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Mass anomalies in green toads *(Bufotes viridis)* at a quarry in Roßwag, Germany: inbred hybrids, radioactivity or an unresolved case?

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Abstract. Amphibian anomalies have attracted human curiosity for centuries and the literature on this issue is very extensive. The vast majority of publications on natural populations refer to less than ten affected individuals. Recent observations of mass anomalies in amphibians and increased environmental awareness reignited the interest in amphibian anomalies as potential indicators of environmental perturbations and triggered concerns about environmental conditions and human health. A particularly severe case of mass anomalies in a natural amphibian population was discovered in a quarry close to the village of Roßwag in southern Germany in 1980. Until now, only very brief preliminary data have been published on this case and the cause(s) of the anomalies have remained controversial. Here we provide a detailed account of the anomalies observed and on the studies undertaken to assess the potential cause(s). Based on an extensive literature review on experimentally induced anomalies in amphibians, we evaluate all known causes of amphibian anomalies as potential explanations for the observations in Roßwag.

At least 245 recently metamorphosed individuals and 1,000–2,500 tadpoles of the green toad (*Bufotes viridis*) exhibited anomalies, thus scoring fourth highest among the 2782 cases from natural populations compiled by us for which concrete data were available. Thirty-two different types of anomalies were observed, which exceeded the next extreme case known (25 types of anomalies in a population of *Rana arvalis* that was exposed to a nuclear accident; 2990 cases with data available). Therefore, the anomalies observed clearly do not represent a normal natural occurrence. The green toad was the only species spawning in the pond and, with the exception of three white clutches of the same species in a neighbouring quarry, no anomalies were found in any other population of amphibians in the vicinity of the quarry.

Based on an evaluation of results from 1025 publications of experimental studies, we show that all potential non-genetic factors taken together could at most explain a fraction of the observed types of anomalies, except for a cocktail of chemicals. Only one individual with a squashed head can be attributed with certainty to a non-genetic cause: traumatism. Chemical analyses of water samples from the breeding pond revealed a copper concentration at a level at which it might cause bent tails and edema in tadpoles. The water samples did not provide any other indication of chemical pollution and no pesticides were detected in abnormal toads. Notwithstanding, pesticides aerially sprayed on neighbouring vineyards might have contaminated the breeding pond well before water samples were taken.

The appearance of various anomalies in tadpoles and recently metamorphosed individuals raised from eggs transferred to the laboratory indicates that several types of anomalies were of genetic origins. The successful breeding of one abnormal individual verified this for one colour anomaly. Inbreeding, hybridization, as well as radioactivity and other mutagenic factors can explain genetically-based anomalies. Hybridization followed by inbreeding was put down as one explanation resulting from an official inquiry. However, genetic and morphological analyses as well as amphibian surveys conducted in the area of Roßwag clearly contradict hybridization as a potential cause. The great interindividual variability and high number of different types of anomalies are not compatible with inbreeding as an explanation. No evidence of mutagenic chemicals was found, though it cannot be excluded with absolute certainty that such substances had been present but were already degraded at the time of chemical sampling. In contrast, high levels of radioactivity were measured at the opening of cracks at a deposit of earth bordering the breeding pond. Alerted officials rejected these measurements as artefacts but did not take any measurements at the opening of cracks. Here it is shown that all radioactivity measurements are consistent with one another and with the hypothesis of radioactive material being burrowed in the deposit of earth and accessible to toads via cracks. This hypothesis is the only explanation that is consistent with all observations and measurements taken and with the results from a huge body of literature on experimental studies. There is no evidence for any other potential cause for the anomalies and biological knowledge clearly contradicts all of them with the exception of mutagenic substances.

Keywords. Anura: Bufonidae: *Bufotes viridis*, Germany, chemicals, genetic causes, hybridization, inbreeding, mass anomalies, radioactivity, teratogenetic causes.

Zusammenfassung. Anomalien bei Amphibien haben seit Jahrhunderten Aufmerksamkeit erzeugt. Entsprechend existiert eine umfangreiche Literatur über dieses Thema. Allerdings beziehen sich die meisten Veröffentlichungen zu freilebenden Populationen auf Beobachtungen von weniger als zehn betroffenen Individuen. In jüngster Zeit wurde jedoch wiederholt ein massenhaftes Auftreten von Missbildungen bei Amphibien beobachtet. Dies sowie ein erhöhtes Umweltbewusstsein haben das Interesse am Auftreten von Anomalien als Indikator für Umweltbelastungen erneut geweckt und zu Sorgen über den Umweltzustand und Gesundheitsrisiken für den Mensch geführt. Ein besonders extremer Fall von Anomalien wurde im Jahre 1980 in einem Steinbruch bei Roßwag, Baden-Württemberg, Deutschland, entdeckt. Bisher wurden nur vorläufige Beobachtungen über den Fall publiziert und die Ursache(n) blieben kontrovers. In diesem Beitrag geben wir erstmals eine ausführliche Übersicht über die beobachteten Anomalien sowie die Untersuchen, die zur Aufklärung der Ursachen unternommen wurden. Basierend auf einer Auswertung von 1025 Publikationen über Experimente diskutieren wir alle Faktoren, die als Erklärung vorgeschlagen wurden oder experimentell Anomalien verursachen können.

In den Jahren 1980–1984 wiesen mindestens 245 metamorphosierte Jungtiere und 1000–2500 Kaulquappen der Wechselkröte (*Bufotes viridis*) Anomalien auf. Abgesehen von drei weißen Laichschnüren der Wechselkröte in einem benachbarten Steinbruch konnten in keiner Amphibienpopulation der weiteren Umgebung von Roßwag Anomalien gefunden werden. Bezüglich der Anzahl missgebildeter Individuen stellt die Roßwag-Population den viert-extremsten, je im Freiland beobachteten Fall dar (n=2782 Fälle mit expliziten Daten). Insgesamt wurden an Kaulquappen und Kröten 32 verschiedene Typen von Anomalien beobachtet, was alle anderen 2990 Fälle übertrifft, für die wir Daten haben (maximal 25 verschiedene Typen innerhalb einer Population von *Rana arvalis*, die radioaktiver Strahlung ausgesetzt war). Diese Zahlen belegen, dass die Anomalien kein normales, häufig auftretendes Phänomen darstellen.

Fast alle betroffenen Individuen verstarben spätestens wenige Monate nach der Metamorphose. Nur ein Individuum mit einer Farbanomalie (großer gelber Fleck an der Flanke) konnte zur Fortpflanzung gebracht werden. Ein Viertel der Nachkommen wiesen dieselbe Anomalie auf, d.h., die Anomalie war vermutlich dominant vererbt. Das Auftreten unterschiedlicher Typen von Anomalien bei der Entwicklung von Eiern und Kaulquappen, die ins Labor transferiert wurden, spricht dafür, dass auch weitere Anomalien eine genetische Ursache hatten.

Die Auswertung der Literatur über experimentell erzeugte Anomalien zeigt, dass alle bekannten, nicht-genetischen Ursachen von Anomalien bei Amphibien zusammengenommen höchstens einen kleinen Anteil der beobachteten Anomalien erklären könnten, abgesehen von einem Cocktail an Chemikalien. Lediglich ein Individuum mit Quetschungen des Schädels kann mit Sicherheit auf eine nicht-genetische Ursache zurückgeführt werden. Chemische Analysen von Wasserproben aus den Laichgewässern haben eine Kupferkonzentration ergeben, bei der gekrümmte Schwänze und Ödeme auftreten können. In den umliegenden Weinbergen wurden handelsübliche Pestizide mit Helikoptern versprüht, jedoch ließen sich Rückstände von Pestiziden weder in den Wasserproben noch in gesammelten Jungkröten nachweisen.

Für eine genetische Erklärung der Anomalien kommen Hybridisierung, Inzucht und mutagene Faktoren in Frage. Hybridisierung gefolgt von Inzucht wurde von der zuständigen Landesregierung als Erklärung angenommen. Inzucht kann ausgeschlossen werden, da bei Inzucht alle Individuen dieselbe Art von Anomalien aufweisen (sowohl theoretisch als auch empirisch umfassend belegt), was im krassen Widerspruch zum beobachteten breiten, von Individuum zu Individuum stark unterschiedlichen Spektrum an Anomalien in Roßwag steht. Alle genetischen und morphologischen Analysen widersprechen der Hypothese einer Hybridisierung mit einer weiteren heimischen Art. Außerdem war die Wechselkröte die einzige im Steinbruch laichende Amphibienart, das Steinbruchgewässer ist als Laichgewässer für die Erdkröte (*Bufo bufo*) ungeeignet und die nächsten Populationen der Erdkröte und der Kreuzkröte (*Epidalea calamita*) sind durch Barrieren und eine Entfernung von Roßwag getrennt, die ein kurzzeitiges Zuwandern einer höheren Anzahl an Individuen dieser Arten ausschließen.

Hinweise auf mutagene Chemikalien liegen nicht vor; es kann jedoch nicht mit absoluter Sicherheit ausgeschlossen werden, dass solche Substanzen zur Fortpflanzungszeit vorhanden, aber zum Zeitpunkt der Wasseranalysen bereits abgebaut waren. Dagegen wurden an einer Erddeponie, die in das Laichgewässer ragte, erhöhte Werte an Radioaktivität gemessen, die das zweifache von Kontrollwerten an anderen Stellen des Steinbruchs betrugen; an der Öffnung von Spalten stiegen sie bis zum 100-fachen der Kontrollwerte an. Die alarmierten Behörden fanden ebenfalls eine Verdopplung der Werte an der Erddeponie, nahmen jedoch keine Messungen unmittelbar an der Öffnung von Spalten vor; stattdessen wiesen sie die hier gemessenen hohen Werte als Artefakte zurück. Hier zeigen wir, dass die verschiedenen Messergebnisse zu Radioaktivität nicht im Widerspruch zueinander stehen, sondern sich decken, soweit es die an vergleichbaren Stellen durchgeführten Messungen betrifft. Sie stimmen auch mit der Hypothese überein, dass in der Erddeponie radioaktive Stoffe verborgen waren, zu denen die Wechselkröten über Spalten Zugang hatten. Diese Erklärung ist die einzige, die mit allen Beobachtungen und Messungen sowie der sehr umfangreichen Literatur zu experimentell erzeugten Anomalien bei Amphibien in Einklang gebracht werden kann.

Schlüsselwörter. Anura: Bufonidae: *Bufotes viridis*, Deutschland, Chemikalien, genetische Ursachen, Hybridisierung, Inzucht, massenhafte Anomalien, Radioaktivität, teratogene Ursachen

1 Introduction

Amphibian anomalies have attracted human curiosity for centuries (e.g., Vallisneri 1706, Geoffroy Saint-Hilaire 1832, 1836). Amphibian anomalies also played a pivotal role in the emerging science of developmental biology, aiming to understand morphogenesis (e.g., Hertwig 1892, Sladden 1930, Woerdeman 1936) and they still assume this role today (e.g., Kovalenko & Kovalenko 1996, Nye et al. 2003).

Increased environmental awareness, the global decline in amphibians (e.g., Henle & Streit 1990, Blaustein & Wake 1990, Mendelson et al. 2004) and recent observations of populations exhibiting mass anomalies (Burkhart et al. 2000, Meteyer et al. 2000, Souder 2002, Lannoo 2008) have prompted renewed interest in amphibian anomalies as potential indicators for environmental perturbations (e.g., Tyler 1983, 1989, Read & Tyler 1990, 1994, Ouellet et al. 1997, Pyastolova & Vershinin 1999, Burkhart et al. 2000, Ouellet 2000, Souder 2002, Vershinin 2002). One species, the African clawed frog (*Xenopus laevis)* is even used as a standard test system for the teratogenicity of chemicals and natural water bodies (Bantle et al. 1989) and, in Russia, newts (*Triturus* spp.) are used as a standard test system for potentially carcinogenic substances (Pliss & Khudoley 1979). A micronucleus test has been used on several amphibian species for *in vivo* monitoring of genotoxic aquatic pollution (Gauthier 1996).

In response to findings of mass anomalies in North America, the North American Reporting Centre for Amphibian Anomalies (NARCAM) was established (Lannoo 2008). Mass anomalies and/or high genetic loads have also been reported in other organisms from polluted environments, for example, plants (Yablokov et al. 2009), invertebrates (Vuori & Parkko 1996, Saura et al. 1998, Hesse-Honegger & Wallimann 2008, Taira et al. 2014), reptiles (Ernst 1995, Bishop & Gendron 1998), birds (van der Schalie et al. 1999, Møller et al. 2007, Yablokov et al. 2009), mammals (Yablokov et al. 2009) and humans (Gilberston et al. 1991, Savchenko 1995, Guizard et al. 2001, Yablokov et al. 2009). However, these organisms, with the exception of humans (e.g., Winter et al. 1988, Savchenko 1995, Feshchenko et al. 2002, Scherb & Weigelt 2003, Lannoo 2008, Yablokov et al. 2009) and domestic animals (Taruffi 1881–1886, Noden & de Lahunta 1985, Yablokov et al. 2009), have still not received the same level of attention as amphibians.

Not surprisingly, the literature on amphibian anomalies is enormous (Henle 2014). Many factors have been shown to cause developmental anomalies in amphibians (reviewed e.g. by Hertwig, O. 1894, Hertwig, G. 1918, Rostand 1971, Dubois 1979, Tyler 1989, Ferraro & Burgin 1993, Ouellet 2000, Blaustein & Johnson 2003), but the relevance for natural populations of many of them remains obscure. Likewise, a considerable number of reviews of amphibian anomalies have been published – the first ones more than a century ago (Geoffroy Saint-Hilaire 1832, 1836, Taruffi 1881–1886). Most reviews focussed on narrow topics and, with few exceptions (noticeably Rostand 1971), did not discuss to any extent the potential and limits to infer causes from the observed patterns of anomalies. Linking cause to pattern is a central issue in ecology (Wiegand et al. 2003) and crucial for the use of amphibian anomalies as an indicator of the nature of an environmental perturbation that has occurred in the habitat.

The large majority of publications refer to malformations in less than 10 individuals per species at a particular site (Ouellet 2000, Henle 2014; this publication). However, this is not the phenomenon of concern. It is only when frequencies and types of abnormalities grossly exceed the baseline level (currently usually assumed to be below 1–5%: e.g., Hoppe 2000, Ouellet 2000, Johnson et al. 2010) that there is any reason to suspect the presence of an unusual factor (Burkhart et al. 2000, Henle et al. 2017a). Whatever the actual causes of mass anomalies, the biggest challenges will be to determine whether they are natural or anthropogenic and to assess their relevance for environmental hazards and human health (Tyler 1989, Burkhart et al. 2000, Souder 2002).

A particularly striking case of mass anomalies in a natural amphibian population was discovered by Rimpp and Henle in a green toad (*Bufotes viridis*) population in a quarry in southern Germany in 1980 (Henle 1981, 1982, Rimpp 1981, Dubois 1984). This discovery received intensive public attention in the mass media (e.g., Kneissler 1981, Kovacsics 1981, Henle et al. 2017b) and resulted in a highly controversial debate about its potential causes with major environmental and political implications (Henle 1981, Rimpp 1981, Flindt 1985a, Henle et al. 2017b). Suggested explanations ranged from a completely normal observation to inbred hybrids and victims of radioactive contamination but only preliminary observations of the anomalies and very limited assessments of potential causes have been published (Henle 1981, 1982, Rimpp 1981, Flindt 1985a).

Elsewhere in this volume, we present the first detailed history of the discovery and its treatment in the scientific, public and political arena and discuss its political and environmental implications (Henle et al. 2017b). Here we describe the affected population and provide a comprehensive description of the observed anomalies. We continue with the investigations into potential causes. We then compare the Roßwag case to other cases of anomalies in natural populations of amphibians and review experimental studies taking into account factors that caused the types of anomalies observed in Roßwag. After evaluating all the causes suggested for the Roßwag population as well as all other causes put forward for other cases of mass anomalies in amphibians, we conclude with a parsimonious explanation that is consistent with all of the observations made.

2 Habitat of the abnormal toad population – The Roßwag quarry

The abnormal toads were found at the end of August 1980 by Rimpp and Henle in a quarry owned by the company Steinbruch Zimmermann KG near a small village called Roßwag approximately 25 km NW of Stuttgart in the State of Baden-Württemberg, southern Germany. A road and the river Enz border the quarry to the South, while the rest of the quarry is bordered by agricultural land (mainly vineyards, but also an extensively-used orchard, fields and a small copse) (Fig. 1). In an abandoned part of the quarry, there was a large water body approximately 100 m × 25 m in size, with an average depth of approximately 0.5 m, a maximum depth of approximately 1 m and shallow shores. One side of the pond had been filled with earth as part of restoration activities (Fig. 2). The pond was free of vegetation and had a soft silty bottom. The shore was sparsely covered with herbs (*Atriplex* sp.).

Within three weeks of discovering the abnormal toads – a great controversy about radioactivity as a potential cause was already raging at that time (Henle et al. 2017b) – the quarry management pumped out all of the water, destroying the pond, but in 1981 some water collected again in the same place forming a small puddle of less than 200 m² and a maximum depth of less than 0.3 m. In addition, there were also two shallow (< 0.3 m depth) depressions of approx. 35–100 m² at the border of the quarry in 1980 and 1981. They were completely free of vegetation and had a loamy respectively silty bottom. In 1982 and 1983, these water bodies were still present and another depression within the quarry and a concrete pool at the entrance of the quarry contained water (Flindt 1985a). In 1984, none of the water bodies contained sufficient water to allow a successful completion of larval development of green toads (Flindt 1985a).

The original pond area was continually filled with soil material and by 2005 this fill had reached a height of approximately 100 m, making it impossible for water to collect at this location. On 15.5.2005, the only existing water body was a very small depression in a more recent part of the quarry. On 28.5.2005, there were two small water bodies with an estimated size of 10 m × 5 m and two road ditches with an estimated size of 10 m × 3 m and 5 m × 3 m, respectively. Furthermore, the concrete pool at the entrance of the quarry contained water.

3 Amphibian surveys in the Roßwag quarry

Before the pond was destroyed in September 1980, one detailed survey of tadpoles and four surveys of the terrestrial habitat in the vicinity of the pond were carried out by Henle, Rimpp and Weiss. On 30.8.1980 and 6.9.1980, tadpoles were visually counted on five plots (each 3 m in length) extending from the shore 2 m into the pond. The total population size was estimated by multiplying the average of the counts with the number of plots, which visually contained similar densities. Toadlets were surveyed within the abandoned part of the quarry by systematically inspecting accessible potential hiding places, such as cracks in the earthen deposit and objects providing cover. In addition, Henle surveyed the area surrounding the existing water bodies (up to a distance of 1 km) on 7.5.1981 and 30.5.1981. The surveys of 1980 were carried out during the day, whereas those of 1981 were carried out during the evening and early night.

Amphibians in the quarry were also surveyed by Christaller and Körbele from May to August 1981 (Christaller 1983) and by Flindt from 1982 to 1984 (Flindt 1985a). No further information is available for these surveys.

Only the green toad (*B. viridis*) was detected in the Roßwag quarry (Rimpp 1981, Henle 1982, Christaller 1983, Flindt 1985a). According to quarry workers the population had existed since 1976 and had presumably been founded by individuals originating from a quarry 1–1.5 km away (Christaller 1983). In May 1981, Christaller (1983) observed 30–50 calling males alone in the remnant of the destroyed pond and conservatively assumed a population size of approximately 200 adults. [Note that Christaller (1983) inconsistently called the Roßwag quarry 'quarry A' in one part of his publication but normally referred to it as 'quarry B']. Over the same period, Henle (unpubl.) counted a minimum of 50 males and 12 females during two surveys. Flindt (1985a) observed 46 adult males and eight adult females in 1982 and 23 adult males and six females in 1983. Given the limited survey days and the fact that most *B. viridis* females only stay one day at the breeding site (Nehring 1988), the population size must have been considerably larger than the minimum counts. The minimum numbers observed show that the population was large to very large, falling within the upper 10% of known population sizes for Baden-Württemberg (Laufer & Pieh 2007).

Data on the number of clutch strings are not available for any year. Tadpole numbers on 6.9.1980 were conservatively estimated as a minimum of 2,000–5,000 individuals in the main pond of the quarry, with the real size probably having been an order of magnitude higher. No tadpoles were observed in the two shallow depressions at the border of the quarry in 1980, but in May 1981 tadpoles were found living in one of them, roughly estimated at 1,000–5,000 individuals (Henle unpubl.).

Minimum numbers of toadlets are available for 1980–1983: > 300 in 1980 (Henle & Rimpp unpublished), 191 in 1981 (Christaller 1983), 361 in 1982 and 675 (591 from water depressions within the quarry plus 84 from the depression at the border of the quarry) in 1983 (Flindt 1985a). In 1984, all water bodies dried out before larval development was completed. In 2005, the population still existed, with a minimum of 150 tadpoles observed in the concrete pool. The water was very turbid; therefore, the total number of tadpoles must have been much higher.

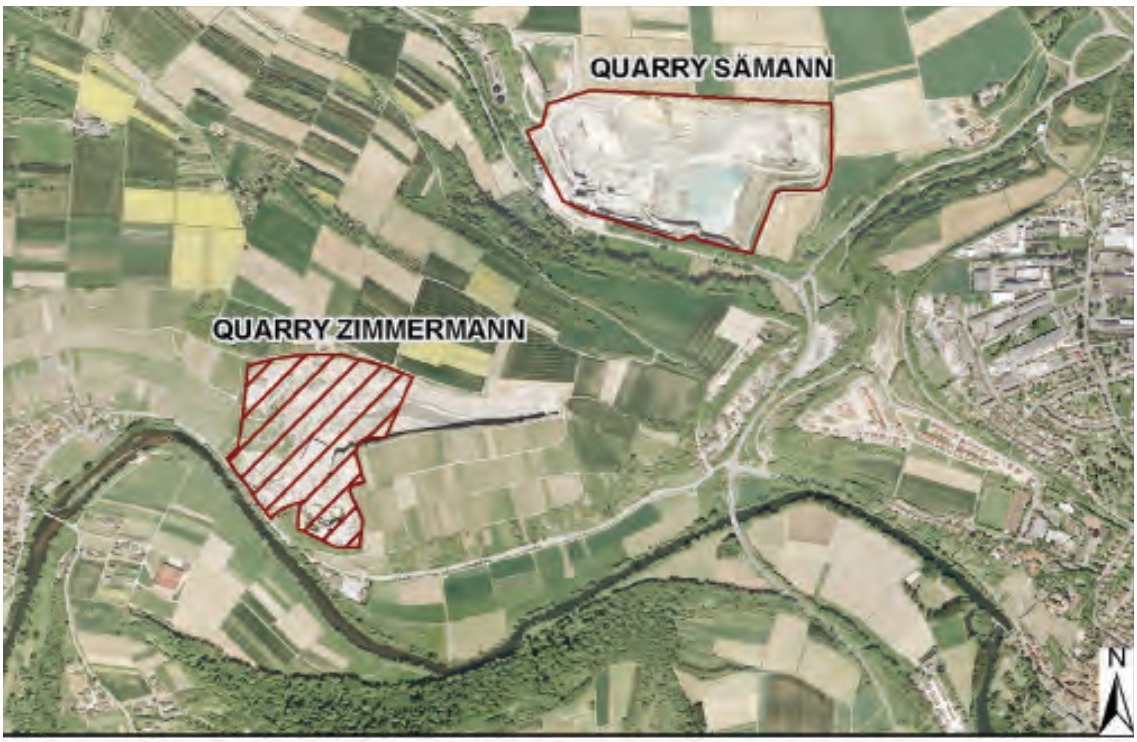

Fig. 1: Landscape structure in the surroundings of the Roßwag quarry and Roßwag's location within Germany.

