *Ribeiroia ondatrae* flatworms is a widespread cause of limb abnormalities in amphibians in the U.S. (Johnson et al., 2002, 2003). In addition, it is likely that many of the abnormalities result from the interaction of multiple factors (Ouellet, 2000; Kiesecker, 2002; Carey et al., 2003; Sessions, 2003).

As relatively low agrochemical concentrations can be related to abnormalities in amphibians (Cooke, 1981; Ouellet et al., 1997), it has been proposed that amphibian populations in agro-ecosystems may be particularly prone to malformations. This expectation is reinforced by the fact that breeding in these habitats often coincides with the timing of fertilizer and pesticide application, and hence, the aquatic developmental stages of amphibians are likely to be exposed to these chemicals (Boone and Bridges, 2003). In the few studies focusing on North American farmland areas published thus far, abnormalities have (Ouellet et al., 1997) or have not (Harris et al., 1998a, b) been found to be more common in anurans in agricultural sites. Similar studies from Europe are lacking (Ouellet, 2000).

The aim of our study was to investigate if the frequency of morphological abnormalities in *R. temporaria* in agricultural habitats is above the expected background frequency of 0–5%, and whether the incidence of morphological abnormalities differs among different types of breeding habitats within agro-ecosystems. *Rana temporaria* is a medium-sized anuran frog, and it is the most widespread amphibian species in Europe (Gasc et al., 1997). It is a generalist species breeding in both temporal and permanent water bodies and in a wide range of habitats (Beebee, 1981). During the breeding season, which in southern Finland begins after the melting of snow in late April-early May, each female lays one egg clutch and the larvae hatch within 9–15 days. The aquatic embryonic and larval development lasts approximately 50–70 days in total, after which the metamorphs start a terrestrial life. Reports of morphologically abnormal *R. temporaria* individuals date back to 1865, and over 20 reports have been published to date. However, most of these reports are concerned with single malformed frogs (Ouellet, 2000). To our knowledge, this is the first large-scale study on the occurrence of amphibian abnormalities carried out in European agricultural habitats.

Materials and Methods

Study sites.—In Finland, intensive agriculture is concentrated in the southern parts of the country, and our study sites covered a large part of this area (Fig. 1). The study sites were situated within 17 randomly chosen 100-ha quadrats composed of a minimum of 35% arable land (our definition of an agricultural habitat). We obtained the habitat compositions of the study quadrats from digitized aerial photographs and by performing habitat descriptions in the field during the breeding season.

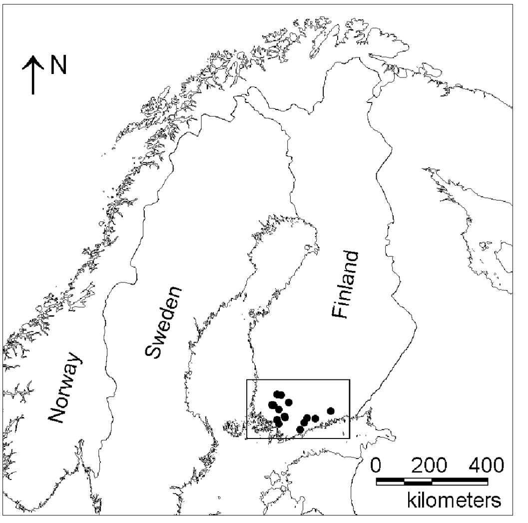

Fig. 1. The study area in southern Finland. Each dot marks the location of a 100-ha study quadrat (n = 17), within which the study sites (n = 26) were situated.

From within these quadrats, we chose the three following habitat types based on classification of the surrounding habitat within a 50-m radius of the breeding site: plowed field (spring cereal), agricultural grassland (lay or pasture including set-asides), and forest (mixed coniferous forest). Originally each habitat type was represented by ten breeding sites, but due to problems with finding an adequate number of metamorphs (and to the early drying up of one breeding site), we ended up with nine breeding sites in plowed fields, seven in agricultural grasslands, and ten in forest habitats. Following this division, there should be an agrochemical gradient with chemical concentrations being highest in the plowed field, intermediate in the agricultural grassland, and lowest in the forest sites (McGuckin et al., 1999; Mander et al., 2000). For most of the year, plowed fields lack vegetation cover, and are thus sensitive to surface runoff, whereas agricultural grasslands have vegetation cover all year round. In addition, agricultural grasslands are not treated with pesticides. The forests have permanent vegetation and no agrochemicals are directly applied to these habitats.