Fig. 2: The breeding pond of *Bufotes viridis* in the Roßwag quarry photographed on 8.9.1980 before its destruction. Photo: K. Henle.

4 Assessment of anomalies

4.1 Methods

Our assessment of anomalies is based on our own field notes of sampled individuals, photographs and 12 malformed voucher specimens deposited at the National Museum of Natural History in Paris (MNHN 1984.2316–2326, MNHN 1984.2331). We also include data presented by Christaller (1983) and Flindt (1985a,b).

Tadpoles briefly described below were staged according to the table of Gosner (1960). We measured snout-vent length (SVL) and total length (TL) of preserved tadpoles using a ruler and of live tadpoles from photographs placed on a mm grid.

Table 1: Summary of anomalies observed in recently metamorphosed individuals of *Bufotes viridis* from Roßwag. *N*: Number; +++: > 25 individuals, ++: > 10 individuals, +: ≤ 10 individuals registered; +(D): anomaly not reported by Flindt (1985a) but detected by Alain Dubois in individuals collected by Flindt (collection year unknown); *: based on individuals raised in the laboratory, **: figures in the body of the table may not add to these sums because of multiple anomalies in some individuals and because anomalies were not specified for all individuals, ***: includes all individuals inside and at the border of the quarry; †: average of three samples; ††: figures in parentheses count different types of ectromely and ectrodactyly separately. Sources: 1980 data – Henle 1981, 1982, Rimpp 1981, Henle, Vershinin & Dubois unpubl.; 1981 data – Christaller 1983, Henle unpubl.; 1982–1984 data – Flindt 1985a.

	1980	1981 Christaller	1981 Henle*	1982	1983	1984*
N sampled	295	191	21	361	675***	87
N malformed**	173	4	4	6	50***	8
Frequency	53±9%†	2%	19%	1.7%	7.4%	9.2%
Polymely	+++	≥1		1	18	
Polydactyly (including schizodactyly)	+				1	
Ectromely: amely	++	1(?)		?	1	2
Ectromely: hemimely	++		1	2	3	
Ectromely: apody	+			1	21	
Ectrodactyly: oligodactyly	+		1	1	3	
Ectrodactyly: brachydactyly	+					
Phocomely	+				1	2
Syndactyly			?	+(D)	+(D)	
Clinodactyly				+(D)	+(D)	
Stiff legs	+++		1		1	
Rotation of legs	+++		?		1	
Vertebral column truncated and stiff	++					
Brachycephaly	+					
Pointed snout	+					
Mandibular hypoplasia	++					
Supernumerary bone at the angle of the jaw	1	1?				
Upper and lower jaw differ in shape and do not close	+					
Head injury (squashed)				1		
Swollen finger	+					
Atrophied finger musculature	+					
Partial melanism	+					
Novel colours	++					
Abnormal pattern	++					
Edema	+++		1			5
Tumours	++					
N types of anomalies††	20 (23)	∑1981: 5 or 6 (6 or 7)		4–6 (5–8)	6–8 (9–11)	3

The terminology for describing amphibian anomalies has not yet been standardized in the literature (Henle et al. 2017c). Whereas some terms are used consistently, such as polymely and anophthalmy, others are applied to different types of anomalies by different authors. Similarly, the same type of anomaly may be given different names. In this publication, we follow the terminology outlined in the companion paper by Henle et al. (2017c).

We group anomalies that occurred on the same body part of an individual (e.g., ectrodactyly or polydactyly on supernumerary legs) as a single anomaly (in this case polymely) and also merge some categories of anomalies that are tallied separately by some but not all authors. Thus, we combine apody, hemimely and amely in the category ectromely and brachydactyly, brachyphalangy and oligodactyly in the category ectrodactyly.

To quantify anomalies, Henle, Rimpp and Weiss counted all observed metamorphosed and adult individuals during their surveys in 1980 and 1981 and noted whether they had any easily visible external anomaly. They listed all types of anomalies observed but counted only the most obvious anomalies. Other anomalies were placed in classes of abundance only. To avoid double counting, individuals were kept in containers and released at the end of sampling. Henle and Rimpp also took four samples of > 80 tadpoles and one sample of 50 tadpoles from different parts of the pond. Sampled individuals were classified with the naked eye as normal or abnormal (e.g., whitish, giant, strong edema, strongly bent bodies).

During two surveys conducted in June and August 1981, Christaller (1983) counted the number of toadlets with very obvious external anomalies. In 1982 and 1983, Flindt (1985a) examined all adult and recently metamorphosed individuals that he found in the quarry for externally visible malformations. Furthermore, he sampled tadpoles and examined them for anomalies.

4.2 Frequency of anomalies

From a minimum of 149 adult males and 26 females examined from 1980 to 1983, only a single male collected by Christaller (1983) in 1981 showed an anomaly: an approximately 7–8 mm long supernumerary bone at the lower angle of the jaw. According to Flindt (1985a), it is a duplication of the upper arm. The accompanying photo does not allow a clear distinction between these two interpretations.

At least 245 toadlets exhibited anomalies. Most of them were found in 1980, when the percentage of abnormal individuals was approximately 50% (Tab. 1; see also Henle 1981, 1982, Rimpp 1981). The frequency of anomalies was 19% in 1981 (in individuals raised in the laboratory) and below 10% in the years 1982–1984 (Tab. 1). The figures for 1982–1984 are minimum numbers since one of us (AD) detected anomalies in individuals conserved by Flindt that had been overlooked by him.

The frequency of abnormal tadpoles in the sample of 6.9.1980 ranged from 30% to 55% (Henle 1982) or 35–55% (Henle 1981), if the smallest sample with 50

Table 2: Summary of anomalies observed in tadpoles. *N*: Number; n.a.: not assessed; +++: > 250 individuals; ++: > 100 individuals; +: > 10 individuals; *: based on individuals raised in the laboratory; **: figures in the body of the table may not add to these sums because of multiple anomalies in some individuals and because anomalies were not specified for all individuals; †: average of five samples taken in September. Sources: 1980 data – Henle 1981, 1982, Rimpp 1981, Henle, Vershinin & Dubois unpubl.; 1981 data – Henle unpubl.; 1982–1984 data – Flindt (1985a).

	1980	**1981**	**1982**			**1984***
			Sample 1	Sample 2	Sample 3	
N sampled	2,000–5,000	80	153	59	286	103
N malformed**	1,000–2,500	27	25	1	1	12
Frequency	35–55%†	33.8%	16.3%	1.7%	0.35%	11.7%
Ectrodactyly	+					
Arm remaining within branchial cavity	10					
Mouthparts malformed	+++	n.a.	++			
Curvature of tail	+++	6	5			
Spiraculum mid-ventrally	1					
Giant tadpoles	+++					
Nanism	++					
Partial albinism	++		2			
Novel colours	+					
Edema	+++	27	20	1	1	12
Tumours	1					
Other anomalies: reduced tail fin	1					
Other anomalies: asymmetric bodies	++					
N types of anomalies	13	2	4	1	1	1

individuals is excluded. Given the estimated number of tadpoles, at least 1,000 and up to 2,500 individuals were abnormal (Tab. 2). On two later occasions before the destruction of the pond, approximately 300 and 100, respectively, abnormal tadpoles were captured. In the 1982 field surveys, the percentage of abnormal tadpoles was 16.3% in the first sample falling within two and five weeks to 1.7% and 0.35%, respectively, due to mortality of abnormal individuals (Flindt 1985a). In 2005, no conspicuous anomalies could be detected but tadpoles could not be captured for detailed inspection.

4.3 Types of anomalies observed in the Roßwag population

In total, 23 different types of anomalies were observed in toads (26, if different types of ectromely and of ectrodactyly are counted separately), 13 in tadpoles and 32 (35) in total. In 1980, 20 (23) different types of anomalies were found in recently metamorphosed toads (Tab. 1) and 13 in tadpoles (Tab. 2). These numbers dropped considerably in subsequent years.

4.3.1 Recently metamorphosed and adult individuals

A) Skeletal anomalies of limbs

A1) Polymely (Figs. 3a–c & 4): From 1980 to 1983 at least 50 recently metamorphosed individuals with supernumerary limbs were found. In 1980, approximately 20% of the abnormal individuals showed polymely. Combining data from 1982 and 1983, 1.8% of all toadlets exhibited polymely. Duplications differed considerably among individuals. Flindt (1985a) found five different types of 5-limbed toadlets alone and two types of 6-limbed toadlets. Additionally, an individual with seven limbs was found (Rimpp 1981). Most common in 1980 were complete but stiff supernumerary limbs originating from the shoulder girdle, which may or may not be split at the elbow or the hand (e.g., Fig. 3a; MNHN 1984.2325), movable supernumerary limbs originating from the shoulder girdle but with both limbs covered to the elbow by the same skin sheet (e.g., Fig. 3b; MNHN 1984.2322) and small additional stumps originating from the shoulder region (Fig. 3c).

Two cleared and stained individuals (Fig. 4 and another one shown in Kneissler 1981) and X-rays of MNHN 1984.2325 demonstrated that at least some supernumerary limbs were associated with a duplication of elements of the shoulder girdle. The shoulder girdle of the individual pictured in Figure 4 was grossly malformed and the duplicated bones of the lower arm on the right side were partly fused. Supernumerary limbs were usually smaller in size and thinner than the normal ones (Flindt 1985a), but in a few cases, they were similar in size (e.g., figure in Klein 1981). The incidence of limb duplication was similar for the left side (registered for 10 specimens) and right side (9 specimens) (Flindt 1985a). It is remarkable that all cases of polymely except for one found in 1983 by Flindt (1985a) affected the front legs.

A2) Polydactyly (including schizodactyly) (Flindt 1985a: Fig. 6H): Polydactyly only occurred on supernumerary limbs (not counted as separate anomaly), except for two individuals unilaterally exhibiting six fingers and seven toes, respectively (Flindt 1985a). The cleared-and-stained individual figured by Kneissler (1981: 15) shows schizodactyly of the terminal phalangeal bone of finger three on the supernumerary right arm.

A3) Ectromely (Fig. 5): Individuals without legs (amely; Fig. 5a; see also Kneissler 1981: figure on page 18), lacking a limb from the elbow or knee (hemimely; Fig. 5b; MNHN 1984.2323, MNHN 1984.2326) or lacking a foot or hand (apody) occurred. Over the period 1980–1984 at least 50 toadlets were observed with incomplete

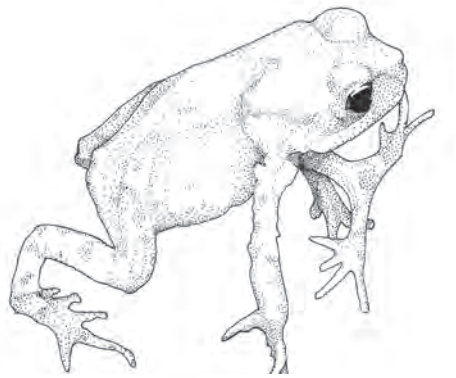

Fig. 3a

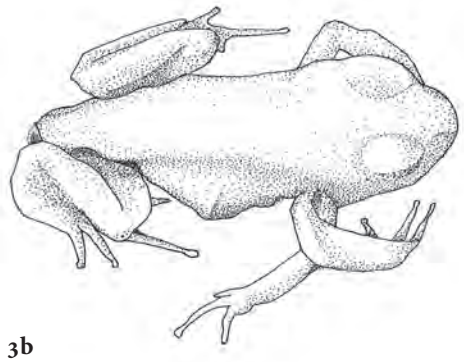

Fig. 3b

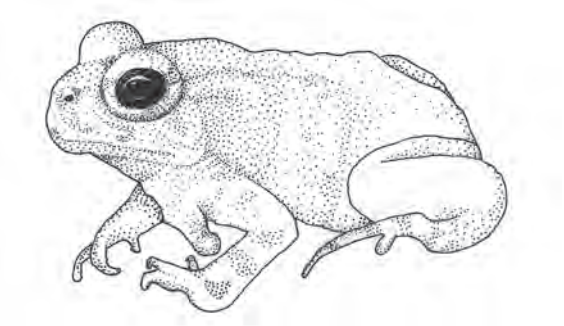

Fig. 3c

Fig. 3: Three common types of polymely observed in recently metamorphosed individuals in September 1980; a) complete stiff supernumerary arm originating from the shoulder girdle and split at the elbow; b) supernumerary arm originating from the shoulder girdle but covered to the elbow by the same skin sheet as the normal leg; and c) bony stump. Drawings from photographs (individuals not preserved) by R. Lange.

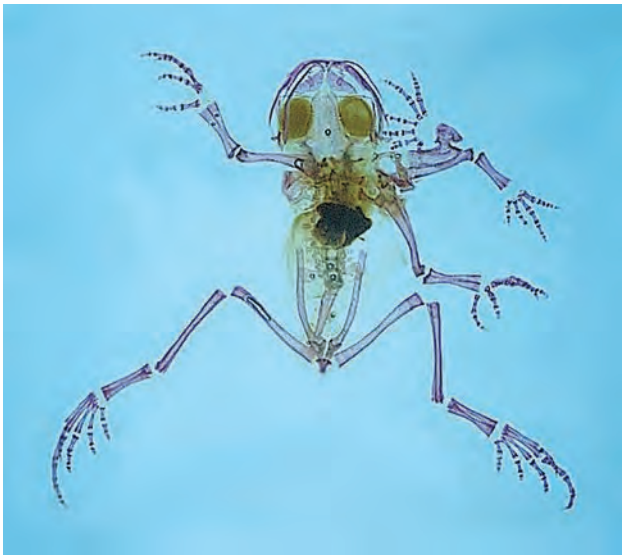

Fig. 4: Cleared and stained specimen of a six-legged frog showing duplications of elements of the right shoulder girdle; specimen collected in September 1980.

Fig. 5a

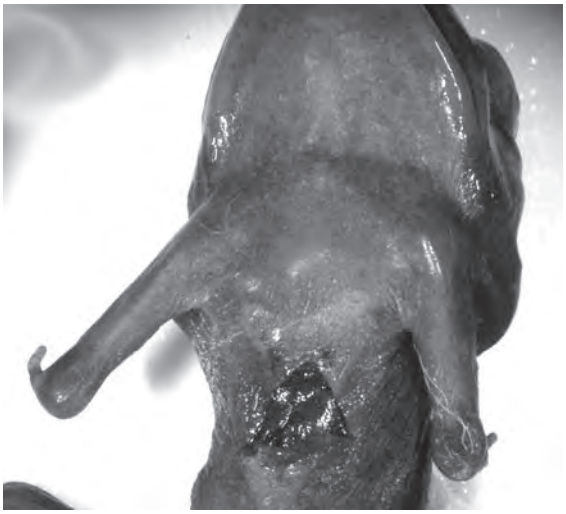

Fig. 5b | Fig. 5: Different types of ectromely observed in September 1980; a) amely (individual not preserved). Photo: K. Henle; b) hemimely (MNHN 1984.2323). Photo: H. Steincke.

hind or front legs. In 1982 and 1983, the frequency was 2.7%, but Flindt (1985a) assumed that such individuals were underrepresented in his samples (Tab. 1) since they had difficulties leaving the water and easily drowned. This is probably also the reason why apody occurred more frequently than hemimely. Both types of anomaly occurred mainly in front legs in 1980, but mainly in hind legs in the following years. In 1983, Flindt (1985a) found eight individuals without a right hind foot and ten individuals without a left hind foot; two individuals lacked both feet. In addition, three individuals were lacking a lower shank. Because Flindt (1985a) assumed that these malformations were due to cannibalism and predation by dragonfly larvae (see discussion), he did not count them as anomalies.

A4) Ectrodactyly (oligodactyly and brachydactyly) (Fig. 6): We did not take note of any individuals with a digit completely lacking (oligodactyly) in our field notes except for the case of incomplete supernumerary legs, but an examination of the voucher specimens revealed one case in an individual with a normal number of legs (MNHN 1984.2325: Fig. 6a). Flindt (1985a) reported such individuals in 1982 and 1983. Individuals with digits reduced in length (brachydactyly) were also not listed in our field notes or by Flindt (1985a), although an examination of voucher specimens revealed two individuals with brachydactyly (MNHN 1984.2319: Fig. 6b; MNHN 1984.2322). In addition, digits on supernumerary limbs were often shortened.

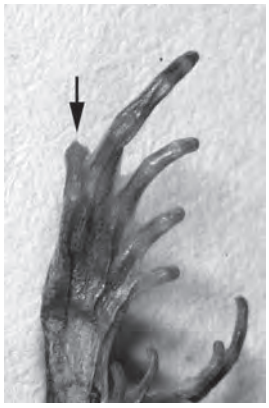

Fig. 6a

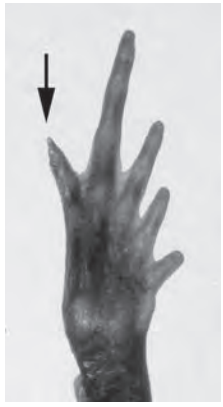

Fig. 6b

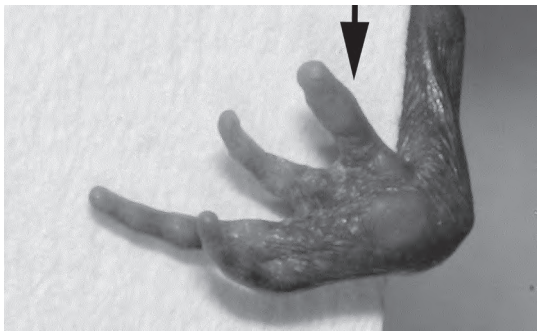

Fig. 6c | Fig. 6: Anomalies of toes and fingers observed in September 1980; a) oligodactyly (MNHN 1984.2325); b) brachydactyly (MNHN 1984.2319); c) swollen first finger (MNHN 1984.21). Photos: H. Steinicke.

A5) Phocomely (Fig. 7): In 1980, we found a few toadlets with one or several fingers or the hand attached directly to the shoulder. Flindt (1985a) observed phocomely in individuals raised in the laboratory from eggs that he had collected in 1984. He assumed that such individuals were not represented in his 1982 and 1983 samples (Tab. 1) because they experienced difficulties leaving the water and easily drowned.

A6) Syndactyly: Dubois (unpubl.) detected cases of syndactyly in recently metamorphosed toads collected by Flindt. This anomaly was overlooked by Flindt (1985a). Henle & Rimpp did not assess this type of anomaly in the field.

A7) Clinodactyly: Dubois (unpubl.) detected cases of clinodactyly in recently metamorphosed toads collected by Flindt. This anomaly was overlooked by Flindt (1985a). Henle & Rimpp did not assess this type of anomaly in the field.

A8) Stiffness and rotation of limbs (see Henle et al. 2017c for terminology) (Fig. 8): Stiffness and rotation of limbs were one of the most frequently observed appendicular anomalies in 1980. Only hind limbs showed rotations, invariably combined with stiffness and vice-versa. Stiffness also occurred in front legs but only when they were supernumerary. Except for one case reported by Flindt (1985a), the stiffness of legs was due to a reduction or an absence of musculature. Rotations were either caused by twisted long bones or an anomalous insertion at the joints.

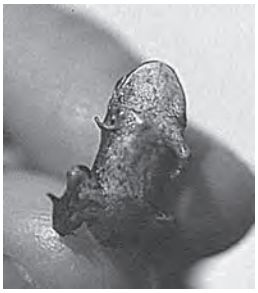

Fig. 7: Phocomely (right arm) and hemimely (left arm) (individual not preserved); September 1980. Photo: K. Rimpp.

Fig. 8: Combination of stiffness and rotation – a specific form of torsion – of both hind legs (individual not preserved); September 1980. Photo: K. Henle.

B) Skeletal anomalies of the body and head

B1) Stiff and shortened vertebral column (Fig. 9): Several individuals exhibited a shortened and stiff vertebral column in 1980, but the number was not quantified. These individuals had a very stout appearance. In most of them, this anomaly was combined with a shortened head.

B2) Brachycephaly (Fig. 9): A shortened head was observed in several individuals in 1980, but their number was not quantified. In most, but not all cases, brachycephaly was combined with a stiff and shortened vertebral column.

B3) Pointed snout (Fig. 10): In 1980, at least ten individuals with an acuminate instead of a broad trapezoidal snout were observed.

B4) Mandibular hypoplasia (Fig. 11): The lower jaw was more or less severely reduced in several individuals and in extreme cases it was completely lacking (agnathia).

B5) Other skeletal anomalies: Christaller (1983) collected one male in 1981 that showed an approximately 7–8 mm long supernumerary bone at the lower right angle of the jaw (figured in Flindt 1985a). According to Flindt (1985a), this bone is a supernumerary limb. The accompanying photograph indicates that this may be the case but does not allow an unequivocal discrimination between both interpretations. In any case, in MNHN 1984.2325, there is a very short bony protuberance present at the angle of the left jaw, an additional protuberance on the right side of the head and the shoulder blades are strongly expanded. In MNHN 1984.2324, the upper jaw is almost quadrangular, but the lower jaw round and thus not completely closing.

Fig. 9: Individual with a stiff and shortened vertebral column, brachycephaly and very few small dark green spots on a grey back (individual not preserved); September 1980. Photo: K. Henle.

Fig. 10: Individual with a strongly pointed snout and a large yellow spot on the back; September 1980; drawing from photograph (individual not preserved) by R. Lange.

C) Colour anomalies

C1) Partial melanism (Fig. 12): In some individuals, the head and body was very dark grey to black dorsally with the exception of small red dots on the warts. In one individual, this colouration extended to the dorsal surface of the hind legs, with arms and feet showing normal colouration and pattern. All partially melanistic individuals exhibited other anomalies as well. Some individuals had blackish but slightly transparent patches on the upper surface of the legs, in the groin or on the back. These individuals were not counted as partial melanistic, but were instead allocated to the category "abnormal pattern".

C2) Appearance of novel colours (Figs. 10 & 13): In 1980, many individuals showed patches of ochre to an orangey yellow colour. The normal colour and pattern of *B. viridis* does not include such colours. The patches were either present on the flanks or on the dorsum and of a variable size. The crossing of one such individual with a normal individual originating from the vicinity of Renningen in Baden-Württemberg, Germany, resulted in 25% ($n = 20$) of the offspring raised beyond metamorphosis exhibiting the same orange yellow patch as their parent. Thus, the anomaly must have had a genetic basis.

C3) Abnormal patterns: Some individuals had transparent blackish patches on the upper surface of their legs, in the groin or on the back. With few exceptions, they were also affected by skeletal anomalies.

D) Edema and tumours

D1) Edema (Figs. 12–14; see also Kneissler 1981: Photo on p.18): In 1980, this was the most common anomaly observed in recently metamorphosed individuals. In mild cases, it was mainly the upper shank that was distended (Figs. 12 & 13). In more severe cases, edema also affected the lower shank and in extreme cases, the whole legs looked like water-filled balloons (Fig. 14).

D2) Tumours (Fig. 15; see also Klein 1981: Photo on p. 24; Kneissler 1981: Photo on p. 18): Tumours were frequent in 1980. They occurred mainly on the head and in a few cases also on the back.

E) Other anomalies

E1) Flindt (1985a) observed one individual with a squashed partly regenerated head in 1982. In the voucher specimens collected in 1981, two additional anomalies were observed: thumb of right hand broadly swollen (MNHN 1984.2321) (Fig. 6c); a phalange of one toe with degenerated musculature (MNHN 1984.2322).

Fig. 11: Mandibular hypoplasia (individual not preserved); September 1980. Photo: K. Rimpp.

Fig. 12: Individual with black dorsal surfaces and edema on the hind legs (individual not preserved); September 1980. Photo: K. Henle.

Fig. 13: Individual with a very large yellow patch on the right flank extending to the back and onto the base of the shank; mild edema on the upper hind leg (individual not preserved); September 1980. Photo: K. Henle.

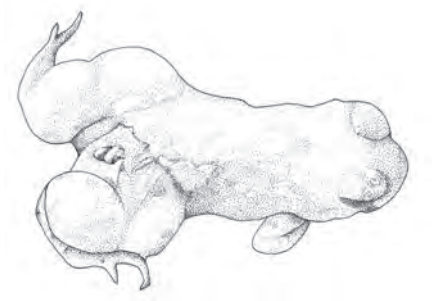

Fig. 14: Severe edema of the hind legs observed in September 1980. Drawing from photograph (individual not preserved) by R. Lange.

Fig. 15: Head tumour observed in September 1980. Drawing from photograph (individual not preserved) by R. Lange.

4.3.2 Tadpoles

A) Morphological anomalies

A1) Ectrodactyly: This anomaly was observed in the field in 1980 in one slightly oversized tadpole (total length approx. 50 mm).

A2) Forelimb enclosed in the opercular fold of skin (Fig. 17): In 1980, more than 10 tadpoles with this type of anomaly were observed in the field. One tadpole transferred to an outdoor tank developed this anomaly, but died before completing metamorphosis.

A3) Mouthparts malformed (Fig. 16): In 1980, many tadpoles of all sizes and colouration showed various irregularities of mouthparts, mainly labial tooth rows interrupted in places where they are normally complete (Rimpp 1981). In a few cases, only a few scattered keratinized denticles were present. In addition, labial papillae were reduced or completely absent in many specimens (Fig. 16a). Furthermore, in a few specimens, one or both beaks lacked keratinization. Flindt (1985a) reported that tadpoles with bent tails and/or (?) edema had unusually broad gaps in the second upper labial tooth row (Fig. 16b).

A4) Curvature of tail (Rimpp 1981: Photo 1): Curvature of tail was observed frequently in 1980. It occurred in giant tadpoles, in whitish tadpoles and in tadpoles that had normal body size and colouration. Some of these individuals had two bents in the tail or strongly asymmetric bodies. Flindt (1985a) also reported individuals with bent tails (Tab. 2).

A5) Mid-ventral spiraculum: One whitish tadpole examined in 1980 had its spiraculum mid-ventrally positioned instead of laterally on the left side as in the majority of Anura (McDiarmid & Altig 1999).

A6) Giant tadpoles (Fig. 17, see also Klein 1981: Photo on page 25; Rimpp 1981: Photo 3): *B. viridis* tadpoles normally reach a maximum total length of 52 mm (Günther & Podloucky 1996). In 1980, many tadpoles had a total length of approximately 40–50 mm. Only those individuals were counted as giant tadpoles that reached a total length of 60 mm and over. More than 100 such individuals were observed in 1980. The largest measured tadpole reached a total length of approximately 85 mm (Henle 1981). None of the giant tadpoles developed forelegs and all ten individuals transferred to outdoor water tanks died without attempting to metamorphose. In 1983, tadpoles reached a total length of only 38 mm (Flindt 1985a).

A7) Stunted growth (Klein 1981: Photo on page 25; Rimpp 1981: Fig. 2): Many tadpoles showed stunted growth with a body length of approximately 5 mm and a total length below 15 mm (Rimpp 1981), a size that tadpoles usually attain soon after hatching. These individuals were blackish – the typical colouration of small tadpoles of that size – and most of them had abnormal mouthparts (various forms of interruptions or lack of one or several labial tooth rows, lack of papillae and lack of keratinized beaks).

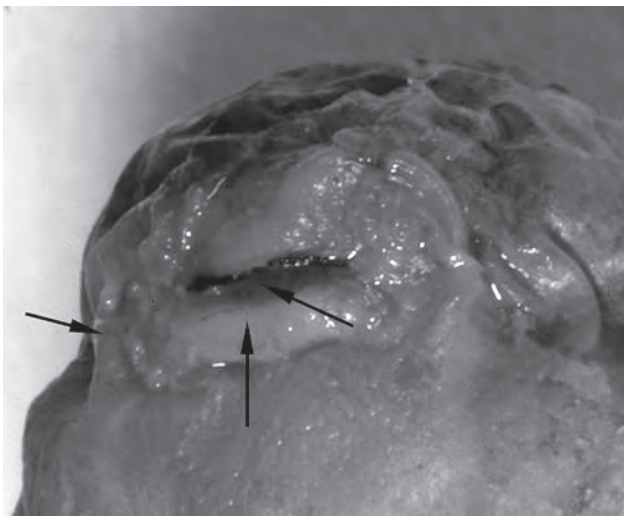

Fig. 16a

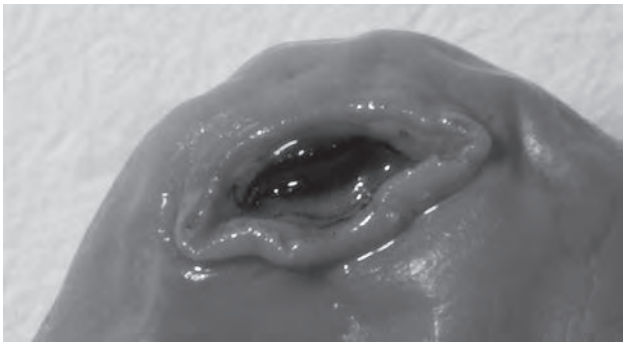

Fig. 16b

Fig. 16: Abnormal mouths of tadpoles collected in September 1980; a) grossly malformed (see arrows), with lower beak lacking keratinization, labial teeth rows with few scattered denticles only and labial papillae reduced and partly absent (MNHN 1984.2317); b) minor deviation: abnormally broad gap of the second upper labial tooth row and reduced keratinization of the teeth (MNHN 1984.2316). Photos: H. Steinicke.

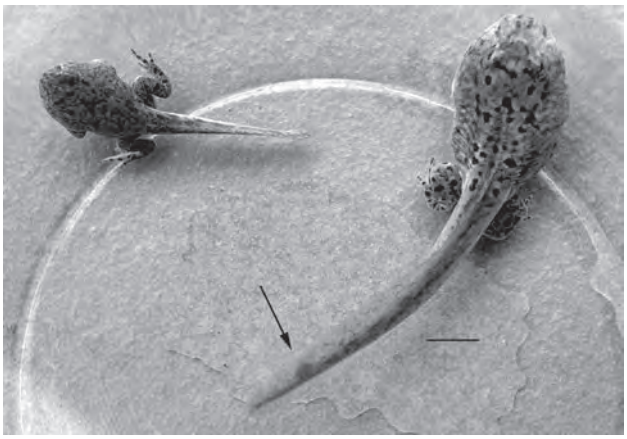

Fig. 17: Giant (> 80 mm total length) and large (approx. 50 mm total length) tadpoles (individuals not preserved), September 1980; the giant tadpole has a small yellow spot on the tail; a skin fold covers the left arm of the large tadpole. Black bar: 1 mm. Photo: K. Henle.

B) Colour anomalies

B1) Whitish tadpoles (Fig. 18): More than 100 whitish tadpoles were observed in 1980 (Henle 1981, 1982, Rimpp 1981) but only two conspicuously light-coloured tadpoles in 1982 (Flindt 1985a). In most individuals, the background colour was dirty white, but in some individuals it had a more or less pronounced brownish tinge (Rimpp 1981: photo 1). In whitish tadpoles, eyes were normally coloured and fine light grey dots and reticulation were present (Fig. 18). In some individuals, parts of the body were translucent. All individuals examined in 1980 were slightly oversized (total length 45–55 mm). Several whitish individuals were affected by slight to strong torsions of the tail and asymmetric bodies. In one individual, the spiraculum was positioned mid-ventrally.

B2) Appearance of novel colours (Fig. 17): In 1980, several tadpoles including giant ones, showed small to large patches of ochre to an orangey-yellow colour. The normal pattern of *B. viridis* tadpoles does not include such colouration. The colouration appeared on the body, the tail fin and the musculature of the tail.

Fig. 18: Large whitish tadpole (approx. 50 mm total length) (individual not preserved); September 1980. Photo: K. Henle.

C) Edema and tumours

C1) Edema (Flindt 1985a: Fig. 4): Edema was very common in 1980 and 1981. Its expression was highly variable. In most individuals, only a small part of the body was affected and individuals were still able to swim under water but some individuals had a balloon-like appearance and floated on the surface of the water. Flindt (1985a) observed edematous tadpoles in the field in 1982 and in tadpoles reared in the laboratory from a clutch in 1984.

C2) Tumours (Fig. 19): A photograph of a giant tadpole taken in September 1980 shows a tumour-like knot in the distal part of the caudal musculature. This anomaly was only noticed while the photograph for the preparation of this publication were being closely examined.

Fig. 19: Tumour-like knot in the tail tip of a giant tadpole (individual not preserved); September 1980. Photo: K. Rimpp.

D) Other anomalies

D1) Other anomalies: In 1980, some tadpoles exhibited strongly asymmetric bodies. Whereas extreme cases were clearly abnormal, it is difficult to establish at which degree of asymmetry it should be classed as an anomaly. Therefore, we did not include asymmetric individuals in the tally of the number of abnormal individuals.

4.4 Individual variability in anomalies

Many individuals were affected by several types of anomalies (up to 4). The average number of different types of anomalies per abnormal individual in the specimens preserved at MNHN is 1.8 (n = 12).

In 1980, few abnormal individuals resembled each other even if they showed the same type of anomaly. This is well exemplified by the following descriptions of voucher specimens:

MNHN 1984.2318 (toadlet): Dorsum completely black without any pattern; this is no longer visible in the preserved individual (examined June 2008).

MNHN 1984.2319 (toadlet): 4th finger of left hand and 5th toe of right foot without terminal phalange (brachydactyly) (Fig. 6b); strong edema with expanded skin on the upper shanks of both hind legs still obvious after preservation (examined June 2008).

MNHN 1984.2320 (toadlet): Edema at the hind leg shanks; in the preservative only visible as wrinkled skin (examined June 2008).

MNHN 1984.2321 (toadlet): 1st finger (thumb) of right hand broadly swollen (Fig. 6c).

MNHN 1984.2322 (toadlet): Supernumerary right arm, both arms covered to the elbow by a single skin sheet, supernumerary arm twisted at the elbow, both hands with four fingers but on one hand a very short thumb (brachydactyly); a phalange of one toe with degenerated musculature.

MNHN 1984.2323 (toadlet): Hemimely on both arms, with a finger-like protuberance at the elbow (Fig. 5b); the individual represents one of the few cases of symmetrical anomaly.

MNHN 1984.2324 (toadlet): Snout slightly malformed (upper jaw almost quadrangular, lower jaw rounded and not completely closing).

MNHN 1984.2325 (toadlet): Supernumerary left and right arms inserted at the shoulder; shoulder blades strongly expanded and probably duplicated; one arm on both sides normal; both supernumerary arms stiff; left supernumerary arm formed by a single long bone with a strongly broadened asymmetrical hand attached to it; hand with seven fingers, five of them brachydactylous; right supernumerary arm split at the elbow (probably two humeri present but fused); both elbows forming a 90° angle with the upper arm; one hand almost normal, the other one strongly reduced but with four fingers, only one of them normal; the remaining fingers partly or completely lacking terminal phalanges (brachydactyly); musculature of the penultimate phalange of one finger absent; outer toe of the left foot (almost) completely

absent (oligodactyly) (Fig. 6a); a bony protuberance present at the angle of the left jaw; an additional protuberance on the right side of the head.

MNHN 1984.2326 (toadlet): Left arm missing from elbow onwards (hemimely), with a tiny finger-like protuberance.

MNHN 1984.2316 (tadpole, Gosner stage 35): TL approx. 45 mm; SVL 18 mm; whitish (light brown in the preservative); eyes completely covered by skin and slightly reduced; the right posterior part of the body with strong edema (only clearly visible in radiographs); mouthparts slightly abnormal: only a few scattered keratinized denticles present on the upper lip, in two rows, the second broadly interrupted medially (gap covering approximately 50% of the row: Fig. 16b).

MNHN 1984.2317 (tadpole, Gosner stage 38): TL approx. 52 mm; SVL 20 mm; upper shanks slightly swollen (edema); mouthparts grossly abnormal (Fig. 16a): only upper beak keratinized (with serrate denticles), lower beak completely lacking keratinization; no tooth row on the upper lip; only a few keratinized denticles scattered across two tooth rows on the lower beak, the first with a narrow median gap, the second complete and almost as broad as the keratinized upper beak; only a few labial papillae showing at the lateral margin of the beak.

MNHN 1984.2331 (tadpole, Gosner stage 25): TL approx. 18 mm; SVL approx. 6 mm, retarded growth; upper and lower beak well keratinized (normal); no tooth row visible but preservation condition is poor and it is not certain that tooth rows were really absent (abnormal).

5 Investigations into potential causes

In short, the investigations undertaken can be summarized as follows. Tadpoles and clutches were transferred to the laboratory to study the further development of abnormal individuals and to find out whether anomalies also developed under laboratory conditions. Surveys on potential predators that could inflict injuries were undertaken in the Roßwag quarry. In the vicinity of the quarry amphibian surveys were carried out to assess whether anomalies occurred in other populations and to search for species that could have possibly hybridized with *B. viridis* from the Roßwag population. Additionally, *B. viridis* sampled at the Roßwag quarry were morphologically and genetically screened for markers characteristic of other toad species. Water samples were taken for chemical analyses and collected toads were analysed for residuals of pesticides. Finally, various radioactivity measurements were taken. Most measurements were severely constrained due to the great controversy about potential causes and the major alterations to the breeding habitat in the Roßwag quarry that followed soon after the discovery of the toads (see Henle et al. 2017b, for relevant background information).

5.1 Amphibian surveys in the wider vicinity of the Roßwag quarry

Amphibian surveys were conducted in the vicinity of the Roßwag quarry to assess whether anomalies were present in other populations and whether other toad species that might hybridize with *B. viridis* were present in the vicinity of Roßwag. Surveys were carried out in the area between the village of Roßwag, the country road between Roßwag and Vaihingen and the major road B10, i.e. approximately 1–1.5 km around the quarry, by Rimpp and Henle in September 1980 and by Henle in May 1981. Flindt (1985a) surveyed an area that extended approximately 8 km to the north and 1.5 km to the south of the quarry in 1982 and 1983. In addition, since 1978, amphibian surveys had been carried out in the administrative district to which Roßwag belongs and in surrounding areas (Christaller 1983).

Within a ca. 6 km radius around the Roßwag quarry, breeding populations of the following amphibians were found: fire salamander (*Salamandra salamandra*), Alpine newt (*Ichthyosaura alpestris*), crested newt (*Triturus cristatus*), midwife toad (*Alytes obstetricans*), yellow-bellied toad (*Bombina variegata*), common toad (*Bufo bufo*), green toad (*B. viridis*), water frogs (*Pelophylax* synkl. *esculentus*), agile frog (*Rana dalmatina*) and grass frog (*R. temporaria*) (Christaller 1983, Flindt 1985a). The only other population of *B. viridis* was found in a quarry (Steinbruch Sämann) approx. 1–1.5 km NNW of the Roßwag quarry. In the early 1970's, the population supposedly comprised several hundred individuals but in 1979, only 30 calling males could be heard (Christaller 1983). In 1982, approximately 25 adults were still present (Flindt 1985a). In 2005, a visual inspection of the quarry from the distance revealed that it still contained a large pond of approx. 75 m × 75 m, two medium-sized ponds and a very small pond, all of which were suitable for *B. viridis* to breed. No amphibian surveys were conducted during this brief visit. This was the only other population in which anomalies were observed: three clutches of white eggs and edema in 13% (*n* not given) of the free-living tadpoles of the same species. Two of these clutches were found in 1982 but were destroyed when the water was deliberately pumped out of the pond (Christaller 1983). For the third clutch and the edematous tadpoles the year of observation was not provided (Flindt 1985b).

5.2 Laboratory studies of anomalies

Eggs, tadpoles and recently metamorphosed individuals were transferred to the laboratory to study the development of anomalies and to assess whether they would develop irrespective of the quarry environment.

On 6.9.1980, Henle transferred eight small blackish tadpoles that otherwise appeared to be normal, ten large (approx. 50 mm TL) whitish, ten giant and three average-sized normal tadpoles to an outdoor elliptical water tank 2 m × 1 m in diameter. Commercial aquarium sand

was used as a substrate and the tank was filled with tap water to a depth of 0.4 m. Half of the water was changed every week. None of the abnormal tadpoles survived, but two of the normal tadpoles metamorphosed without developing any anomaly. The third individual died during metamorphosis. Its right forearm did not emerge from the opercular chamber. All small black tadpoles developed strong edema and died within a few days. The whitish and the giant tadpoles did not develop any signs of front legs and in some whitish individuals hind legs were also completely absent. All whitish and giant tadpoles died before the onset of metamorphosis.

On 7.5.1981, Henle collected part of an egg string that was approximately 20 cm long from a pair spawning at the small water-filled depression on the border of the quarry and transferred it to an outdoor tank. Eggs hatched within 14 days but hatching embryos soon died. Several of them showed torsions of the body, anencephaly, microcephaly, gross distortions and edema.

On 30.5.1981, Henle collected 80 tadpoles with a normal appearance and raised them under the same conditions as in 1980. Of these, 34% developed edema (Tab. 2) and died. In all affected tadpoles, edema rapidly increased in severity until, in the final stage, tadpoles had an inflated balloon-like appearance (as in Fig. 4 of Flindt 1985a). These individuals were unable to dive and soon died. In six of these tadpoles, the tail was bent. Of the 21 individuals that metamorphosed successfully, one showed hemimely of one arm, one had stiff hind legs, one showed ectrodactyly and one had mild edema of the hind legs (Tab. 1).

In 1982, Flindt (1985a) transferred 45 tadpoles to the laboratory, of which 20 showed edema. Of the latter, the last two died during metamorphosis whereas all but one of the 25 tadpoles that appeared normal metamorphosed successfully. In the field, the mortality of edematous tadpoles also appeared to be very high as reflected by a seasonal decline in their frequency (Tab. 2).

In 1984, Flindt (1985a) transferred part of a recently deposited egg string containing approximately 120 eggs to the laboratory, of which 103 individuals hatched. Of these, 11.7% showed severe edema (Tab. 2) and 87 survived to metamorphosis, of which eight developed anomalies (Tab. 1).

Flindt (1985b) also transferred approximately 120 eggs to the laboratory from a white egg string found in the Sämann quarry. Approximately 20% of the embryos developed gross distortions of the body and died. Survivors started to develop pigmentation two days after hatching and although they appeared normal one week after hatching, edema developed in approximately 40% of them. Some tadpoles showed abnormal screw-like swimming behaviour. Ten individuals metamorphosed successfully and were reported not to show any obvious anomaly.

Flindt (1985a) and Rimpp (unpubl.) attempted to raise metamorphosed toads exhibiting polymely, ectromely, lacking the lower jaw or showing large yellow patches on the flanks. Most individuals were severely handicapped, had difficulties or were unable to catch prey and showed uncoordinated movements. As a consequence, most individuals died soon after metamorphosis (Flindt 1985a, Henle 1982) and only a single individual with a large orange yellow patch on the flank could be raised to maturity (Rimpp unpubl.). This individual was crossed with a specimen collected in the vicinity of Renningen, Baden-Württemberg, Germany. Of the 20 offspring 25% showed the same anomaly as their parent.

In summary, the laboratory studies show that several types of anomalies also developed under laboratory conditions, indicating that they might have been of genetic origin. By crossing, a genetic origin could be confirmed for the yellow patch of one individual.

5.3 Hybridization

Flindt (1985a) analysed the serum albumin variation of nine adult *B. viridis* that he had collected in the Roßwag quarry in 1982–1983 to test the hypothesis that the anomalies were caused by hybridization. Additionally, he assessed species-specific patterns and morphological characteristics of – presumably 60 – adults.

The serum albumin bands of all nine individuals were diagnosed as *B. viridis* (Flindt 1985a). Likewise, pattern, colouration and the index "body length/hind leg length" of all individuals were typical for this species. In the survey years no other species of toads was found in the quarry (Henle 1981, 1982, Rimpp 1981, Christaller 1983, Flindt 1985a) and in 1980 none of the malformed individuals showed patterns typical for *Bufo bufo* or *Epidalea calamita* (Rimpp 1981). Only one adult examined by Flindt (1985a) and MNHN 1984.2321, a recently metamorphosed individual with a swollen finger, showed a divided instead of a single subarticular tubercle, as is the case in *E. calamita*. However, this characteristic occurs in about 5% of individuals in *B. viridis* populations (Flindt & Hemmer 1969).