In Finland, the most frequently used herbicides are glyphosate and MCPA, the most sold fungicide and insecticide being mancozeb and dimethoate, respectively (Savela et al., 2003). In a two-year study conducted in southern Finland in the years 2004–2005, the most frequently found pesticides in surface waters were MCPA, dichlorprop, and mecoprop (K. Siimes, unpubl.

TABLE 1. LIST OF ALL THE STUDY SITES WITH THEIR EXACT POSITIONING, AND THE NUMBER OF COLLECTED AND ABNORMALLY DEVELOPED INDIVIDUALS PER SITE. n_{tot} = total number of studied individuals, n_{abn} = number of morphologically abnormal individuals.

Habitat type	Study site	N	E	n_{tot}	n_{abn}
Plowed field	F1	60°25′10	24°35′80	124	3
	F2	60°32′23	25°15′00	152	1
	F3	60°14′14	24°19′80	137	0
	F4	60°44′59	26°13′24	188	1
	F5	60°33′34	23°22′60	101	0
	F6	60°54′32	22°32′32	154	0
	F7	61°10′37	22°51′51	145	0
	F8	60°53′28	22°39′29	137	4
	F9	60°46′22	22°58′38	136	3
Agricultural grass	G1	60°33′40	24°45′47	108	0
	G2	60°32′57	24°46′37	147	4
	G3	60°32′52	24°45′39	140	0
	G4	60°58′36	23°35′35	157	6
	G5	60°58′40	23°35′90	152	0
	G6	60°53′38	22°35′39	148	1
	G7	60°54′17	22°36′50	174	2
Forest	F1	60°25′28	24°34′43	166	0
	F2	60°32′37	25°14′39	138	1
	F3	60°32′50	24°45′45	176	0
	F4	60°35′20	23°22′40	321	5
	F5	60°30′28	22°53′33	125	3
	F6	60°58′40	23°35′21	155	2
	F7	60°58′48	23°35′52	153	1
	F8	60°58′14	23°35′33	227	0
	F9	61°9′58	23°8′59	108	1
	F10	60°23′23	22°58′25	245	2

data). The maximum concentrations detected were 8.8 µg/L for MCPA, 4.4 µg/L for dichlorprop, and 1.6 µg/L for mecoprop.

The breeding sites were chosen randomly from all the suitable sites found within the study quadrats. Only breeding sites which had at least ten egg clutches during the breeding season were accepted in order to minimize possible family effects on the frequency of morphological deformities. Due to logistic reasons, we were unable to analyze water samples from our study sites, and thus the actual agrochemical concentrations could not be verified.

Collection and evaluation of samples.—The metamorphs were gathered during June and July 2002. Approximately 150 individuals were collected from each of the 26 breeding sites (Table 1). They were caught with dip nets and by hand. Metamorphosis was determined as the appearance of both of the forelimbs (Gosner stage 42; Gosner, 1960). The captured metamorphs were transferred into the laboratory in 10-L buckets (with moist moss on the bottom) and stored in a cold room (8–10 C) until examination, after which the individuals were returned to capture site. If it was not possible to catch all the metamorphs from a site on a single occasion, the site was revisited after a couple of days. To avoid sampling same metamorphs multiple times, the animals were held in the laboratory until the site was sampled completely, and then all metamorphs were released together. The morphological abnormalities were evaluated by examining anesthetized individuals under a stereomicroscope. The individuals were anesthetized in MS-222 (tricaine methane sulfonate) dissolved in water. Only external morphological abnormalities were examined. As the terminology used in describing amphibian abnormalities varies, and because the distinction between malformations and deformities can be vague, we use the term morphological abnormality (according to Sessions, 2003), which includes both types of abnormalities. We identified only three cases of abnormalities to be clearly trauma-related. One metamorph lacked digits from its right forelimb. The skin of the limb was badly torn, which led us to believe the abnormality was most likely caused by a predator or a mechanical injury. In the other two cases, the metamorphs dragged their right hind limb, which led us to suspect that the limbs were more probably injured than suffering from an abnormality. These individuals were excluded from the analyses.

Statistical analyses.—The probability of being abnormally developed was analyzed with generalized linear mixed models using GLIMMIX macro of SAS statistical package. In the models, the type of breeding site (plowed field, agricultural grassland, or forest) was considered as a fixed effect, whereas the study quadrat was considered as a random effect to account for non-independence of individuals from the same study quadrats. The abnormality frequencies were analyzed as means per breeding site, and the data were arcsin-square-root transformed before the analysis.