The closest breeding population of *B. bufo* was located at approximately 1–1.5 km SSE of the quarry in cut-off oxbows of the River Enz (Flindt 1985a), with two country roads and the river Enz between the populations (Fig. 20). The next closest population was located NNW of Illingen at a distance of approximately 2.5–3 km from the Roßwag quarry (Christaller 1983), with a village and a major road separating the population from Roßwag. Before 1980, the occasional calling of the natterjack toad (*E. calamita*) was reported at the Roßweiher near Maulbronn at a distance of approximately 10 km from the Roßwag population and isolated from it by two major roads and several country roads (Christaller 1983). The nearest extant population known was very small and located close to Heimsheim (Betzenbuckel), approximately 15 km SSW of Roßwag. The nearest existing large *E. calamita* population known was located approx. 18 km away near Bretten.

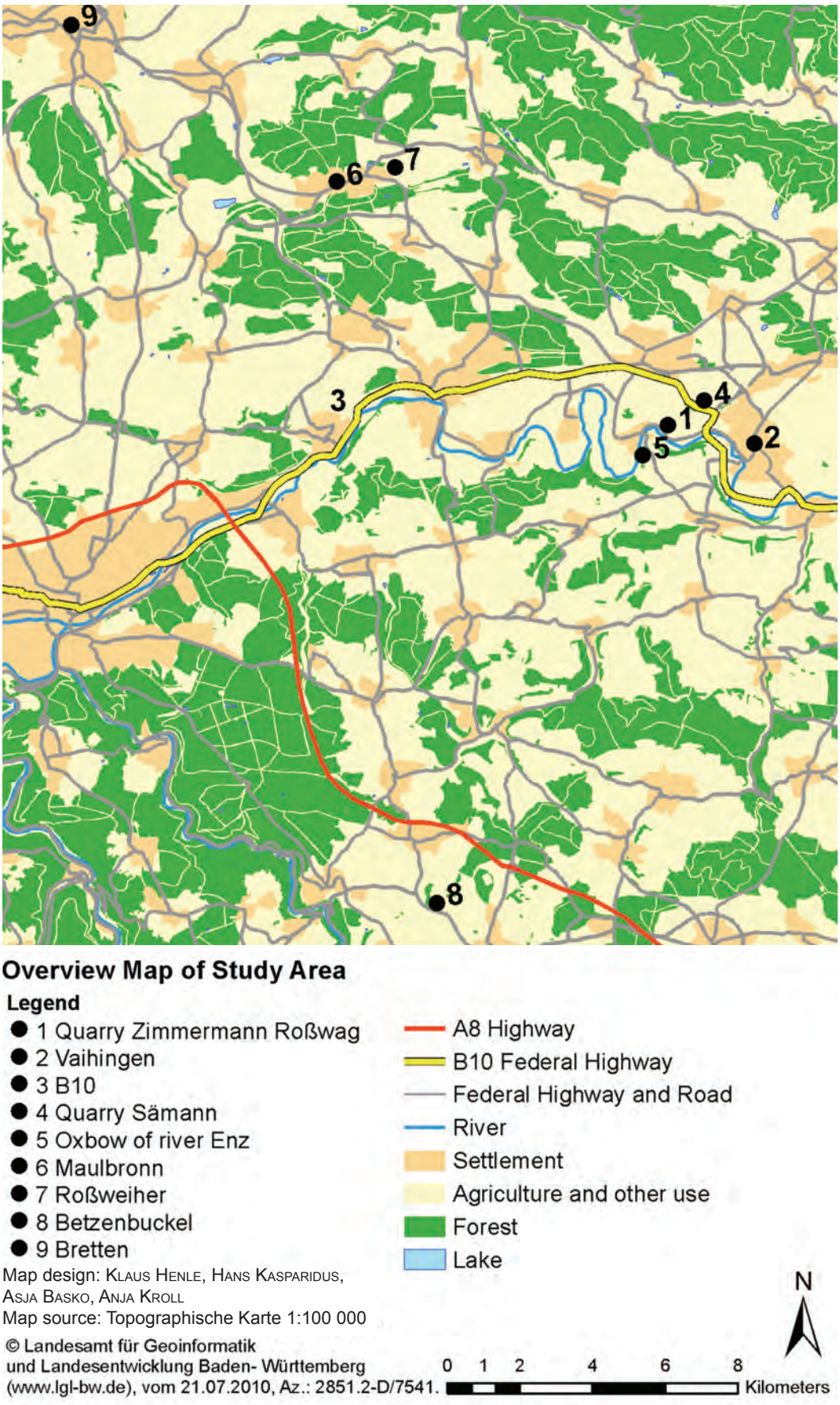

Fig. 20: Locations of landscape barriers and breeding populations of toads in the Roßwag region.

5.4 Potential predators

In 1980, Henle & Rimpp surveyed the shoreline of the pond visually and with dip-nets for dragonfly larvae, dytiscid beetles, leeches and other potential predators. The clear water of the pond enabled good visibility to the bottom of the pond even at its deepest part. The pond was not connected with any other water bodies and free of fish. Neither dragonfly larvae nor dytiscid beetles, leeches or any other potential predator species could be observed in any of the surveys.

In 1983, Flindt (1985a) observed many dragonfly larvae in a pond on the border of the quarry but did not report any predators for the remaining four water bodies. He implied that the dragonfly larvae had bitten off the hind legs of tadpoles. In addition, he explicitly stated that in the same pond he had observed cannibalistic attacks directed at the tails and hind legs of other tadpoles.

5.5 Chemical analyses

Rimpp collected water samples from the main pond on 8.9.1980 and sent them to the chemical laboratory of a private company. Three additional water samples were taken on 10.9.1980 by the Police Department of the City of Pforzheim and analysed by the Chemical Laboratory of the City of Pforzheim as well as by the Landesamt für Umweltschutz, Karlsruhe (LfU; State Institute for Environmental Protection, Baden-Württemberg). These samples were screened for metals, nitrate, nitrite, ammonium, phosphate and calcium. Gas chromatography was carried out by the Chemical Laboratory of Pforzheim to assess the presence of pesticides. Teratogenicity or mutagenic tests (see e.g. Birge et al. 1983, Lowcock et al. 1997) were not carried out by any of the laboratories. Abnormal toads collected in 1980 by Rimpp were also analysed for residues of chlorinated hydrocarbons by the Institute of Animal Hygiene at the University of Freiburg.

In the vineyards surrounding the quarry, the following pesticides were sprayed using helicopters: pomuran (i.e., captan + mancozeb), rovral [i.e., iprodion = 3-(3,5-dichlorophenyl)-N-(1-methylaethyl)-2,4-dioxo-1-imidazolidincarboxamid], copper sulphate and sulphur (Buck pers. comm.). In the early 1980s these pesticides were commonly applied to vineyards.

The analysis of the water samples collected by Rimpp did not provide any suspicious results but no concrete data are available. Gas chromatography by the Chemical Laboratory of the City of Pforzheim did not detect any traces of pesticides (neither the methods nor the minimum detection levels were given). Copper was below the detection level and no excessive concentrations of any other metals were found although sulphate concentration was very high (Tables 3 & 4). No residues of chlorinated hydrocarbons could be detected in the abnormal toads analysed by the Institute of Animal Hygiene at the University of Freiburg (Kneissler 1981, Henle 1982, Schnorbach 1982).

Table 3: Results of the chemical analyses of water samples from the Roßwag pond collected by the Police Department Pforzheim at 15:00 on 10.9.1980. Bdl: below detection level (detection level not given). Source: unpublished protocols of the Chemical Laboratory of the City of Pforzheim, dated 7.10.1980.

Chemicals	**Concentration (in mg/l)**		
	Sample 1	Sample 2	Sample 3
$KMnO_4$ consumption	8.7	6.0	6.7
Chloride	43.4	41.5	43.4
Nitrate	19.70	23.66	23.82
Nitrite	0.12	bdl	bdl
Ammonium	0.32	bdl	bdl
Phosphate	0.22	0.05	0.11
Iron	23.0	72.5	87.0
Manganese	0.8	1.35	1.6
CaO	250.5	245.0	240.0
MgO	135.5	123.7	135.9
Copper	bdl	0.1	0.1
Cadmium	bdl	bdl	bdl
Chromium	bdl	bdl	bdl
Nickel	bdl	bdl	bdl
Zinc	0.2	0.6	0.5
Plumbum	0.05	0.15	0.1
Mercury	bdl	bdl	bdl
Silver	bdl	bdl	bdl
Tin	bdl	0.4	0.85

Table 4: Results of the chemical analyses of water samples from the Roßwag pond that were received by the Institute of Water and Waste Management, State Agency for Environmental Protection, Karlsruhe (LfU) on 11.9.1980. Bdl: below detection level (detection level not given). Source: unpublished protocols, dated 9.10.1980.

Chemicals	**Concentration (in mg/l)**
pH	7.5
Ammonium	< 0.1
Calcium	90
Fe (total)	approx. 0.1
K	10
Mg	80
Na	25
Cyanide (total)	bdl
Chloride	43
Sulphate	approx. 400

5.6 Radioactivity studies

5.6.1 Assessment methods

The first assessment was made by Henle, in the presence of Rimpp, on 8.9.1980 using a Geiger counter sensitive to α and β radiation (type Minicont, trade mark of Herfurth). Measurements took place at the shore of the pond and across the basis of the earthen deposit within the quarry as well as at control sites outside of the quarry. All measurements were taken approximately 1 m above the ground. Large cracks at the base of the earthen deposit were selected as additional sampling points since they provided potential hiding places for toads. At these locations, measurements were taken at various distances above the opening of the cracks (Tab. 5a). Before and after the measurements, batteries were checked and showed sufficient charge. Because unexpected high levels of radioactivity were indicated, the instrument used was tested for potential artefacts and malfunctioning after terminating the assessments by taking measurements while holding the instrument vertical, upside down, at different angles, by shaking it heavily, by placing it upon the ground and upon stones collected from the earthen deposit and by rapidly approaching the surface of the earthen deposit at sites without cracks. Each test was repeated at least twice.

Upon a request made by Rimpp to repeat the measurements (Henle et al. 2017b), the Institute for Radiation Assessment at LfU measured the level of radioactivity in the quarry around mid-day of 10.9.1980. Rimpp but not Henle was informed and asked to attend immediately. The field protocol of LfU does not contain any information about the concrete locations or any other details regarding the measurements taken (Tab. 5c). A stone from the quarry wall, one water sample from the pond and one sample of rubble, presumably from the earthen deposit, were collected and spectrally analysed in the laboratory. No sediment samples were taken, which is unfortunate since some radionuclides are readily filtered out of the solution (Olympic Dam Operations 1990) and the tadpoles of *B. viridis* mainly inhabit the bottom of the pond.

On the evening of 10.9.1980, Henle once again measured the level of radioactivity at the shore of the pond, across the base of the earthen deposit and at three distances above the opening of cracks in the earthen deposit using a Xenon Geiger counter sensitive to α and γ radiation (Tab. 5b). The same instrument was used for additional measurements by Schreiber, Professor of Physics at the University of Stuttgart-Hohenheim, in the presence of Henle on 13.9.1980. They were stopped by the intervention of the quarry manager so that only very preliminary measurements at the shore of the pond and above the earthen deposit could be made and no field notes were taken.

The Institute for Energy and Environmental Research (Institut für Energie- und Umweltfragen – IFEU) in Heidelberg compared the level of radioactivity at three sites of the earthen deposit with five control points within and outside the quarry on 28.9., 9.11. and 7.12.1980. All measurements were taken just above the surface of the earthen deposit. It is important to note that, after the measurements taken by Henle, the earthen deposit had been covered by 3–10 m of fill. Large cracks with openings to the surface were no longer present. Due to a continual covering of the earthen deposit, measurements were abandoned thereafter (Franke et al. 1981).

5.6.2 Assessment results

Results of the field measurements are summarized in Table 5. All measurements that compared the level of radioactivity between control sites outside the quarry and/or the vicinity of the pond with the earthen deposit in September 1980 showed approximately 2–3 times higher levels at the earthen deposit, a difference that is within the expected normal range of variation (LfU in lit., Landtag von Baden-Württemberg 1981, Landesregierung von Baden-Württemberg 1984). In November and December 1980, the relative intensity at the earthen deposit compared to the control sites was lower (Tab. 5d).

Measurements close to the openings of cracks were made only by Henle in September 1980. These measurements showed an exponential increase of radiation intensity when approaching the opening of the cracks. They were 1–2 orders of magnitude higher directly at the opening of the cracks compared to 1 m above them (Tab. 5a,b). Tests carried out before and immediately after the measurements showed that the batteries were sufficiently charged. All tests for artefacts made for the Herfurth Minicont instrument were unable to elicit any elevated values. When returned to the laboratory none of the instruments showed any damage or malfunctioning.

The laboratory results of the three samples taken by LfU did not reveal any traces of artificial radionuclides (internal protocol by Funsch LfU, Landtag von Baden-Württemberg 1981, Landesregierung von Baden-Württemberg 1984) but showed considerably higher levels of natural radionuclides in the sample of the earthen deposit compared to stones from the quarry (Tab. 6).

Table 5: Summary of field measurements of radioactivity in the Roßwag quarry

a) Henle, 8.9.1980: Herfurth Mincont (α and β sensitive). Note, the tests for the correct functioning of the instrument are not included.

Location	**Measurements**	**Intensity: min – max (Impulses per second)**
Car park	once at 17:00 h	5–7
Entrance to the quarry	10 times, starting 17:15 h	5–7
Along the pond	repeatedly while moving along the shore; during the period 17:30-18:30	5–7
Across the basis of the earthen deposit	continuously while moving across the deposit; 30 min	12–15
Close above 2 cracks at the left side of the earthen deposit	3 times, ≥ 2 min each	100
Close above 3 cracks at the right side of the earthen deposit	2 times, ≥ 2 min each	500–1000
At the surface of the lowest of the 3 cracks	once, 2 min	1000–5000
5 cm above the lowest crack	2 times	100
25 cm above the lowest crack	2 times	10–15
At the surface 1 m away from the lowest crack	once	10–15
Opposite side of the pond	5 times	5–7
1 m above the earthen deposit	2 times	10–15
20 min later at the surface of the largest crack	2 times	1500-2000; values decreased exponentially with distance from crack opening
Car park	once	5–7

b) Henle, 10.9.1980: Xenon Geiger counter (α and γ sensitive)

Location	**Measurements**	**Intensity: min – max (Impulses per second)**
At the pond	5 times, 2 min each	300–500
At the basis of the earthen deposit (3 locations)	2 times, 2 min each	700–1000
30 cm above the same crack as on 8.9.1980	2 times, 2 min each	1000
20 cm above the same crack as on 8.9.1980	2 times, 2 min each	1200
10 cm above the same crack as on 8.9.1980	2 times, 2 min each	1500–1800
Opposite side of the pond	2 times, 2 min each	300–500

c) LfU, 13.9.1980; S: scintillation counter; C: contamination counter. Source: unpublished protocol of the LfU, dated 11.9.1980

Location	**Measurements**	**Intensity: min – max (Impulses per second)**
Within the quarry	Not recorded	S: 70–150; C: 15–25
Location unspecified: "Normal level (plain)"	Not recorded	S: 50; C: 10

d) IFEU, various instruments and dates. Relative sensitivity: Earthen deposit / control site; Source: Franke et al. (1981)

	Intensity (Impulses per second)		
Date	**Control sites**	**Earthen deposit**	**Relative intensity**
28.9.1980	0.25±0.02	0.47±0.021	1.88±0.19
9.11.1980	0.49±0.02	0.72±0.02	1.47±0.10
7.12.1980	0.44±0.02	0.61±0.03	1.39±0.11

Table 6: Summary protocol of the laboratory measurements made for radioactivity of samples taken in the Roßwag quarry by the LfU. Source: unpublished protocol of the LfU, dated 11.9.1980.

Sample	Elements	Activity
Stone of the quarry wall	Ra-226	≈ 0.65 pCi/g
	AC-228	≈ 0.12 pCi/g
	K-40	≈ 2 pCi/g
Earthen deposit	Ra-226	≈ 1.5 pCi/g
	AC-228	≈ 1.8 pCi/g
	K-40	≈ 38 pCi/g
Pond water	Ra-226	< 0.01 pCi/ml
	Ac-228	< 0.01 pCi/ml
Pond water (residues)	total α	≈ 0.03 pCi/ml
	Rest β	≈ 0.06 pCi/ml
	K-40	≈ 0.008 pCi/ml

5.6.3 Experimental studies on the effects of radioactivity on amphibians

The Federal Ministry of the Interior commissioned a study on the effects of radioactivity on amphibian development (Henle et al. 2017b) carried out by the Zoological Institute of the University of Bonn. The common toad (*B. bufo*) was used as a test species. Three unpublished Masters theses resulting from this study are known to us. Schneider (1984) analysed the effects on the nervous system, Trux (1985) studied the histopathology of the developing visual system and Oerter (1985) assessed general developmental anomalies in tadpoles. Since the latter that is particularly relevant as a comparative basis for the field observations in Roßwag is unpublished and in German, we provide a brief summary of the study.

Embryos at tailbud stage III [after Cambar & Gipouloux (1956); equivalent to Gosner (1960) stage 19] (5.5–8 days old) received 320, 640 or 800 rad Co-60 γ irradiation (distance of irradiation source: 50 cm). The number of embryos used ranged from 200 to 1400 depending on the treatment. At each stage usually 10 individuals were conserved for analyses of anomalies. The study terminated with the completion of metamorphosis but the results were only presented up to stage IV_{13} (Gosner stage 40).

Growth retardation (total body length) of 2.6, 4 and 5.7 mm was observed for the 320, 640 and 800 rad treatments, respectively. Mortality increased strongly in the 640 rad treatment group. Histological examinations of individuals in stage III_8–IV_1 (Gosner stages 23–26) showed that all structures were disorganised or destructed. The brains showed incomplete cellular arrangements as well as disorganisation and necrosis of cell nuclei due to chromatin coagulation. The pronephros was degenerated and the epidermis exhibited necrosis, a condensation of pigment granula and abnormally distributed cells. The histological anomalies were less pronounced in later stages. A summary of the external anomalies recorded is provided in Table 7. They included among others brachymely, ectromely, clinodactyly, syndactyly, polydactyly, polymely, torsion of legs, malformed mouthparts and depigmentation.

Table 7: Anomalies of common toad (*Bufo bufo*) embryos and tadpoles after Co-60 γ-irradiation of stage III5; stages after Cambar & Gipouloux (1956) and, in parenthesis, after Gosner (1960); *n*: sample size; Source: Oerter (1985: Tab. 5–7) and text; note minor differences exist between her text and her tables.

Stage	Anomaly	Exposure (rad)	Frequency (*n*)
III_8	–	320	0% (10)
(23)	Gills deformed and underdeveloped; proliferation of epidermis	640 800	11% (9) 30% (10)
IV_1	–	320	0% (10)
(26)	Mouth closed, denticles missing on the denticle bearing labial ridges	640 800	50% (10) 100% (10)
	Hind leg buds retarded or missing	640 800	50% (10) 100% (10)
	Edema	640 800	50% (10) 50% (10)
IV_{12} (39)	Labial papillae abnormal, rows of denticles abnormal, broken or missing, mouth closed, horny beak partially missing	320 640 800	10% (10) 78% (9) 67% (15)
	Massive edema	640 800	89% (9) 33% (15)
	Depigmentation and transparency	640 800	78% (9) 33% (15)
	Brachymely, ectromely, clinodactyly, syndactyly, polydactyly, torsion of legs	320 640 800	10% (10) 67% (9) 20% (15)
	Dorsal torsions, hyperplasm of skin		not given
IV_{13}	Labial papillae abnormal, rows of denticles abnormal, broken or missing, mouth closed, horny beak partially missing	640	84% (25)
(40)	Edema	640	100% (25)
	Depigmentation and transparency	640	36% (25)
	Torsion of legs; polydactyly, polymely (tarsus), femur absent and lower leg attached to body (phocomely)	640	12% (25)

6 Discussion

The observations in the Roßwag quarry can be summarized as follows. A large number, high frequency and broad range of anomalies were found in *B. viridis,* all declining from 1980 to 1984. Anomalies could not be detected in any other amphibian population in the region except for three white clutches and edema in some tadpoles of the same species in a neighbouring quarry. Breeding showed that at least one type of the observed anomalies (yellow patches) was inherited. The appearance of various anomalies in tadpoles transferred to the laboratory or hatched from transferred clutches indicates that other anomalies are also of a genetic origin, although a teratogenic factor present at the time of egg deposition but not showing any effects until later on in development cannot be excluded with certainty.

With respect to potential causes for amphibian anomalies, all tests for hybridization failed and no other toad species was present in the same quarry. Potential predators were only found in one small water body and only in one year. Pesticides were sprayed by helicopter on vineyards bordering the quarry but no unusual chemical contamination of pond water or animals could be observed. High levels of radioactivity were found although disputed (see below).

Numerous hypotheses about the causes of the anomalies have been put forward, including "normal occurrence of a natural phenomenon" (Tab. 8). Some of these hypotheses would not normally appear in a scientific paper but because of the highly controversial debate about potential causes (Henle et al. 2017b) we scrutinise all of them.

The official report commissioned by the Ministry of Food, Agriculture, Environment and Forestry of the State of Baden-Württemberg excluded most of the potential causes from consideration, suggested different explanations for each type of anomaly and concluded that the anomalies are a normal natural phenomenon that have existed in the Roßwag quarry since at least 1908 (Landesregierung von Baden-Württemberg 1984). It acknowledged that the commissioned study could not provide a sound final explanation of the factors causing the anomalies but regarded past hybridization combined with atavism and inbreeding as the likely cause. It compared the anomalies observed to a small number of other cases in natural populations and experimental studies (Flindt 1984, 1985a, Landesregierung von Baden-Württemberg 1984). No review of the relevant literature was attempted. Such an approach is common in the literature on natural occurrences of anomalies in amphibians but it is not sufficient (see Souder 2002 for a parallel in recent mass anomalies in North America).

Whereas some authors (e.g., Reaser & Johnson 1997) suggest that, albeit limited, inferences can be made from the percentage of individuals affected, others disagree (e.g., Kovalenko 2000a,b). Numerous experimental studies have shown that the frequency often, but not always, depends on the strength of the causative factor (e.g., Nishioka et al. 1981, Power et al. 1989, Johnson et al. 1999) and differs among stages (e.g., Tschumi 1954, Kovalenko 2000a,b) and species (e.g., Johnson et al. 2001a, Vershinin 2002, Dubois 2014, 2017). The only conclusion from high incidences of anomalies that can be made is that something unusual must have happened and that the causative factor was strong.

Inferences are made even more difficult by the fact that all types of anomalies can have different causes (see

Table 8: Hypotheses suggested as explanations for the anomalies observed in *Bufotes viridis* from Roßwag.

Hypothesis	Suggested by (Reference)
Normal regular occurrence	Landesregierung von Baden-Württemberg 1984
Traumatism: falling stones	Flindt 1985a
Traumatism: failed predator attacks	Flindt 1985a
Traumatism: picking of eggs	Ewe, Institute of Zoology, University of Hohenheim (see Kovacsics 1981)
Parasites	Not suggested previously
Same cause as that of anomaly P	Landratsamt Enzkreis (see Schmid 1981, Henle et al. 2017b), Landtag von Baden-Württemberg 1981, Flindt 1985a
Virus	Landratsamt Enzkreis (see Schmid 1981), Flindt 1985a
Sound pressure from detonations	Landratsamt Enzkreis (see Anonymous 1980a,b)
Temperature extremes or shock	Not suggested previously
Electromagnetic fields	Once suggested in a discussion but never considered seriously
Overripeness of eggs	Flindt 1984, 1985a
Chemicals	Anonymous 1981a, Klein 1981, Flindt 1985a
Hybridization	Flindt 1984, 1985a, Landesregierung von Baden-Württemberg 1984
Inbreeding and genetic drift (recessive gene)	Flindt 1984, 1985a, Landesregierung von Baden-Württemberg 1984
Atavism	Flindt 1984, 1985a
UV-B (increased ambient levels)	Not suggested previously
Radioactivity	Henle 1981, 1982, Kneissler 1981 Rimpp 1981

Tab. 9) and by the lack of comprehensive reviews covering many types of anomalies and attempts to extract a pattern from a comparison of experimental studies in spite of at least 236 reviews on amphibian anomalies. We therefore conducted a thorough review of the pertinent literature to provide a sound basis for evaluating potential causes in the Roßwag case. We tried to trace any citation on amphibian anomalies starting with our own extensive literature collection, the literature on amphibian anomalies compiled by The North American Reporting Centre for Amphibian Malformations, the literature on field herpetology compiled by Schriftenschau für Feldherpetologie and key word searches in the Zoological Record for 1945–1985 and in Biosis for 1945–1985 and 1996–1999. More recent volumes and additional references were searched through the internet. Only publications that we could read were retained. This resulted in an evaluation of 3117 publications involving 3183 cases with natural populations; 1025 publications addressed experimental studies.

We regarded each species as a separate case if a publication contained information on several species. The same applies for several populations of a species, if sample sizes and the anomalies were counted separately for each population. Otherwise, we lumped data for all populations. If authors provided data for concrete populations and across sites, we only used data for concrete populations. For comparison with Ouellet (2000), we summed data across years and authors for the same population, if there was no data overlap. Otherwise, we only used the data from the most recent publication.

In many experimental studies no statistical comparisons with controls were made or different anomalies were lumped for tests. Therefore, we accepted a potential causative relationship, if (a) a mechanistic causative pathway between the factor and the anomaly in question is known, (b) at least one study showed a statistical significant difference between treatment and control, (c) there was a significant correlation between the rate of anomalies and the intensity of the causative factor even if no control data were provided, (d) more individuals were affected in the experiment treatment than in the control in more cases than expected at random or (e) 100% (n>50) of individuals were affected if no data of the controls were available.

6.1 Evaluation of hypotheses suggested for the Roßwag population

6.1.1 Normal natural occurrence

Observations of single or a few malformed individuals are nothing new or exceptional and the Roßwag population of *B. viridis* shares several anomalies with other populations. The State Government of Baden-Württemberg regarded the anomalies observed in the Roßwag population of *B. viridis* as a normal natural occurrence (Landtag von Baden-Württemberg 1981, Landesregierung von Baden-Württemberg 1984). However, the natural background rate of anomalies in "healthy" environments is usually around or well below 1% (Tab. 10), with previous authors assuming a baseline value of 5% or lower (reviewed by Johnson et al. 2010, Lunde & Johnson 2012, Reeves et al. 2013, Borkin et al. 2012 and Borkin 2014). Notably, von Humboldt (1797) pointed out that anomalies of frogs are exceedingly rare in spite of thousands of frogs having been dissected. Only ten other cases out of 2782 cases with concrete data available exceed 1000 abnormal individuals (Fig. 21). Of these, two comprise pooled data for several locations. Similar numbers within a population like Roßwag were found in *Ambystoma tigrinum* (tumours, edema) from a sewage pond (Rose 1976, 1977, Rose & Harshbarger 1977), in *Ambystoma macrodactylum* (ectrodactyly, syndactyly, polydactyly, ectromely, polymely and a further unspecified anomaly) in a pond with high prevalence of the trematode *Ribeiroia ondatrae* (Sessions & Ruth 1990, Johnson et al. 2003, 2006), in an *Anaxyrus boreas* population (ectrodactyly, ectromely, micromely, polymely, tail injuries) with the introduced fish *Gasterosteus aculeatus* being the cause (Bowerman et al. 2010) and in *Rana pirica* (ectromely, edema, microcephaly, anophthalmy, tumours) from a sewage pond (Mizgireuv et al. 1984). Considerably more individuals were affected only in a population of *A. tigrinum* (swollen legs and venter, hemorrhage) presumably dying from bacterial infection [Worthylake & Hovingh (1989); but see Docherty et al. (2003) who suggest that an iridovirus may have been the cause].

With 32 different types of anomalies detected, the Roßwag population of *B. viridis* far exceeds any other case (n=2990). The vast majority (81%) involved less than three types of anomalies; only for five other cases at least 15 different types of anomalies were reported for a single species (Fig. 22). One of them pooled data from 38 sites from across Bermuda and mentioned 20 different types in *Rhinella marina* (Linzey et al. 2003, Bacon et al. 2006a,b, 2013, Fort et al. 2006a,b). In terms of a single affected population, 15 types of anomalies were observed in the *Lithobates pipiens* population at Ney Pond, USA, which sparked off renewed interest in mass anomalies in North America (Helgen et al. 1998, 2000, Canfield et al. 2000, Garber et al. 2001, 2004, Souder 2002, Lannoo et al. 2003, Vandenlangenberg et al. 2003, Lannoo 2008). Parasites and chemical pollution were suggested as alternative explanations (e.g., Helgen et al. 1998, Lannoo 2008). For a *L. sphenocephalus* population at Colin's roadsite ditch, Great Swamp National Wildlife Refuge, USA, 20 types of anomalies were reported (Reeves et al. 2013). Of 583 metamorphosed individuals and tadpoles sampled 2003–2009, 11% were abnormal, with most types of anomalies being observed in one or very few individuals only. No potential cause was provided. Only one case approaches the observations in Roßwag. After the nuclear accident at the Siberian Chemical Combinat at Seversk (Tomsk, Russia), at least 25 different types of anomalies were observed in clutches, embryos, tadpoles and juveniles of *Rana arvalis* collected in the radioactive trace zone (Kuranova & Saveliev

Table 9: Overview of the factors that caused anomalies in tadpoles or recently metamorphosed juveniles in experiments. ++: significant relationship experimentally shown (see text for criteria) or 100% of individuals affected and $n > 50$ if no data of controls are available; (+): anomaly observed in treatments but not significantly more frequent than in controls, confounding factors not excluded or no statistical tests available; +?: association suggested but contradicted by other studies; –?: anomaly observed in an experimental treatment but a causative association with the treatment is unlikely; –: never observed in ≥ 10 experiments or never more frequent than in controls, with ≥ 10 experiments and ≥ 2 species; 1: see text for discussion; 2: epigenetic effect; 3: only reddish skin or haematonoms; 4: correlational evidence; 5: elevated rate in hybrid zone; 6: fused vertebrae; 7: only in specific host-parasite combinations; negative in most combinations; 8: apart from rare exceptions, only reddish skin or haematonoms. Source: References in text and Henle et al. (2017a).

	Non-genetic									**Genetic**			
Type of anomaly	Traumatism	Parasites	Mucous or excrements of fish	Virus	Other diseases	Temperature	Electrosmog and magnetic field	Overripeness of eggs	Chemicals	Hybridization	Inbreeding	Increased natural level of UV-B	Radioactivity & other mutagenic factors
Polymely	++[1]	++	(+)[1]		–	+?[1,2]	(+)	+?	++[1]	–?		–	++
Polydactyly	++	++	++			–		1	++[1]	–	++	–	++
Ectromely	++	++[7]	–	(+)[4]	++	++[2]	++	(+)	++	(+)[1]	++	++	++
Ectrodactyly	++[1]	++[7]	++	(+)[4]		++	+?	–	++	(+)	++	++	++
Phocomely	–		(+)[1]	–	–	–		–	++[1]	–?	–?	–	++
Clinodactyly	–	(+)	(+)[1]			(+)			++	–			(+)
Syndactyly	++	(+)	++			(+)			++	–?			(+)
Stiffness and rotation of limbs	(+)[1]	(+)	(+)[1]			++[1]			++	++	++	+?	++
Arms remaining within branchial cavity	–	–	–	–	–	–	(+)	–	++	(+)	(+)	–	(+)
Stiff and truncated vertebral column	–	–	–					–		–	++[6]	–	++
Brachycephaly & microcephaly	–		–					(+)		++	++	–	++
Mandibular hypoplasia	–		–		++	–		–	++	–	++	–	++
Mouthparts malformed in larvae	–		–		++				++	(+)[5]			++
Swollen fingers or body (solid)		–	–		(+)[1]	–		–		–	–	–	
Torsion of tail or body		(+)						(+)	++	(+)	++	++	++
Spiraculum misplaced	–	–	–	–	–	–		–	–	(+)		–	–
Giant larvae	–	–	–	–	–	–		–	++	(+)	++	–	–
Stunted growth				(+)					++	(+)	++		++
Melanism and darkening	–	(+)[4]	–	–	(+)	–		–	++	–?	++	++	++
Albinism and depigmentation	–	–	–	++	++	–		–	++	(+)	++	–	++
Other divergent patterns or colouration	–	++[8]	–	(+)[3,4]	++[3]	–		–	++		++	–	++
Edema		(+)	(+)[1]	++	++	+?	++	(+)	++	++	++	+?[1]	++
Tumours			(+)[1]	++	++				++	–			++

1997, unpubl.; Kuranova 1998, 2003). In small mammals, the rate of anomalies was 3.3. times higher than in control areas (Moskvitina et al. 2011). Concomitantly, 21 different types of anomalies were detected in *Salamandrella keyserlingii* embryos and larvae (Saveliev et al. 1996, Kuranova 1998, 2003). Thus, the Roßwag population clearly outruns all other natural populations in terms of the spectrum of anomalies.

Table 10: Background rates of anomalies in amphibians; only studies with samples sizes $N > 5{,}000$ included. We exclude cases that address only one type of anomalies; see Henle et al. (2017a) for a review of such cases.

Types of anomalies	Frequency	*N*	Species	Reference
–	0%	19,802	*Ambystoma tigrinum*	Rose 1981
Albinism, ectrodactyly	0.01%	25,000	*Necturus maculosus*	Hutt 1945
Oligodactyly & syndactyly	0.01%	10,000	*Rana temporaria*	Koskela 1974
Polymely of hind legs, shizodactyly, syndactyly, duplication of parts of the tail	0.02%	17,935	*Ambystoma talpoideum*	Semlitsch et al. 1981
Ectromely, deformed limbs	0.03%	36,151	8 anuran species in Michigan	Skelly & Benard 2010
Ectromely, deformed limbs	0.07%	22,482	*Lithobates sylvaticus*	Skelly & Benard 2010
Ectromely, oligodactyly, mandibular hypoplasia, polymely, digits malformed	0.4%	9,987	*Acris crepitans*	Gray 2000a,b, 2002
Unspecified	< 0.5%	> 5,300	*Rana pretiosa*	Bowerman & Johnson 2003
Ectromely, ectrodactyly, syndactyly, polydactyly, polymely, anophthalmy, microphthalmy	0.7% (range 0.2–1.7%)	21,050	*Lithobates sylvaticus*	Eaton et al. 2004
Clinodactyly, polydactyly	0.13%	5,350	*Rana temporaria*	Borkin & Pikulik 1986
Polydactyly, polymely, ectrodactyly, clinodactyly, brachydactyly, syndactyly, hump-shaped tarsus (bony triangle?), iris lacking, anophthalmy	1.02%	44,000	*Bufo bufo*	Rostand 1949, 1951a,b
Ectromely, ectrodactyly, broken bones, nasal bones destroyed, blindness, edema (adults and juveniles)	0.97%	99,992	*Bufo bufo*	Wolf 1994

Moreover, many of the anomalies observed in Roßwag were rarely reported from natural populations: phocomely, swollen fingers, atrophied musculature on digits, supernumerary bone at angle of lower jaw, spiraculum misplaced, stiff limbs, rotation of limbs, arms covered underneath a fold of skin, stiff and truncated vertebral column, mandibular hypoplasia and yellow patches as a novel colouration (Henle et al. 2017a). Notably, the last six types were frequent in Roßwag. In conclusion, the Roßwag case is the most extreme one ever reported far beyond any normal natural occurrence.

6.1.2 Non-genetic factors

6.1.2.1 Traumatism. ***Evidence put forward for this hypothesis.*** Flindt (1985a) found an individual with head injuries and, without supporting evidence, explained all cases of ectromely and ectrodactyly in juveniles as injuries inflicted by stones. He attributed ectromely and ectrodactyly in tadpoles to cannibalism and failed predation attacks by dragonfly larvae, because he observed dragonfly larvae in one of the water-filled depressions in 1983.

Anomalies known to be caused by traumatism. Anomalies caused by traumatism are summarized in Table 9. While ectromely is common following experimental amputations (e.g., Girvan et al. 2002), Loeffler et al. (2001) and Lannoo (2008) considered it as unlikely for a predator to amputate the limb bud of a tadpole without causing fatal injury to the tadpole itself. Loeffler et al. (2001) assumed that non-lethal limb bud injuries are more likely to be due to intraspecific interactions and abrasions against plants or the substrate. In line with their idea, the only case known to us in which staged predation caused ectromely without other injuries is an adult *Notophthalmus viridescens* (n=14), whose front leg was amputated by a *Chelydra serpentina* (Hurlbert 1970). However, we do not know of any experimental study that demonstrated abrasion as the cause of limb injuries.

Ectromely occurred in a few staged predation experiments but results were often inconsistent with one another or only occurred under specific conditions. The leech *Erpobdella octoculata* can cause high incidences of ectromely in *Bufo bufo* tadpoles, and additionally tail injuries, but not in *Rana temporaria* tadpoles (Viertel & Veith 1992, Bohl 1997). It may also cause limb anomalies in the newt *Ichthyosaura alpestris* (Kneitz et al. unpublished report, fide Hachtel 2011). Whereas sticklebacks (*Gasterosteus aculeatus*) did not inflict ectromely in *B. bufo* tadpoles (Ballengée & Sessions 2009), experiments using *Anaxyrus boreas* resulted in ectromely (or ectrodactyly; it is not stated which one of these) but also high rates of tail damage (Bowerman et al. 2010). Likewise, mosquito fish (*Gambusia holbrooki*) injured tadpoles of *Lithobates capito* at very high rates (mainly the tail) but rarely those of *L. sphenocephalus* (Gregoire & Gunzburger 2008). Experimental exposures of *B. bufo* and *R. arvalis* tadpoles to adult newts (*Lissotriton helveticus, L. vulgaris*), diving beetles (*Dytiscus marginalis* and *D. lapponicus*), water scorpions (*Nepa cinerea*) and various other insects did not result in ectromely (Henrikson 1990, Manteifel & Reshetnikov 2002, Ballengée & Sessions 2009). For dytiscid beetles this

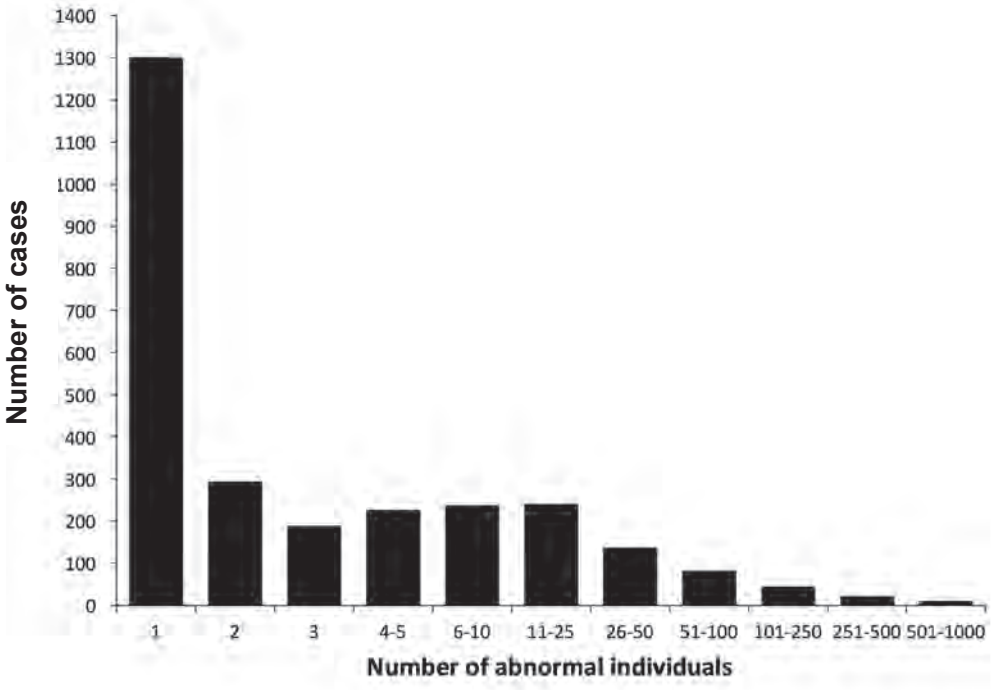

Fig. 21: Frequency distribution of the number of abnormal individuals reported from natural populations.

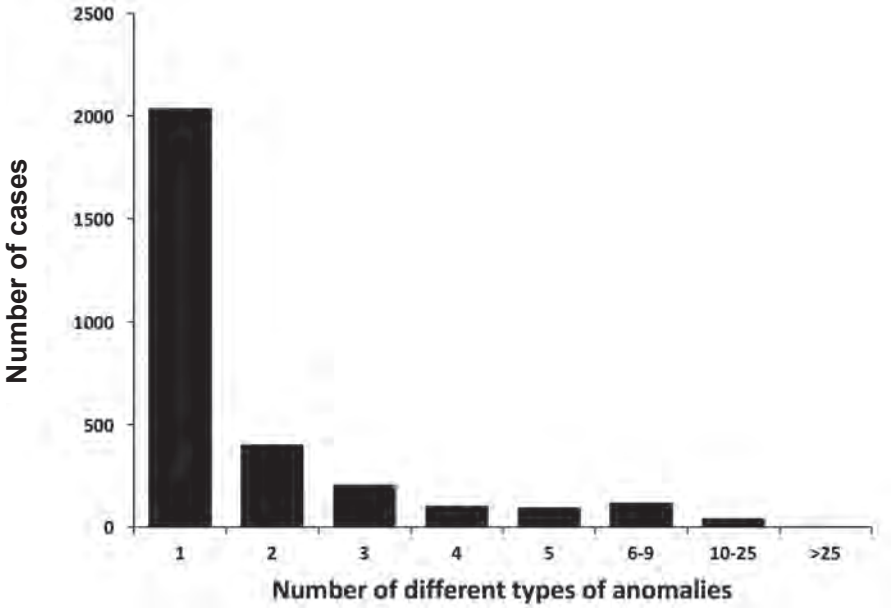

Fig. 22: Frequency distribution of the number of different types of anomalies reported from natural populations.

may be explained by them grapping tadpoles by the body. In contrast, crayfish (*Cambarus diogenes* and/or *Orconectes propinquus*) may capture tadpoles either by the body or by the hind legs (Formanowicz & Brodie 1982) but these authors did not mention whether crayfish attacks resulted in ectromely in larval amphibians. Attacks by crayfish may cause ectromely in adult frogs – although it remains unclear as to whether the observed attacked individual managed to escape predation (Niemiller & Miller 2005).

With two exceptions (Ballengée & Sessions 2009, Bowerman et al. 2010), ectromely or ectrodactyly was never reported from staged predation experiments with odonate nymphs, whereas tail injuries occurred frequently (e.g., Caldwell 1982, Morin 1985, Petranka 1989, Henrikson 1990, Richards & Bull 1990, Wilbur & Semlitsch 1990, Chovanec 1992, Parichy & Kaplan 1992, Thiesmeier 1992, Manteifel & Reshetnikov 2002, Casanovas & Úbeda 2006, Malkmus 2009). The exceptions were nymphs of *Aeshna mixta, Libellula depressa* and especially *Sympetrum* (probably either *S. striolatum* or *S. sanguineum*) that amputated parts of the limbs of Gosner (1960) stage 32–37 tadpoles of *B. bufo* without killing them (Ballengée & Sessions 2009)

and *Somatochlora albicincta* that caused missing and abnormal limbs in *Rana cascadae* (Bowerman et al. 2010). However, in all experiments with *B. bufo* tadpoles, tail injuries and other injuries were very common as well (no data is available for *R. cascadae*) – which unfortunately is not assessed in most studies on hotspots of amphibian anomalies. Moreover, in staged experiments, other species of the same dragonfly genera only inflicted tail injuries without amputating or mutilating limbs: *Sympetrum nigrifemur* in *Pelophylax perezi* tadpoles (Malkmus 2009), *A. cyanea* and *Aeshna* spp. in *B. bufo, Bombina bombina, Hyla arborea, R. dalmatina* and *R. temporaria* tadpoles (Hendrikson 1990, Chovanec 1992, Manteifel & Reshetnikov 2002, Van Buskirk et al. 2003). Along these lines, ectromely was extremely rare in Michigan and Connecticut across many ponds that contained high densities (> 1 individual / m^2) of larval *Sympetrum* (*S. internum, S. obstrusum, S. rubicundulum, S. semicinctum, S. vicinum*) (Skelly & Benard 2010).

Likewise, conspecific interactions result rarely in ectromely or ectrodactyly but injuries are common (e.g., *Ambystoma laterale*: Van Buskirk & Smith 1991; *Eurycea cirrigera*: Halliday & Tejedo 1995; *Salamandra salamandra*: Thiesmeier 1992; *Ommatotriton ophryticus*: Kosswig 1951, Raxworthy 1989; *Dendropsophus minutus*: Peixoto & dos Reis Gomes 1997; *Nannophrys ceylonensis*: Wickramasinghe et al. 2005). Exceptions are the carnivorous larval *Ambystoma talpoideum* and *A. macrodactylum,* which inflict significantly elevated rates of ectromely and ectrodactyly in staged interspecific interactions (Semlitsch & Reichling 1989, Johnson et al. 2006). In the former, high rates of tail losses also occurred – in the latter (Johnson et al. 2006) it was not assessed. Also, in captivity *Cryptobranchus bishopi* may frequently bite-off the legs of conspecifics during the breeding season (Ettling et al. 2013) and the same is the case in larval *Triturus pygmaeus* (Malkmus 2007); in the latter case, the limbs regenerated within 1.5 months. The only other case of apody caused by conspecifics known to us is a juvenile *Hynobius tsuensis* (Wallays 1998).