Results

Only 1.0% of the 4115 studied metamorphs had morphological abnormalities. Of all the studied populations, 62.6% had at least one abnormal metamorph, the highest population-specific abnormality frequency being 3.8% found from agricultural grasslands. The abnormalities were more common in agricultural grassland than in plowed field and forest habitats (Fig. 2), but the differences were less than 0.4% and thus statistically non-significant (Table 2). Likewise, the effect of study quadrat was non-significant (Table 2). All the abnormalities were observed in the limbs, and they occurred more frequently in the hind than the forelimbs (Table 3). In all but one case, the observed morphological abnormalities were asymmetric. None of the individuals had multiple abnormality types. The most common type of abnormality in the hind limbs was hemimelia (Table 3), and in the forelimbs brachydactyly, but apody also occurred (Table 3).

Discussion

Although findings of amphibian populations with unexpectedly high incidences of morphological abnormalities have been made in the U.S. (reviewed in Blaustein and Johnson, 2003; Sessions, 2003), similar results have not been published from Europe. It is uncertain whether this reflects a true difference in occurrence of abnormalities in the two continents or simply a lack of studies and data from European populations. In the present study, we examined

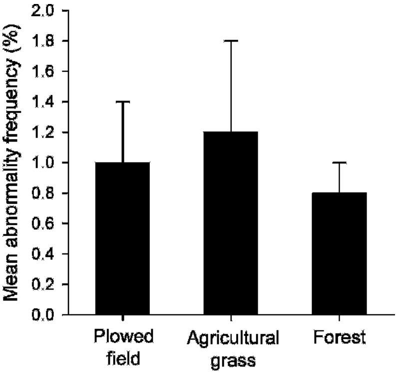

Fig. 2. Mean frequency (+SE) of morphological abnormalities in *R. temporaria* metamorphs within the studied habitat types.

over 4000 *R. temporaria* metamorphs from 26 breeding sites residing in different types of agricultural habitats and found abnormalities only in very low frequencies. Both the average and maximum abnormality frequencies found in our study fall within the estimated baseline frequency of 0–5% and are far from the levels observed in many U.S. populations (Sessions and Ruth, 1990; Johnson et al., 2003). Our results are, however, in concordance with the findings of Gillilland et al. (2001), who did not find abnormalities to be more common in adult, juvenile, and larval green frogs in agricultural sites in the U.S. Hence, if the Finnish agro-ecosystems can be taken as representative, this suggests that the incidence of morphological abnormalities in *R. temporaria* in northern Europe is not alarmingly high.

As for the representativeness of Finnish agro-ecosystems, it is true that pesticides and fertilizers are consumed more in Western and Central Europe than in Nordic countries or Eastern Europe (European Environment Agency [EEA], http://www.eea.europe.eu/). Herbicide consumption per agricultural land area unit in

Table 2. Factors Affecting the Incidence of Morphological Abnormalities in *R. temporaria* Metamorphs.

Abnormalities				
Random effects	*Estimate*	*S.E.*	*Z*	*Pr Z*
Quadrat	0			
Residual	0.07	0.02	3.39	0.0003
Fixed effects	*ndf*	*ddf*	*F*	*P*
Habitat	2	7	0.02	0.98

TABLE 3. MORPHOLOGICAL ABNORMALITY TYPES IN THE HIND AND FORELIMBS OF *R. temporaria* METAMORPHS (MODIFIED FROM JOHNSON ET AL., 2001B; SESSIONS, 2003). n = number of individuals, % = proportion of all abnormalities. The total number of individuals investigated was 4115.

Abnormality type	Description	Hind limb		Forelimb	
		n	%	*n*	%
Apody	Absence of a foot or a hand	2	5.0	2	5.0
Brachydactyly	Abnormal shortness of one or more digits	5	12.5	3	7.5
Brachymelia	Abnormal shortness of a limb	–	–	1	2.5
Clinodactyly	Curvature of one or more digits	1	2.5	1	2.5
Ectrodactyly	Absence of one or more digits	2	5.0	–	–
Ectromelia	Absence of a limb	4	10.0	1	2.5
Hemimelia	Partial or complete absence of distal portions of a limb	9	22.5	–	–
Limb hyperextension	Rigid flexure of a limb joint	4	10.0	–	–
Micromelia	Abnormal smallness of a limb or parts of a limb	4	10.0	–	–
Syndactyly	Complete fusion of digits	1	2.5	–	–
Σ Abnormally developed individuals		*32*	*80.0*	*8*	*20.0*

Nordic countries is between 0–0.5 kg/ha, whereas in France and Britain it is between 1.5–2.0 kg/ha, and in Belgium above 2 kg/ha (EEA). Hence, the frequency of abnormalities might be higher in more southern *R. temporaria* populations, as also indicated by one case study (Cooke, 1981). Nevertheless, because the cold climate of Nordic countries slows down the breakdown of agrochemicals, they may persist in nature for a relatively long time, exposing organisms to potentially harmful levels of agrochemicals.