In conclusion, ectromely caused by predation attempts is more of an exception than the rule in staged predation experiments; i.e. it only occurs for specific combinations of predators, amphibian species and larval stages exposed and potentially other details of the interactions. For example, the opposing results reported for *Sympetrum* and *Aeshna* dragonfly nymphs and for sticklebacks (*Gasterosteus aculeatus*) (see above) suggest that congeneric predators and even the same predator species may have a different potential to amputate or mutilate limbs or that differences in details of the staging experiments may have caused opposing results. In any case, dominance of tail injuries and rarity or absence of ectromely and ectrodactyly in staged experiments with dragonfly larvae and with conspecifics are incompatible with the conclusion of the official inquiry (Landesregierung von Baden-Württemberg 1984, Flindt 1985a) that ectromely and ectrodactyly in the Roßwag population were caused by dragonfly larvae and conspecific attacks. Moreover, ectromely was also observed in the absence of dragonfly larvae in the quarry pond in 1980 (this study) and in the laboratory by Flindt (1985a).

Hyperregeneration following traumatism is often assumed as cause for polymely. While numerous studies (e.g., Tornier 1896, 1905, Lissitzky 1910, Puppé 1925, Brandt 1940, Kleinebeckel 1975, Michael & El Mekkawy 1977, Michael & Hassona 1982, Tsonis & Eguchi 1985, Stopper et al. 2002) demonstrated that experimental injuries, amputation or transplantation of amphibian legs or toes can result in hyperregeneration, i.e., the duplication of the affected part, duplications occur only if injuries cause physical rearrangement of cells or death of some but not all cells in the apical epidermal cap of the blastema (Stopper et al. 2002, Nye et al. 2003). Simple amputation usually does not lead to a rearrangement of cells and thus usually does not induce hyperregeneration (Lecamp 1935, Stopper et al. 2002). To our knowledge, staged predation experiments never resulted in polymely and we know of only two cases in which the partial loss of legs from attacks by conspecifics induced polymely: an *Alytes obstetricans* tadpole (Hellmich 1929, Wolterstorff 1941) and one *Ambystoma mexicanum* (Duméril 1867).

In numerous experiments involving amputations of limbs or parts thereof the torsion of legs was only mentioned once (2 tadpoles in *Hymenochirus boettgeri*) (Girvan et al. 2002). In contrast, traumatism often results in injuries of fins (e.g., Kosswig 1951; dragonfly studies discussed above), eyes (Kluge 1981, Ballengée & Sessions 2009), scars on the body or head in fighting males (e.g., Martins et al. 1998, Kwet & Di-Bernardo 1999) and on the head and body of tadpoles attacked by dragonfly nymphs (Ballengée & Sessions 2009), as well as schizodactyly and syndactyly (Semlitsch et al. 1981). These anomalies were, however, rare or absent in Roßwag.

Evaluation. It is difficult to imagine how stones could cause the amputation of fingers and legs without inflicting any other injuries to metamorphosed individuals. Toad tadpoles avoid injured conspecifics (demonstrated for *B. bufo*: Kulzer 1954, Pfeiffer 1966, Kiselva & Manteifel 1982) and cannibalism has never been observed in tadpoles of European toad species. Likewise, there is no evidence that dragonfly larvae may have caused ectromely or ectrodactyly in the tadpoles. Only few species of dragonflies are known to amputate limbs of anuran tadpoles without killing them. Significantly, tail injuries that are frequently inflicted when predators attack tadpoles did not occur in the Roßwag population of *B. viridis*. Moreover, no potential predators were detected in the pond in the Roßwag quarry in 1980 or in 1981 and in 1983 dragonfly larvae were only observed in one of five ponds (Flindt 1985a). Finally, ectromely also occurred in laboratory raised individuals in the absence of predators. In conclusion, traumatism is only a likely explanation for the one individual with head injuries or rare cases of partially missing legs.

6.1.2.2 Parasitism. ***Evidence put forward for this hypothesis.*** This cause was never suggested for the Roßwag population but widely discussed for recent incidences of mass anomalies in North American amphibians and therefore evaluated here as well.

Anomalies known to be caused by parasitism are summarized in Table 9. Anomalies strongly differ among the parasites involved and species differ strongly in their sensitivity. The largest range of anomalies is caused by the trematode *Ribeiroia ondatrae*. In most experimental infestations, polymely, polydactyly, taumely (bony triangles), femoral projections and cutaneous fusion were significantly more frequent than in controls (Johnson et al. 1999, 2001b, Stopper et al. 2002, Schotthoefer et al. 2003, Johnson & Hartson 2009). Ectromely, ectrodactyly, brachymely, rotation of limbs, clinodactyly, syndactyly, reduced or misshaped ilia and edema occasionally appeared in experiments. However, the rates of these anomalies were usually similar to controls; so the causal relationship is not certain. Two studies conducted on *Pseudacris regilla* are an exception (Johnson et al. 1999, Romansic et al. 2011), in which ectromely was frequent, presumably statistically significantly higher than in controls but the method of counting anomalies was not explained sufficiently for a rigorous formal test. In contrast, experimental infestations of limb bud stages (Gosner stages 27 and 28) of Sri Lankan *Polypedates crucifer* tadpoles with monostome-type cercariae (*Acanthostomum* species according to Jayawardena & Rajakaruna 2013) resulted in significantly elevated rates of ectromely, ectrodactyly, micromely, deformations of the vertebral column, translucent skin and partially or completely lacking pigmentation (Rajakaruna et al. 2008). While a few individuals also showed skin webbing and polydactyly, the difference to controls was not significant. The results were stage-specific. If pre-limb bud stage tadpoles (Gosner stages 25 and 26) tadpoles were exposed, edema, bent tails and ulcers of the skin occurred whereas metamorphosed individuals showed bent bodies and skin ulcers (Jayawardena et al. 2010a). Experiments with the genus *Alaria* failed to produce anomalies (Johnson et al. 1999).

The parasitic fly *Lucilia bufonivora* deposits its eggs in the nasal opening of anurans (mainly in *Bufo bufo*; other species are rarely attacked) and the larvae destroy the nasal openings, leading to the death of the parasitized toad (reviewed by Neumann & Meyer 1994 and Henle et al. 2017a). The North American species *L. illustris* is able to destroy the eyes (Anderson & Bennett 1963) and trombiculid mites of the genus *Hannemannia* may encyst in the skin of amphibians in North-, Central and South America (reviewed by Henle et al. 2017a). The only deviating colour patterns known to be caused by parasites are haematonoms, e.g. by the small ostracod parasite *Argulus foliaceus* that clinged to the digits in a breeding population of *Pelobates fuscus* (Schäperclaus 1954).

Evaluation. Although the trematode *Ribeiroia ondatrae* can cause several of the limb anomalies observed in Roßwag, the pattern differs considerably, with three typical anomalies caused by the trematode – taumely, femoral projections and cutaneous fusions – being absent and another typical one, polydactyly, being rare in Roßwag. Furthermore, stiff and truncated vertebral columns, phocomely, colour anomalies (except for haematonoms), tumours and giant larvae are not induced by *Ribeiroia ondatrae* or any other parasite. Moreover, *R. ondatrae* does not occur in Europe. All types of anomalies known to be caused by parasitism could together explain at most a small fraction of the types of anomalies observed in Roßwag (Tab. 9). Moreover, symptoms of parasitic infections are similar among individuals in contrast to the expression of anomalies in the Roßwag population of *B. viridis*. Finally, the development of anomalies in tadpoles from eggs transferred to the laboratory in the absence of parasites is not consistent with parasitic infections as an explanation of the anomalies.

6.1.2.3 Skin mucus and fish excrements (anomaly P factor). ***Evidence put forward for this hypothesis.*** Suggestions to consider anomaly P by Nöthiger (in lit.) was taken as a proof by the responsible administration that the anomalies of the Roßwag toads are caused by the same factor (see Henle et al. 2017b for details). Flindt (1985a) was vaguer but regarded the anomalies as sufficiently similar to justify such an explanation. He further justified this link by his assumption that the anomalies observed in Roßwag were due to hybridization and the fact that anomaly P affects species within the *Pelophylax esculentus* synklepton, which includes taxa of hybridogenetic origin. He faild to provide an explanation for the role of skin mucus and fish excrements in this context.

Anomalies known to be caused by the anomaly P factor. The anomaly P syndrome is characterised by mass polydactyly of the hind limbs often combined with massive edema (reviewed by Rostand 1971, Dubois 2014, 2017). Other morphological peculiarities include a good, although not always perfect, bilateral symmetry (i.e., the number of additional digits was always roughly the same on both sides, with sometimes one more toe or finger on one side, but never an excess of several digits on one side) and a first toe longer than the second and following. The most severely affected tadpoles may additionally show polymely, brachymely and in the most extreme cases phocomely, various types of bony excrescences and tumours, especially in the inguinal region, but these anomalies never occurred without severe polydactyly (Rostand 1955, 1958, 1971, Surlève-Bazeille et al. 1969b). No quantitative data are available for these anomalies. Exposure of *Pelophylax esculentus* and *Rana temporaria* tadpoles to skin mucus or feeding them with fish excrements from ponds in which anomaly P occurred caused significantly elevated rates of polydactyly (Rostand & Darré 1968, 1969) and ectrodactyly plus syndactyly, respectively (Surlève-Bazeille et al. 1969a).

Evaluation. The only similarity between anomaly P and the anomalies in Roßwag are the very high percentage of animals affected. Polydactyly was rare in the Roßwag population but is invariably present in anomaly P. Most of the anomalies observed in Roßwag have never been detected in populations affected by anomaly P. Moreover, anomaly P is known only in green frogs of the genus *Pelophylax* and only in large ponds inhabited by tenches (*Tinca tinca*) and eels (*Anguilla anguilla*) (Dubois 2014, 2017). Fish therefore seem to be vectors of the causal factor of anomaly P (whatever it is) but it is not known whether this factor can also be active in the absence of fish. In any case, fish were absent from the Roßwag quarry.

6.1.2.4 Viral and other diseases. ***Evidence put forward for this hypothesis.*** Schmid (1981) and Flindt (1985a) suggested a viral cause for the Roßwag anomalies based on the argument that the anomalies observed in Roßwag were supposedly similar to those observed in anomaly P and that a virus is probably the cause of anomaly P.

Anomalies known to be caused by viral and other diseases are summarized in Table 9. Many pathogens cause diseases, with hemorrhage, skin lesions and edema being commonly observed non-specific clinical manifestations (Densmore & Green 2007, Hemingway et al. 2009). For example, viral (herpes virus, iridovirus and tadpole edema virus), bacterial (e.g., *Aeromonas hydrophila, Flavobacterium indologenes, Chlamydophila* and other gram-negative aeromonads, pseudomonads and enterobacteria) and probably fungal (*Basidiobolus ranarum)* infections may lead to edema (Emerson & Norris 1905, Wolf et al. 1968, Newcomer et al. 1982, Olson et al. 1992, Taylor et al. 1999, Crawshaw 2000, Densmore & Green 2007, Gray et al. 2009), skin lesions and the loss of eyes (Polder 1973, Rose 1976, Bennati et al. 1994, Taylor et al. 1999, Densmore & Green 2007). A reddish colour due to hemorrhage is a symptom called "red leg" disease that may be caused by *A. hydrophila* (Emerson & Norris 1905, Hunsaker & Potter 1960, Nyman 1986, Bradford 1991, Carey 1993), other gram-negative aeromonads, pseudomonads and enterobacteria (Caselitz 1966, Densmore & Green 2007) and *Ranavirus* (Gray et al. 2009) and is also found in individuals affected by *Batrachochytrium dendrobatidis* (e.g., Brodman & Briggler 2008, Venesky & Brem 2008). Often, but not always, bloatedness or edema are noted as well (e.g., Emerson & Norris 1905, Densmore & Green 2007, Gray et al. 2009). Note, however, that bloatedness and hemorrhage may also be caused by supersaturation of the water with gases and, according to Colt et al. (1984), may be the primary etiology in many observed cases, as supersaturation commonly occurs in lakes in the spring (Harvey 1967).

Partial or complete depigmentation has been observed in infected individuals of *Ambystoma mexicanum* (Brunst 1968), *Pipa carvalhoi* (Leja 1981), *Xenopus laevis* (Newcomer et al. 1982), *Agalychnis callidryas* (Wright & Whitaker 2001) and tadpoles of *Ptychohyla hypomykter* (Mendelson et al. 2004). Symptoms of infection with *B. dendrobatidis* are malformations of the mouthparts in tadpoles, especially depigmentation of keratinized parts (e.g., Fellers et al. 2001, Lips et al. 2004, Rachowicz & Vredenburg 2004), discolouration (usually brown) and in some species reddening of the skin, usually of the ventral body and toes and feet (reviewed by Carey et al. 2003) and sometimes grossly visible lesions (reviewed by Carey et al. 2003) or black flakes of unshed skin (Cummer et al. 2005). However, depigmented mouthparts are not diagnostic for *B. dendrobatidis* (Rachowicz 2002); likewise, infected tadpoles may appear normal (Blaustein et al. 2005, Padgett-Flohr & Goble 2007). An abnormal deposition of melanin may be an inflammatory reaction to skin infection with helminths (Mutschmann & Manzke 2006).

Wisniewski (1984) noticed atrophy of the face, jaw and legs in the urodeles *Cynops ensicauda popei* and *Paramesotriton chinensis* after infection by *Saprolegnia.* Correlational evidence also exists that poxvirus, *B. dendrobatidis* and bacterial infection with *Aeromonas* may cause ectromely and oligodactyly due to necrosis, ulceration, as well as a reddening of skin (Frye 1985, Cunningham et al. 1993, Brodman & Briggler 2008).

Tumours are further well-known pathologies of viral infections in amphibians (reviewed by Balls & Ruben 1964, Mizell 1969, Balls et al. 1978, Asashima et al. 1987) and other organisms (Gross 1961). Experimental infections with fungi of the genus *Mycobacterium* and other unidentified fungi may also cause tumours in amphibians (Dhaliwal & Griffiths 1963, Inoue & Singer 1970). Green (2001) reported a swelling on the caudal half of the body and the proximal half of the tail in *Notophthalmus viridescens* that was associated with an *Ichthyophonus*-like fungal infection. Infection with *Saprolegnia* may also result in ulcerated skin and erythema (Densmore & Green 2007).

Evaluation. All known diseases cause a narrow range of anomalies. Most types of anomalies observed in Roßwag have never been seen in any infected amphibian (compare Tables 1 and 9). Furthermore, no evidence for any disease is available for the Roßwag population and infected animals always show the same symptoms whereas the Roßwag individuals differed strikingly in their anomalies. Thus disease can be excluded as a potential cause.

6.1.2.5 Temperature extremes and shocks. ***Evidence put forward for this hypothesis.*** This was never suggested as a cause for the Roßwag population but for a few other natural occurrences of mass anomalies, although never proven for any of them (Woitkewitsch 1961, Worthington 1974, Harkey & Semlitsch 1988).

Anomalies known to be caused by temperature extremes and temperature shocks are summarized in

Table 9. Most studies have been conducted on embryos (reviewed by Hertwig 1894, 1898, Lillie & Knowlton 1897, Hoadley 1938). Under permanent high temperature, a typical haploid syndrome manifests itself and almost all individuals die in early embryonic stages. Temperature shock led to triploids in hybrid eggs of *Bufotes viridis* × *Bufo bufo* and *B. viridis* × *Epidalea calamita,* most of which died as embryos although a few developed normlly or showed edema and giant sizes (Hertwig & Weiss 1955). Temperature also acts synergistically with overripeness in early embryonic anomalies (Briggs 1941, Witschi 1952, Rugh 1965, Mikamo 1968, Mikamo & Hamaguchi 1973).

Temperature effects on larvae have been less well studied. At a constantly low temperature (6–9°C), larval *Ambystoma mexicanum* reabsorbed digits, resulting in brachydactyly or oligodactyly (Blount 1950). Low temperature also resulted in a slightly albeit significantly elevated (1.8%) rate of abnormal limbs in *Pseudacris ornata* (Harkey & Semlitsch 1988). Under an elevated temperature (18°C), a high proportion of *Euproctus asper* larvae developed bent tails (Clergue-Gazeau 1971). A constantly high temperature caused a significantly elevated frequency of ectrodactyly in the developing limbs of *B. b. formosus.* Clinodactyly and edema was occasionally observed, but at low frequency and only in a few experiments (Muto 1969a,b, 1970, 1971). Likewise, in *Pseudacris ornata* the frequency of limb anomalies was significantly increased (1.3%) (Harkey & Semlitsch 1988). Under a constantly high temperature, regenerating limbs of *A. mexicanum* showed a fusion of tarsal bones and occasionally syndactyly (Schmalhausen 1925). In *Pleurodeles waltl,* temperature acted epigenetically on an inherited anomaly causing ectromely, ectrodactyly, twisted feet and knee anteroversion (Dournon 1983, Dournon et al. 1998). At high temperature (30°C) and when the treatment started early in development, the frequency, severity and variability of anomalies were greater than at control temperatures (20°C) (Dournon 1983, Dournon et al. 1998). Epigenetic effects of temperature are also known for some embryonic anomalies of the same species (Conter & Jaylet 1974, Fernandez & Beetschen 1975).

Witschi (1920, 1925) reported that *Rana temporaria* raised at a constantly high temperature (27–28°C) from overripe eggs developed polymely. An epigenetic effect is likely because most polymelous individuals were derived from a single female and never appeared in batches raised at lower temperatures (19–20°C).

Ensinck (1978) suggested that constantly high temperatures may cause stiffness of legs in *Gastrotheca marsupiata* but confounding effects cannot be entirely excluded.

Evaluation. There is no possibility that the Roßwag population received artificial temperature shocks. The large volume of the pond where the largest number of abnormal individuals was found excludes the possibility of exposure to exceptionally high temperatures during development. It is not known whether the temperature of the small water bodies in which *B. viridis* spawned after 1981 was exceptionally high. However, the years when anomalies were observed were not exceptional and anomalies also appeared in individuals transferred to the laboratory. Although it is highly unlikely, it cannot be excluded with certainty that high temperature contributed to the few individuals with ectrodactyly, syndactyly or clinodactyly found by Flindt (1985a). In any case the vast majority of anomalies detected in Roßwag cannot be explained by temperature extremes (see Tab. 9).

6.1.2.6 Electro smog and strong magnetic fields. *Evidence put forward for this hypothesis.* This cause was once raised in a non-public discussion of the Roßwag toads. It was also suggested by a layperson for the mass anomalies observed in North America (Souder 2002) and there is a great deal of controversy over the potential health hazards of electromagnetic radiation for humans (Coleman & Beral 1988, Ross 1988). Anomalies in the strength of the magnetic field have never been suggested as a cause for amphibian anomalies.

Anomalies known to be caused by electromagnetic fields and abnormal magnetic fields are summarized in Table 9. The effect of the electromagnetic field on amphibian development has, to our knowledge, never been studied. We only know of two studies on the regeneration of limbs in newts. Landesman & Douglas (1990) observed a significantly ($\chi^2 = 6.47$; $\alpha < 0.01$) elevated rate, compared to controls, of abnormal limb regeneration in *Notophthalmus viridescens* when amputated individuals were exposed for 30 days to a pulsed electromagnetic field. Anomalies reported are fused carpal bones, a lower or larger number of carpal bones than normal variation, oligodactyly, ectromely and malformed long bones. In contrast, Smith & Pilla (1981) did not obtain elevated rates of anomalies using similar waveforms (for 21 days) but regeneration was completely inhibited when they used other waveforms.

The effects of abnormally strong magnetic fields in amphibian devleopment was studied by Levengood (1969). When de-jellied eggs of *Ambystoma maculatum* or *Lithobates sylvaticus* were exposed to strong magnetic fields (6300–17700 Gauss), hatching larvae showed edema, scoliosis, microcephaly and stunted growth at rates higher than controls. Tadpoles of *L. sylvaticus* that reached climax stages of metamorphosis showed various (non-specified) leg anomalies, edema, two cases of polmely, one case of one arm not emergig from the branchial cavity, one case of unilateral anophthalmy and one case of neoteny. The percentage of animals affected was significantly higher for both levels of the strength of the magnetic field.

Evaluation. The lack of experimental studies makes evaluation difficult. The few studies only show a small range of the types of anomalies observed in Roßwag. In any case, no potential sources of pulsed electromagnetic fields or for a severely elevated strength of the magnetic field existed in the quarry. Thus, it is an unlikely cause

for anomalies of *B. viridis* in Roßwag although it merrits studies on its teratogenic and mutagenic potential in amphibian larvae.

6.1.2.7 Sound pressure and shock waves. ***Evidence put forward for this hypothesis.*** Launched by the district president, newspapers reported that citizens with sound knowledge and interest in the Roßwag case believe that the anomalies of the Roßwag toads were due to detonation shock waves from quarry operations (Anonymous 1980a,b).

Anomalies known to be caused by sound pressure and shock waves are summarized in Table 9**.** Only a few studies are available on the teratogenic effects of sound pressure in amphibians. Bombardement with ultrasound (0.7 WA/cm², 1000 kHz, 5–15 min) caused duplication of body, bicephaly, microcephaly, acephaly, anophthalmy, torsion of the body, torsion of the tail and edema in embryos of *Ichthyosaura alpestris* and *Lissotriton helveticus* (Bonhomme & Pourhardi 1957, Bonhomme et al. 1960, Pourhardi et al. 1968). The majority of individuals died at the neurula stage at the latest and no anomalies developed when post-gastrula stages were treated. We do not know of any study that experimentally exposed late larval stages to ultrasound or to explosion shock waves.

Evaluation. If detonations were responsible for the anomalies, we would expect anomalies to occur in all active quarries since detonations are part of regular operations of active quarries. More than 100 quarries have been surveyed in Baden-Württemberg by herpetologists without reporting elevated rates of anomalies (Laufer & Pieh 2007). Furthermore, the eggs transferred to the laboratory were not exposed to any sound pressure waves but developed anomalies and no mechanism is known by which sound pressure waves can induce mutations (though this deserves study). Moreover, sound pressure is ineffective at late embryonic stages and lethal at earlier stages. Therefore, detonations cannot have contributed to the anomalies observed in Roßwag.

6.1.2.8 Overripeness of eggs. ***Evidence put forward for this hypothesis.*** Flindt (1985a) argued that the malformations of the front legs are astonishingly similar to the symptoms that develop when raising overripe eggs. Although he raised the open question as to why females would have produced overripe eggs only in the Roßwag population, he concluded that this could have been due to warm water in the quarry (which is unlikely: see section on temperature extremes above).

Anomalies known to be caused by overripeness of eggs are summarized in Table 9. Overripeness of eggs leads to a range of anomalies in cleaving eggs and embryos resulting in the death of most individuals at early embryonic stages. Anomalies include irregular cleavage, incomplete blastopore closure with a persistent yolk-plug, exogastrula, incomplete gastrula, abnormal neurula, tumour-like growth, spina bifida, microcephaly, acephaly, microphthalmy and anophthalmy. The frequency of anomalies increases with the time of retention and temperature (Briggs 1941, Witschi 1952, Rugh 1965, Mikamo 1968, Mikamo & Hamaguchi 1973). O_2-deficiency and CO_2 excess (Witschi 1952) and degeneration of egg cytoplasm as well as the disturbance of chromosome distribution during meiosis (Zimmerman & Rugh 1941, Witschi 1971) have all been suggested as mechanisms leading to the observed anomalies. Witschi & Chang (1954) speculated that mutations could also be caused by overripeness of eggs but did not provide an explanation for this hypothesized effect.

The majority of the few individuals surviving beyond embryonic stages are normal (Briggs 1941, Rostand 1951c). Anomalies reported for very early tadpole stages are bent bodies and microcephaly in *Lithobates pipiens* (Briggs 1941). Witschi (1920, 1922, 1925, 1952) obtained *R. temporaria* with polymely and individuals with polydactyly of duplicated hands that developed from overripe eggs. Witschi (1952) counted the total as seven polymelous individuals and two polydactylous individuals. He also made reference to ectromely but did not provide any figures. Polymely only appeared in batches raised at high temperatures (27–28°C) (Witschi 1925) but not in batches raised at 10–20°C and all the affected individuals were the offspring of a single female. Data on controls (non-overripe eggs) were not provided. Thus, skeletal anomalies in individuals developing from overripe eggs are rare, contrary to the statement by Flindt (1985a). Later experiments (e.g., Rostand 1951c) were unable to induce any skeletal anomalies by overripeness of eggs in *R. temporaria*. Therefore, it is very likely that in the study of Witschi (1925) temperature exerted an epigenetic effect on a recessive mutation borne by the parental female.

Evaluation. All results contradict the hypothesis of overripeness as a cause of anomalies in the Roßwag population: the low number of surviving embryos, the absence of anomalies, such as giant tadpoles or colour anomalies, the rarity of skeletal anomalies, the inability to reproduce early reports of polymely as a consequence of overripeness and the lack of an explanation as to why females of the Roßwag population and only them, should retain eggs so long that overripeness comes into play.

6.1.2.9 Chemicals. ***Evidence put forward for this hypothesis.*** Anonymous (1981a) and Klein (1981) suggested that chemicals sprayed on adjacent vineyards or illegally dumped in the quarry caused the anomalies but did not provide any supporting evidence. The chemical teratogenic literature was apparently unknown to Flindt (1985a) and he therefore did not further discuss chemicals as a potential cause of the anomalies observed in Roßwag.

Anomalies known to be caused by chemicals. There is a considerable body of literature on the teratogenicity of a wide range of chemicals for developing amphibians. In addition, partial reviews of anomalies caused by a range

of different types of chemicals are available: acidification (Pierce 1985, 1993, Ferraro & Burgin 1993), alkalinity, salinity and metals (Ferraro & Burgin 1993), LiCl (Hall 1942, Pasteels 1945, Bustuoabad et al. 1977, Kao & Elinson 1998), an anaerobic environment (Büchner 1948, 1955, Rübsaamen 1955, Mangold & Peters 1956), endocrine disruptors (Hayes 2000) and various pesticides (Power et al. 1989, Cowman & Mazanti 2000, Johnson et al. 2010).

Chemicals can cause a range of different types of anomalies (Tables 9 & 11). It was only for three types of the anomalies considered that we could not find any report – a stiff and truncated vertebrate column, microcephaly in larval or metamorphosed individuals and a misplaced spiraculum; these are rare anomalies. In addition, only retinoids and vitamin A applied under specific conditions can induce polymely or polydactyly unless the limbs are simultaneously manipulated surgically (amputation or insertion of crystals – see Henle et al. 2017a for discussion). Furthermore, apart from darkening, depigmentation, hemorrhage and the use of dyes, few colour anomalies are known to be induced by chemicals (see Henle et al. 2017a).

Evaluation. *Salts, metals and pH.* The teratogenicity of metals depends on the concentration and differs among organisms and metals (Ferraro & Burgin 1993, Power et al. 1989, Peskova 2000). Of the chemicals analysed in the water samples from Roßwag (Tables 3 & 4), only iron was measured at a concentration that could potentially cause anomalies in embryos or tadpoles of anurans (Miller & Landesmann 1978, Dawson et al. 1988, Haidacher & Fachbach 1991, Nechitailo & Peskova 1999, Peskova 2000). When eggs of *Ommatotriton vittatus* were exposed to 0.5 mg/l $FeSO_4$ – this is below the value measured for iron by the Chemical Laboratory of the City of Pforzheim (Tab. 3), but above the value measured by the Landesanstalt für Umweltschutz (Tab. 4) – they developed a curvature of the axial skeleton (Nechitailo & Peskova 1999, Peskova 2000). Copper sulphate was sprayed by helicopters in vineyards adjacent to the Roßwag quarry (Buck, pers. comm.). Known teratogenic effects of copper in tadpoles are curvature of the axial skeleton in *O. vittatus* (at 0.5 mg/l $CuSO_4$: Nechitailo & Peskova 1999, Peskova 2000) and ectromely in *Xenops laevis* (significant effects at ≥ 0.25 mg/l copper: Fort & Stover 1997). Both values are above those measured for copper in the water samples from Roßwag (Tables 3 & 4) but below that measured for sulphate (Tab. 4). In any case, *B. viridis* is exceptionally tolerant to high salt concentration (Zavadil & Prikryl 2003). In conclusion, it is unlikely that pH, salts or metals contributed to the observed anomalies.

Nutritional deficiencies. An absence of essential micronutrients has been invoked as a contributor to the mass anomalies of frogs in Minnesota, USA (Fort et al. 1999, Souder 2002). Nutritional deficiencies (vitamin C, calcium, possibly vitamin D and other essential nutrients) may cause rachitis, growth retardation, torsion of the tail and body, changes in head proportions and bleaching in urodelans (Klatt 1927, Krefft 1938), stiff legs, torsion of the legs (Klatt 1927, Krefft 1938, Reinhardt 1939, Vogt 1939, Bruce & Parkes 1950, Marshall et al. 1980, Leibovitz et al. 1982, Pollack & Liebig 1989, Martínez et al. 1992), hypertrophy of the lower jaw and anomalies of the urostyle, pelvic girdle and the spine (Bruce & Parkes 1950) and phocomely and ectromely in *Ambystoma mexicanum* and *Pleurodeles waltl* but not in *Triturus* s.l. spp. (Reinhardt 1939, Vogt 1939). In *Ambystoma* sp., dietary deficits (probably arginine) caused anemia and short and curled gills (Patch 1936). Edema is also often regarded as a symptom of nutritional deficiencies (Grimm 1953, Reichenbach-Klinke 1961, Jara 1963) but few experimental analyses have shown this (e.g., Reinhardt 1939). In anuran husbandry, it has further been suggested that ectromelous offspring (observed in dendrobatids, discoglossids and hylids) are due to deficiencies in vitamins and minerals of the parents or the offspring (e.g., Schmidt 1985, Glaw 1987, Krintler 1988) but this hypothesis has not yet been tested experimentally. Poor nutrition led to the fusion of tarsal bones and occasionally ectrodactyly in regenerating limbs of *A. mexicanum* (Schmalhausen 1925). The results of the analysis of the water samples taken from the Roßwag quarry (Tables 3 & 4) are inconsistent with mineral deficiencies and the large size of many tadpoles and most toadlets further contradicts nutritional deficiencies as a potential cause. Moreover, the patterns of anomalies resulting from nutritional deficiencies differ in most respects from the pattern observed in Roßwag.

Fertilizer. Although the water samples do not provide any evidence for fertilizer contamination (Tables 3 & 4), fertilizer was probably used in fields close to the Roßwag quarry. Wolf (1994) observed inflamed reddish bellies in adult *Bufo bufo* that migrated across fields that had been treated the year before with *N*-fertilizers. Nitrate and nitrite caused edema and bent tails in tadpoles of some but not all species at lethal concentrations (Marco et al. 1999). Hecnar (1995) detected eye and head anomalies, complete depigmentation and digestive tract deformities in tadpoles treated with ammonium nitrate. These types of anomalies clearly differ from those observed in Roßwag.

Pesticides. The water samples and tissue analyses did not reveal any indication for pesticide contamination; however, pomuran (captan + mancozeb) and rovral (= iprodion; [3-(3,5-dichlorophenyl)-2,4-dioxoimidazolidinyl]-N-(methylethyl)carboxamide) were sprayed by helicopter to vineyards bordering the quarry and thus were probably drifted into the water bodies of the Roßwag quarry as well. Mancozeb can cause edema, abnormal notochord, bent tail and gill displacement in embryos (Ghate 1985, Harris et al. 1998) but we do not know any experiment involving tadpoles. We found no studies testing the teratogenicity of the other substances for amphibians. Captan is usually assumed to be weakly mutagenic in vitro but requires

bioactivation and is not mutagenic in vivo (Osaba et al. 2002, Rahden-Staron 2002). For mancozeb, assessments of biomarkers for genotoxicity in pesticide workers have provided positive and negative results (reviewed by Bull et al. 2006). For iprodion, we found no information regarding mutagenicity. Captan and mancozeb are classified as carcinogens and iprodion is classified as a probable carcinogen (Pastor et al. 2003).

Anomalies reported for most other pesticides are mainly of a general teratogenic type, such as exogastrula, abnormal neurula, anophthalmy, microcephaly, bent bodies or tails and edema in embryos and bent bodies and tails, growth retardation, mouth anomalies and edema in tadpoles (e.g., Cooke 1981, Power et al. 1989). In addition, many pesticides can induce pale tadpoles (Henle et al. 2017a). Only few pesticides are known to cause darkening: diamethoate and benzene hexachloride (Pandey & Tomar 1985), dichlorvos (Tomar & Pandey 1988) and lindane (Marchal-Ségault & Ramade 1981). No other pigmentary changes resulting from exposure to pesticides are known. Defenuron is the only pesticide known to us that results in giant tadpoles (Paulov 1977). Several pesticides cause various skeletal anomalies (Tab. 11) and, according to Levy (1958), semicarbazide may induce tumours but this needs confirmation. In any case, at environmentally relevant concentrations, agrochemicals (no differentiation of data among glyphosate, dimethoate, chlorpyrifos and propanil) may cause skin ulcers in tadpoles (Jayawardena et al. 2010b).

In summary, the fungicides sprayed on neighbouring vineyards may potentially have contributed to the tumours observed in the Roßwag toads. However, pesticides are not known to cause any of several conspicuous anomalies, notably polymely, stiff and truncated vertebral columns, yellow spots and abnormal patterns. Also, if pesticides contributed significantly to the anomalies, we would expect higher rates and more severe anomalies in the years 1981–1984 compared to 1980 because the water bodies were much smaller, which in turn should lead to higher and thus more teratogenic, concentrations of pesticides in the breeding sites. This expectation is opposite to the observed frequency and severity of the anomalies. Moreover, the pesticides sprayed on neighbouring vineyards were in common use and one would expect many other populations with anomalies unless their application was unusually careless on vineyards in Roßwag. Finally, treatments with specific pesticides result in similar anomalies in all affected individuals and not in a hige range of individually differing anomalies, as observed in Roßwag.

Industrial waste. Crankcase oil and crude oil can cause edema and a deformed axial skeleton in amphibian larvae (Pyastolova & Danilova 1986, Mahaney 1994), coal combustion waste result in bent tails and missing labial teeth rows (Rowe et al. 1998, Hopkins et al. 2000), PCBs can lead to lordosis, scoliosis and edema in embryos of toads (Birge et al. 1978) and stiff legs in metamorphosed individuals (Qin et al. 2005), whereas tar and benzpyrene can cause high rates of tumours (Koch et al. 1939). These are but a small fraction of the different types of anomalies observed in Roßwag and there were no signs of dumping industrial waste in the quarry.

Endocrine disruptors and endocrine substances (hormones). A range of chemicals, including various pesticides and/or their metabolits, such as DDT (Osborn et al. 1981), maneb 80 (Fort et al. 1999) and methoprene (Ankley et al. 1998, La Clair et al. 1998), as well as medical drugs (e.g. thalidomide: Bazzoli et al. 1977, dumpert & Zietz 1984) may interfere with the endocrine pathways of developing organisms and cause various anomalies in amphibians. Anomalies caused by pesticides are reviewed above. For a review of the effects of endocrine disruptors on amphibian development and their environmental significance see the review of Hayes (2000). The insecticide methoprene, which can mimic natural retinoid acids (Harmon et al. 1995) and chemicals inferring with the thyroid axis have received particular attention and are briefly reviewed below.

Endocrine substances – retinoids. In North America concerns have been raised that mass occurrence of leg malformations may be attributable to the disruption

Table 11: Skeletal anomalies caused by pesticides.

Substance	Species	Effects	Author(s)
Acetyl hydrazine	*Xenopus laevis*	Ectromely and torsion of legs	Fort & Stover 1997
DDT, DDE	*Rana temporaria, Bufo bufo*	Stiff legs, abnormal snouts, mandibular hypoplasia (together with loss of keratinized beaks, torsion of body and tail and transient colour change in tadpoles)	Cooke 1971, 1972, 1973, Osborn et al. 1981
Diquat	*Rana temporaria*	Mandibular hypoplasia	Gelnarová 1987a,b
Endosulfan	*Rana temporaria*	Stiff legs	Gelnarová 1987a,b
Malaoxon	*Xenopus laevis*	Ectromely, bent vertebral column, abnormal acetabular joint	Snawder & Chambers 1989
Maneb	*Xenopus laevis*	Ectromely, torsion of legs	Fort et al. 1999
Methoprene	*Lithobates pipiens*	Ectrodactyly, ectromely and severe axial distortions (at concentrations causing 100% mortality in the laboratory)	Ankley et al. 1998
S-methoprene	*Lithobates sphenocephalus*	Ectromely (in addition anophthalmy, amelanism at non-significant rates) under field conditions	Sparling 2000
Thiosemicarbazide	*Xenopus laevis, Lithobates sylvaticus*	Clinodactyly, abnormal limb articulations (dislocation of joints), bent long bones	Newman & Dumont 1983, Riley & Weil 1986

of retinoid signalling pathways (Gardiner & Hoppe 1999). Retinoids are metabolic derivatives of vitamin A that act as signalling molecules and regulate many processes critical to early embryonic development (Sucov & Evans 1995), in the initiation of pattern formation of limbs and in limb regeneration (Lee et al. 2004). Alterations in retinoid levels, whether excess or deficiency, resulted in developmental anomalies in nearly all of the vertebrate species studied (Degitz et al. 2000, Lee et al. 2004). The teratogenic effects depend on the concentration, the duration of treatment and the developmental stage at treatment. Effects also differ among normal and regenerating limbs (Maden 1983, Niazi 1996) and according to whether tadpoles are raised in retinoid acid solutions or fed retinoid acid.

In regenerating limbs, low doses of retinoids and synthetic activators of retinoid acid receptors cause ectrodactyly, intermediate doses induce polymely and high doses suppress regeneration (Saxena & Niazi 1977, Maden 1983, Thoms & Stocum 1984, Scadding & Maden 1986b). In developing limbs, it suppresses differentiation causing shortened skeletal elements, oligodactyly or ectromely as well as triangular bony excrescences (Scadding & Maden 1986a, Degitz et al. 2000, Gardiner et al. 2003). In contrast to other studies, Bruschelli & Rosi (1971) and Gardiner et al. (2003) also obtained polymely in developing limbs. In rodents polydactyly has also been observed following a reduction in the dose of Hox gene products, which are involved in the signalling pathways of retinoids (Lee et al. 2004). The disparity regarding polymely may be explained by the high stage specificity of the teratogenic effects (Niazi 1996). In the experiment of Gardiner et al. (2003), polymely only occurred when stage 51 (after Nieuwkoop & Faber 1994) tadpoles of *X. laevis* were treated. Limb elements are only sensitive at the beginning of their differentiation and this sensitivity seems to progress sequentially and proximodistally; therefore phocomely can only be produced if retinoid treatment is restricted to the short period of the initial differentiation of shanks or thighs (Niazi 1996).

Increased circulating retinoid acid also caused significantly elevated rates of bony triangles and ectrodactyly but it additionally significantly increased syndactyly, anophthalmy and microphthalmy in *Xenopus laevis* (Alsop et al. 2004). It also caused polyphalangy and duplicated digits (polydactyly), albeit at non-significant levels. Effects further depended on the stages that were exposed, with stages 46 (after Nieuwkoop & Faber 1994) having been more sensitive than later stages for eye anomalies; limbs tended to be more affected when stage 49 or 52 were fed retinoid acid compared to stage 46. Various agrochemicals (Mann et al. 2009, Paganelli et al. 2010), polychlorinated toxicants, estrogen, pharmaceutica and inflammation may increase internal cycling levels of retionoid acids and one study showed that compounds in pulp mill effluents that probably originated from the wood used bound to retinoid acid receptors (Alsop et al. 2004, Lee et al. 2004).

Embryo lethality occurs at much lower concentration than those necessary to cause reductions and deletions of the hind limb (Degitz et al. 2000). This suggests that if retinoid mimics in the environment are causing hindlimb malformations, this would only occur under scenarios of pulsed-chemical exposure or when water bodies became contaminated at later larval stages of development. In any case, neither potential sources of retinoids or retinoid mimics are known from the Roßwag quarry and, importantly, the spectrum of anomalies differ, with bony triangles being absent in Roßwag and neither colour anomalies, nor edema or giant tadpoles being induced by retinoids. Thus, retinoids are unlikely to have been a contributor to the anomalies in the Roßwag *B. viridis* population.

Endocrine disruptors – thyroid axis. In the laboratory, antithyroidal substances are among the most potent teratogenic chemicals. Feeding antithyroidal substances (thymus oil, various plant seed oils or extracts of the thyroidea) to tadpoles of *X. laevis* resulted in 8–13 anomalies (phocomely, oligodactyly, clinodactyly, reduction or absence of bones of the pelvic girdle, torsion of the legs, stiff legs, front legs covered by a skin fold, mandibular hypoplasia, edema, giant tadpoles, tail resorption incomplete, exophthalmy, degeneration of keratinized mouthparts and papillae, dark pigmentation) (Romeis 1918, Pohland 1962, Wurmbach et al. 1964, Wink & Wurmbach 1967). Thymus oil suppresses cell division but it is not yet clear whether it acts directly on the cell or indirectly via the thyroid gland. Thiourea causes similar changes to the basophilic cells of the thyroid as thyroidectomy (Gasche 1946) and estradiol (Nishimura et al. 1997), which blocks the conversion of thyroxine into the more potent triiodothyronine (Maclatchy et al. 1986). Estradiol and the pesticide defenuron (Paulov 1977) cause giant tadpoles with estradioal sometimes also causing goitre. Raising tadpoles in solutions of estradiol benzoate may cause high frequencies of ectrodactyly and clinodactyly and testosterone proprionate may cause syndactyly and edema (Collenot 1965). Thus, contamination by antithyroidal substances or substances that have steroidal effects could have contributed to the anomalies observed in Roßwag. However, no potential source for any contamination with such substances is known for the Roßwag quarry. In any case, they cannot explain all conspicuous and frequent anomalies, especially polymely, ectromely, yellow spots, albinism and tumours. Moreover, these substances do not accelerate growth in some tadpoles but retard it in others and individuals exposed to similar concentrations show similar symptoms.

Mutagenic chemicals. To our knowledge, all studies undertaken to test the mutagenicity of chemicals were made on the cellular level (e.g., Siboulet et al. 1984, Fernandez et al. 1993) except for studies by Armstrong and colleagues (e.g., Armstrong & Gillespie 1980, Hart & Armstrong 1984), who immersed adult males of *A. mexicanum* and *X. laevis* in ethyl methanesulfonate or ethyl nitrosurea and examined anomalies in the offspring. They focused on

chromosomal anomalies and morphological anomalies of embryos. Without providing details they listed edema, torsion of body, microcephaly, microphthalmy, vertebral truncation and reduced pigmentation in hatchlings (Hart & Armstrong 1984). Additionally, Armstrong & Gillespie (1980) showed an individual with ectrodactyly and amely. Since no details for tadpoles or metamorphosed individuals are available, a comparison with the Roßwag population of *B. viridis* is difficult. However, anomalies differed strongly among individuals, which is consistent with the observations in Roßwag and included vertebral truncation, which is an anomaly that has been infrequently reported for amphibians (see Henle et al. 2017a) but had appeared in several individuals in Roßwag.

Summary evaluation. Apart from the chemicals sprayed on the neighbouring vineyards, which may have contributed to edema, ectromely, bent tails and tumours, no other evidence for potential chemical contamination in the Roßwag quarry is available. However, it is conceivable that the earthen deposit in the quarry could have contained undetected contaminants because of degradation at the time samples were taken and because the chemical analyses undertaken were of limited scope.

Different types of chemicals taken together can induce most of the anomalies observed in Roßwag. Notwithstanding, undetected chemical contamination in the quarry could not explain the occurrence of anomalies in individuals developing in rain puddles at the border or outside the quarry. Mutagenic substances are an exception since they may have affected adults that spawned in the rain puddles in later years, but the pesticides sprayed on the neighbouring vineyards are not regarded as potent mutagens (see above). Moreover, mutagenic chemicals excluded, individuals treated with a particular chemical substance show the same types of anomalies, not different ones, which is in contradiction to the observations made in Roßwag. Furthermore, most chemical substances, for which we have data, are able to induce only a small number of different types of anomalies. Embryonic anomalies and mutagenic chemicals excluded, only the feeding of tadpoles on antithyroidal substances (thymus oil, various plant seed oils or extracts of the thyroidea) resulted in more than ten types of anomalies (a maximum of 13; see above). The highest number for any pesticide we found was seven types when newts (*Triturus carnifex*) with amputated legs were exposed to maneb 80, which most likely also interferes with the thyroid axis (Arias & Zavanella 1979, Zavanella et al. 1984). Thus, only a cocktail of pesticides and other contaminants, with individual tadpoles exposed to different chemicals within the cocktail or mutagenic substances could explain a considerable part of the anomalies observed in Roßwag. Still, non-mutagenic chemicals cannot explain yellow spots and abnormal colour patterns, nor the fact that anomalies developed in eggs transferred to the laboratory and that yellow spots were heritable. Finally, it is difficult to imagine how individuals living in the same, partly small water body should become exposed to different chemicals within a chemical cocktail. In conclusion, while mutagenic chemicals would be consistent with the observations of anomalies made, we have no indication that such substances could have contaminated the Roßwag quarry as well as the rain pools on its border.