We found no significant differences in the incidence of abnormalities among different types of agricultural habitats, although we anticipated them to be higher in the cultivated field sites where agrochemicals are applied the most. As we lack data on water chemistry, it is possible that this results from us not sampling across an actual agrochemical gradient. It is also possible that *R. temporaria* is not a sensitive indicator species, as it is widespread and capable of adapting to different environmental conditions. However, increased abnormality incidences have been found in *R. temporaria* tadpoles next to potato fields in Britain (Cooke, 1981). We may conclude that such environmental factors that cause increased levels of abnormalities in *R. temporaria* were not generally present in the studied agricultural habitats.

All the abnormalities found in our study occurred in the limbs, particularly in the hind limbs. As in many previous studies (Ouellet et al., 1997; Johnson et al., 2002), abnormalities were mostly unilateral. However, while the most frequent malformation types reported in literature are extra limbs (polymelia; Sessions and Ruth, 1990; Ouellet et al., 1997; Gardiner and Hoppe, 1999) or partially missing limbs (hemimelia; Johnson et al., 2001a), we observed no case of polymelia or polydactyly. This indicates that causal agents behind these types of malformations, such as retinoids and *Ribeiroia* (Johnson et al., 2002; Gardiner et al., 2003), may be rare in Finnish agricultural breeding sites. Also, exposure to high levels of UV-B radiation can result in increased levels of hind limb deformities, but these are typically symmetrical (Pahkala et al., 2001). Since symmetric abnormalities were almost completely lacking from our data, it seems unlikely that observed abnormalities could be attributed to UV-B radiation. Although we had excluded individuals with clear signs of injuries, it is still possible that traumas resulting from predation early in the development could explain many of the observed abnormalities (Sessions, 2003).

Finally, as we assessed the incidence of abnormalities at the end of the aquatic development, it is possible that abnormality-dependent mortality had occurred before this period. In other words, tadpoles with severe abnormalities may have experienced a higher mortality rate or prolonged development and not reached metamorphosis as frequently as developmentally normal ones. However, as earlier studies have found high abnormality frequencies in metamorphs or similar to those in tadpoles (e.g., Ouellet et al., 1997; Johnson et al., 2002), we do not believe that early life mortality has seriously biased our estimates of abnormality frequencies. Furthermore, under the assumption that limb abnormalities increase mortality rate, one would expect to find hind limb abnormalities to be less frequent than forelimb abnormalities, because tadpoles for this study were collected just when the forelimbs emerged and when the hind limbs had been visible already for several (3–6) weeks. Still, hind limb abnormalities were more common than forelimb abnormalities.

In conclusion, our findings suggest that the incidence of high amphibian deformity frequencies is not a common phenomenon in Finnish agricultural habitats. The low frequency of abnormalities observed in this study is typical for most amphibian populations studied up to date (Meyer-Rochow and Asashima, 1998; Gillilland et al., 2001; Johnson et al., 2002) and gives little reason to suspect that current practices in application of agrochemicals in Finland would pose serious threats to the morphological development of *R. temporaria* populations breeding in agricultural habitats. However, negative effects of agrochemicals alone and in combination with other stressors, e.g., on survival, immune defenses, and sexual development of amphibians, are possible (e.g., Hayes et al, 2002; Boone and Bridges, 2003; Gendron et al., 2003; Relyea, 2003; Broomhall, 2004) as are effects on later life development and performance due to possible delayed effects. Thus, although deformities are easy to detect, they should not be the only end point used in measuring effects of agricultural contamination on amphibians. Likewise, further monitoring over time and examination of incidence of abnormalities in central and southern European *R. temporaria* populations and in other, possibly more sensitive amphibian species, would be needed before broader generalizations are possible.

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ECOLOGICAL GENETICS RESEARCH UNIT, DEPARTMENT OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES, UNIVERSITY OF HELSINKI, P.O. BOX 65, FI-00014 UNIVERSITY OF HELSINKI, FINLAND. E-mail: (HP) henna.piha@helsinki.fi. Send reprint requests to HP. Submitted: 7 Dec. 2004. Accepted: 23 May 2006. Section editor: S. J. Beaupre.