6.1.3 Factors revealing or inducing genetic-based anomalies

6.1.3.1 Traumatic parthenogenesis (pricking of eggs). Parthenogenesis means that the egg develops solely from genetic material from the ovum, without any participation of DNA from a spermatozoon. Parthenogenetic offspring may be haploid, homozygous diploid or polyploid. Parthenogenesis can be achieved in various ways: it is "spontaneous" in nature in many groups of organisms and it can be artificially induced by several kinds of intervention, such as the chemical treatment of the sperm (e.g., inactivation of the nucleus by acriflavine, toluidine blue, methylene blue or thionine) or by physical action (e.g., cold and hot temperature shocks, sub-lethally high temperatures, high hydrostatic pressure, irradiation of the sperm, pricking the egg with a fine glass needle or cross fertilization with distantly related species of the same or a different genus) (e.g., Hertwig 1898, Bataillon 1910, Fischberg 1948, Moore 1955, Miyada 1960, 1977, Weiss 1960, Gallien et al. 1965, Pogany 1976, Gillespie & Armstrong 1979).

Evidence put forward for this hypothesis. This explanation was made by Ewe (Univ. Stuttgart-Hohenheim) in an attempt to suppress a planned publication on the Roßwag toads that nevertheless appeared in a popular science magazine (Kovacsics 1981). No supporting evidence was provided.

Anomalies known to appear in traumatic parthenogenetic individuals. Usually, the pricking of eggs mainly results in haploids (e.g., Parmenter 1933), where anomalies generally manifest at the beginning of the gastrula stage. This is the earliest stage at which paternal genes are activated (Davidson 1976). Most individuals die at this stage (e.g., Hertwig 1923, Volpe & dasgupta 1962). Surviving embryos often develop edema, microcephaly, defective eyes, defects of the circulatory and digestive system, as well as abnormal otocyst and brain formation. Individuals that reach free-swimming larval stages and metamorphose are rare exceptions (Baltzer 1922, Parmenter 1933, Miyada 1960, 1977, Nishioka & Kondo 1978). If individuals survive the embryonic stage, they will usually develop normally (Böök 1941, 1943, Fischberg 1948). One of the reasons for the lethality of haploidy may be that any lethal recessive allele, which in a diploid embryo is "silenced" by a dominant "wild" allele, can be expressed in the haploid and entails death.

Pricking at the 2-cell stage stops further development of the pricked cell and incomplete or partially duplicated embryos develop from the other cell (Endres & Walter 1895).

Evaluation. Traumatic parthenogenesis through the pricking of the egg is very difficult to achieve (e.g., Bataillon 1910, Parmenter 1933). One needs to use a very fine needle, polluted by a few drops of blood, as some material must be pushed into the egg (Maiorca et al. 1975). Hundreds of eggs must be individually pricked before one develops; this is a very painstaking procedure. The idea that birds could prick toads' eggs (but not eat or even kill them!) and induce parthenogenesis is *completely absurd*, both from a biological point of view regarding bird behaviour and from the point of view of traumatic parthenogenesis, as a bird's beak is much too large to have the action of a very fine needle.

6.1.3.2 Atavism. ***Evidence put forward for this hypothesis.*** Being aware of the shaky grounds for hybridization as an explanation of the anomalies in the absence of any evidence for it and the presence of many contradictory results, Flindt (1985a) argued that hybridization in the past combined with atavism caused the anomalies. As evidence, he provided hearsay from an elderly lady (Anonymous 1981b), who soon afterwards supposedly suffered from advanced Alzheimer's disease (Henle et al. 2017b). He cautiously introduced the hearsay but when he came to his final conclusions, it was solid as a rock. He additionally took the adult individual with a supernumerary bone at the angle of the jaw found by Christaller (1983) in 1981 as proof that the anomalies must have existed before 1980 in the quarry. In combination with inbreeding and hybridization, this reasoning was taken as justification to regard the anomalies observed in Roßwag as a normal occurrence by the official inquiry (Landesregierung von Baden-Württemberg 1984).

Anomalies known to be caused by atavism. Only epigenetic processes may lead to the expression of genes that are silenced under normal conditions, which then may lead to abnormal development. For example, high temperatures during larval development may induce ectromely and ectrodactyly in a specific line of *Pleurodeles waltl* that carries a mutation sensitive to temperature (Lauthier 1971, Dournon et al. 1998).

Evaluation. Atavism is the sudden reappearance of ancient characters. It is often used in a metaphysical frame with no reference to any mechanism that may cause this reappearance. Placed in a modern genetic context, one might explain "atavism" under the concept of epigenetics as genes that have been silenced in previous generations and then have been reactivated. Although methylation of the DNA that may silence genes is usually completely erased shortly after fertilization, examples of transgenerational persistence of methylation of silenced genes are meanwhile known for a range of organisms (Gilbert & Epel 2008, Vargas 2009). However, no mechanism is known that would silence many genes coding for a range of different morphological characters and colouration simultaneously for decades and then suddenly erase this silencing in a single year. Beyond that, it would not provide an explanation for the origin of the anomalies, it would only shift the timeframe.

Lethal mutations do not accumulate with time (Falconer 1989, Hedrick 1994, Kirkpatrick & Jarne 2000). On the contrary, in their presence even mildly deleterious mutations are rapidly purged (Gilligan et al. 1997). The malformed adult discussed by Flindt (1985a) may or may not have been born before 1980, the year of the discovery of the mass anomalies. *Bufotes viridis* grows rapidly and may reach adult size within a year (Günther & Podloucky 1996). Of two marked individuals recaptured by Flindt & Hemmer (1970b), one had reached 52 mm in the second year, making it feasible that the adult in question had metamorphosed in 1980, especially since the quarry operated intensively under flood light in 1980 (Henle et al. 2017b); flood light attracts insects and toads, including *B. viridis,* gather under the light to feed on these insects (Baumgart 2003).

6.1.3.3 Hybridization. ***Evidence put forward for this hypothesis.*** Sander suggested this hypothesis in a parliamentary hearing (Henle et al. 2017b). He based his hypothesis on hearsay: the supposedly definitive observation of *B. bufo* spawn in the quarry by the manager of the quarry. Independently, Flindt (1985a,b) explained three phenomena observed in Roßwag by hybridization between *Bufotes viridis* and an unspecified species (*B. bufo* or *Epidalea calamita*): (1) various anomalies (edema, curvature of the tail, malformed mouthparts, etc.) of young tadpoles and the anomalies of the tadpoles that had hatched from the white clutch collected in the neighbouring quarry, because he regarded them as characteristic for interspecific hybridizations of amphibians; (2) the giant tadpoles, which he attributed to heterosis (hybrid vigor); (3) polymely of metamorphosed individuals, which he related in a diffuse way to anomaly P of green frogs (*Pelophylax* synkl. *esculentus*) and the hybrid character of *P. esculentus*. The official position of the State Government of Baden-Württemberg explained all anomalies with hybridization in combination with inbreeding and atavism (Landesregierung von Baden-Württemberg 1984).

Anomalies reported from experimental hybridizations are summarized in Table 9. Anomalies depend on the species involved. Artificial hybridizations between European toad species and other genera fail to develop (Pflüger & Smith 1883, Born 1886, Tchou-su 1931). The results of hybridization experiments among European toad species strongly depend on the species involved, the paternal and maternal species, the geographic origin of the specimens and the individuals involved in the cross (different results may be obtained with different parents of the same species). All crosses between *B. bufo* males and *B. viridis* females died as embryos (Born 1883, 1886, Hertwig 1918, 1930, 1953, Montalenti 1933, Perri 1946, Hertwig & Weiss 1955, Vlcek 1995, Hertwig et al. 1959, Delarue 1982, Zavadil & Roth 1997), with the exception of one of two crosses performed by Blair (1972), in which

6.1% metamorphosed. No further details were provided by Blair (1972). Death at early embryonic stages can be explained by the expression of parental genes, which starts at the gastrula stage (Davidson 1976). Reciprocal crosses also either died as embryos or, if they were viable and reached advanced tadpole stages, at most a few individuals showed anomalies (Born 1883, 1886, Pflüger & Smith 1883, Hertwig 1918, 1953, Lang 1926, Montalenti 1933, 1939, Perri 1946, Hertwig & Weiss 1955, Meusel 1955, Hertwig et al. 1959, Weiss & Ziemann 1959, Flindt & Hemmer 1970a, Delarue 1974, 1982, Kawamura et al. 1980, Zavadil & Roth 1997, Bressi et al. 2000, Zavadil et al. 2003, Duda 2008). The only anomalies repeatedly obtained at elevated rates in experiments are edema, the torsion of tails and microcephaly in embryos and young tadpoles, which do not complete their development. Occasionally, a few albinistic, dwarf or giant tadpoles and missing eyes in metamorphosed individuals were reported but never any skeletal anomalies.

Similarly, crosses between *E. calamita* males and *B. viridis* females either died at early tadpole stages at the latest (Born 1886, Meusel 1955, Blair 1972, Delarue 1982) or metamorphosed normally, with at most a small percentage showing anomalies (maximum frequency reported: 5.9%; $n = 341$) (Hertwig et al. 1959, Flindt & Hemmer 1967b, 1970a,b, Siboulet 1971, Grosse 1976, 1977). The observed anomalies were edema, microcephaly, the torsion of tails, stiff legs, large and dwarf tadpoles and one albinistic individual. Reciprocal crosses almost invariantly died at early embryonic stages (Hadorn 1955, Meusel 1955, Hertwig et al. 1959, Weiss 1960, Flindt & Hemmer 1967b, Flindt et al. 1968, Schipp et al. 1968, Siboulet 1971, Delarue 1982), with a single exception in which nine individuals survived the hatching stage and developed normally (Weiss 1960).

Although gonads appear normal in some juveniles (Perri 1949), the rare hybrids of Europen toads that reached sexual maturity and were tested were sterile (Hertwig & Weiss 1955, Weiss & Ziemann 1959).

Evaluation. Morphologically, the strings of *B. bufo* and *B. viridis* are almost identical and it is practically impossible to differentiate between them, even for specialists (Arnold & Burton 1978, Nöllert & Nöllert 1992, Günther 1996), which seriously questions the only supposed field evidence put forward for a hybrid origin of the Roßwag anomalies. Whereas it is known that hybridization can lead to different types of anomalies (Tab. 9), the anomalies attributed to hybridization by Flindt (1985a) are not at all typical for hybridization. Rather, they are common anomalies that can have a very wide range of causes (Tab. 9). Moreover, only approximately 50% of the different types of anomalies observed in Roßwag have ever been reported from hybridization experiments even if anomalies are included that have been observed only rarely or in a single experiment (Tab. 9; based on 78 different combinations of hybridizing species).

In spite of Flindt's (1985a) insistence on the hybrid hypothesis, it is impossible as the cause of the anomalies observed in Roßwag. First of all, only *B. viridis* was found in the quarry between 1980 and 1983. A large number of individuals of a second species is an essential prerequisite because of the low viability of hybrids (see above) – and even then the frequency of abnormal individuals would be much lower than observed. It is very unlikely that large numbers of *E. calamita* males or *B. bufo* females (only their crosses with *B. virdis* can survive beyond embryonic stages – see above) were overlooked in all surveys given the high visibility at the breeding sites in Roßwag. Moreover, the habitat is typical for *B. viridis* but only marginally suitable for *E. calamita*. *B. bufo* does not occur in similar habitats in the region (own unpubl. observations). In general, it rarely breeds in quarries in Baden-Württemberg and tends to spawn much earlier (Sowig & Laufer 2007).

No mixed populations are known in the region of Roßwag (Christaller 1983, Flindt 1985a). The next population of *B. bufo* lived 1–1.5 km SSE of the quarry (Flindt 1985a), with two country roads and the river Enz separating it from the quarry. The next closest population occurred NNW of Illingen at a distance of approximately 2.5–3 km (Christaller 1983). A village and a major road separate it from Roßwag. Switching breeding sites that are separated by more than 1 km is rare for *B. bufo* even in the absence of barriers (Reading et al. 1991). The closest existing large population of *E. calamita* was separated by a distance of approximately 18 km. This distance is far beyond the known dispersal capacity of the species, ranging to a maximum of 3–5 km, with the movement of most males being restricted to a radius of less than 600 m around the breeding site (Sinsch 2009) – and only males can produce viable offspring when hybridizing with *B. viridis* (see above)! Blab (1986) suggested that under very high density, the maximum distance for founding new populations is 8-10 km although there is no supporting documentation for this dispersal distance. In any case, the closest extant population known in the region was located well beyond that distance (Christaller 1983) and even the closest extinct population was more than twice the documented dispersal distance away from Roßwag. Moreover, it is highly inconsistent to assume that many individuals of other toad species immigrated purely for mating from great distances and to state at the same time that the population was inbreeding and consequently closed to immigration from a much closer conspecific population in the neighbouring quarry.

Importantly, Flindt (1985a) collected eggs from a pair that was genetically and morphologically *B. viridis*. Tadpoles and toadlets raised from the clutch developed anomalies. Moreover, all of Flindt's (1985a) morphological and genetic analyses contradicted hybridization as a cause. Previously, all authors, including Flindt himself, regarded serum albumin

electrophoresis and the selected combination of morphological characters studied by him as reliable for the discrimination of European toad species and their hybrids (Flindt & Hemmer 1973, Kabisch & Engelmann 1975, Siboulet et al. 1975). However, for the official report, Flindt (1985a) rejected the reliability of genetic diagnostics arguing that hybridization may have taken place in the distant past and that all diagnostic characters were lost while genetic incompatibility was maintained – a position that is biologically impossible. The chance of fixation is highest for neutral or mildly deleterious mutations (Lande 1995). It is theoretically impossible that neutral genetic markers would be lost but a large number of (semi-)lethal genes are maintained in the population (Falconer 1989, Kirkpatrick & Jarne 2000) and the studies of Kawamura & Nishioka (1978) and Nishioka (1978) have confirmed this experimentally for amphibians. Moreover, adult *B. viridis* × *B. bufo* hybrids are sterile (Hertwig & Weiss 1955, Weiss & Ziemann 1959).

Finally, it is hard to explain why hybridization should have a decreasing effect from 1980 to 1984, why the Roßwag population should show a much wider range of anomalies than experimental hybridizations and why such anomalies were never reported for populations, in which *B. viridis* lives sympatrically with other toad species and especially from those for which natural hybridizations have been demonstrated (e.g., Flindt & Hemmer 1967a,b, Glaw & Vences 1989, Andrä 1994). In summary, to consider hybridization as an explanation, one must first make several highly unlikely assumptions followed by several biologically impossible ones and even then only a few of the anomalies observed in the Roßwag toads could be explained.

6.1.3.4 Inbreeding and low genetic variability. *Evidence put forward for the cause.* Flindt (1985a) introduced inbreeding as an alternative explanation to hybridization. The official position of the state government (Landesregierung von Baden-Württemberg 1984) combined inbreeding, hybridization and atavism in their explanation of the anomalies. No supporting evidence for inbreeding was given.

Anomalies known to be caused by the factor (Tab. 9). We found only two studies on the effects of genetic variability on anomalies in amphibians. Weyrauch & Grubb (2006) reported a significant influence of genetic diversity (as measured by arbitrary RAPD primers) on rates of bent and wavy tails in tadpoles of *Lithobates sylvaticus*. In contrast, Williams et al. (2008) did not find any significant difference in several measures of inbreeding between abnormal and normal individuals of *Ambystoma tigrinum*.

With the exception of stiff legs, inbreeding has never been a major issue in amphibian husbandry and thus to cause anomalies infrequently even in small founder populations typical for captive breeding. Heselhaus (1983) observed stiff legs in all offspring of all crosses of one pair of *Epipedobates tricolor* (as *Dendrobates*) and in all offspring of a *Colosthetus* sp., but only when sibs were bred. Schmidt (1985) also attributed ectromely and ectrodactyly that appeared in captive offspring of dendrobatid frogs to inbreeding but he obtained the same anomalies in offspring of individuals caught in the wild, making the explanation unlikely. Inbred *Osteocephalus elkejungingerae* exhibited black eyes (Henle 1992). Oeser (1952) obtained albinistic *Phrynobatrachus* sp. from inbreeding and maintained them for several generations.

Stocks of *Pleurodeles waltl, L. pipiens* and especially *A. mexicanum* and *X. laevis* have been maintained in the laboratory for many generations (Moore 1955, Gallien 1969a,b, Armstrong 1985, Gurdon & Woodland 1975). They have been subjected to inbreeding and various other genetic techniques to discover recessive genes. These studies revealed a genetic basis for the following anomalies: polydactyly in *X. laevis* (Uehlinger 1969), ectrodactyly in *A. mexicanum* (Humphrey 1967b) and *X. laevis* (Droin & Fischberg 1980), phocomely in *A. mexicanum* (Humphrey 1975), stiff legs (Krotoski et al. 1985, Kovalenko 2000a, Kovalenko & Kovalenko 2000), fused vertebrae (Kovalenko 1994), truncated vertebral column (Krotoski et al. 1985), lack of otoliths (Droin 1967) and degeneration of the lower jaw in tadpoles (Droin & Beauchement 1975) of *X. laevis*, as well as abnormal eyes of *A. mexicanum* and *X. laevis* (Armstrong 1985, Krotoski et al. 1985). Edema, the torsion of tails and microcephaly in embryos, also frequently appeared in breeding experiments with *A. mexicanum, P. waltl, X. laevis* and *L. pipiens* as well as in other *Xenopus* species (e.g., Elsdale et al. 1958, Gallien & Collenot 1964, Beetschen & Jaylet 1965, Uehlinger & Reynaud 1965, Uehlinger 1966, Humphrey 1967b, Droin et al. 1970, Droin 1978, Droin & Colombelli 1982, Korotoski et al. 1985). Albinistic and melanistic lines were obtained in *A. mexicanum* (e.g., Bagnara & Obika 1964, Humphrey 1967a, Borack 1972, Frost et al. 1984) and in *L. pipiens* (Gibbs et al. 1971, Browder 1972), albinistic ones in *P. waltl* (Lacroix & Capuron 1970), transient albinism in *A. mexicanum* (Humphrey 1975) and *X. laevis* (e.g., Hoperskaya 1975, 1981, Mikhailov & Korneev 1980, Droin & Fischberg 1984) and transient and permanent absence of yellow pigmentation in *Pleurodeles waltl* (Collenot & Collenot 1985, Collenot et al. 1989). Furthermore, maintenance of the brown embryonic colouration to the tadpole stage in *X. laevis* (Uehlinger & Droin 1969) and bright yellow patches of xanthophores on the head of *A. mexicanum* (Tompkins 1970) appeared in breeding stocks. A female descendent from an irradiated male *Pleurodeles waltl* carried a recessive mutation in which melanophores were morphologically normal but lacked the ability of expansion and contraction for matching the brightness of the background (Jaylet et al. 1980).

Apart from embryonic anomalies, it is rare that more than two types of anomalies that form a syndrome occurred in single inbred lines, despite the fact that some of these lines have been maintained in the laboratory for decades (Gurdon & Woodland 1975). In some mutants of *A. mexicanum,* albinism is combined with ectromely, a lack of ribs and edema (Humphrey 1967b, 1973, Martin & Signoret 1968, Malacinski 1978), in others edema is combined with abnormal kidneys and gills, microphthalmy and feet that do not differentiate (Humphrey & Chung 1978) or with abnormal gills and the distribution of melanin (Humphrey 1972). Jaylet (1971) obtained a line of *P. waltl* with abnormal vertebrae, poorly formed eyes and an open mouth. In another line, ectromely, ectrodactly, twisted feet and a reversed knee joint (anteversion) are inherited, with high temperature acting epigenetically, with the severity and variability of the anomalies increasing the sooner the heat treatment began (Lauthier 1971, Dournon et al. 1998). The lethal recessive mutation "ulcer" causes ulcers, reduced gills, weak ventral edema and ectromely (Signoret et al. 1966, Gounon & Collenot 1975). A single recessive gene causes brachymely, syndactyly, polydactyly and brachydactyly in *X. laevis* (Droin & Fischberg 1980) and another one brachydactyly, brachymely, brachycephaly, nanism, kyphosis and bulging eyes in *A. mexicanum* (Lipsett 1941). In the latter case, most individuals die at early larval stages. In all of the cited cases, all individuals show the same type of anomalies (or syndrome of anomalies). Three unlinked mutant genes that cause lethal albinism respectively microcephaly, edema and bent tails have been observed within one family line of *X. laevis* (Droin 1992).

Evaluation. What is fundamental to genetic theory (Falconer 1989) and has been demonstrated in numerous experiments (see above) is that inbreeding reduces variability. If anomalies appear they are always of the same type(s). This is incompatible with the extreme variablility in the Roßwag population of *B. viridis*, with few abnormal individuals resembling each other. Moreover, it is inconsistent to assume that many individuals of other toad species immigrated solely for the purpose of mating from great distances (see previous section) while claiming at the same time that the population was inbreeding and consequently closed to immigration from a much closer conspecific population in the neighbouring quarry. The minimum size of the Roßwag population falls within the upper size classes known for the species for Baden-Württemberg and Germany (Günther & Podloucky 1996, Laufer & Pieh 2007). If inbreeding had caused anomalies in one of the largest populations, then most German populations would now show serious anomalies. To explain the extraordinary variation of anomalies in the Roßwag population, one would need to assume that at least 32 inbred lines existed in the quarry without mixing. This would mean that all individuals selected only closely related individuals for breeding. With males being able to mate multiple times, the probability that 32 females selected a related male from the same inbred line out of 32 co-existing ones is

$$\left(\frac{1}{32}\right)^{32} = 7 \times 10^{-49} \; !$$

Thus, to retain the inbreeding hypothesis, one would have to assume that the Roßwag toads (and only these toads) were able to recognize their closest kins and sought incest instead of avoiding it.

6.1.3.5 UV-B (increased natural levels). ***Evidence put forward for the cause.*** Although this was never suggested for the Roßwag toads, increased awareness of ozone depletion and increased levels of UV-B have triggered concerns that amphibian anomalies could be caused by increased levels of UV-B (Grant & Licht 1995, Blaustein et al. 1997).

Anomalies known to be caused by UV-B are summarized in Table 9**.** UV-B can damage the DNA, especially by producing pyrimidine dimers. Enzymes, such as photolyase, can repair damaged DNA but require UV-A or visible light for this (Licht & Grant 1997). The earliest studies examined the effects of UV-B at environmentally irrelevant high doses. Under such conditions, UV-B deactivates the nucleus in newly fertilized eggs, leading to embryos with twisting, abnormal neurula and a curvature of the medullary plate. UV-B treatment before first cleavage may further interfere with egg reorganization leading to more or less severe reductions of the head and tail proportion of embryos (Malacinski et al. 1977, Scharf & Gerhart 1983). When tadpoles are treated, mainly a variety of skin abnormalities, a curvature of the spine (lordosis) and tail kinks are observed and melanin pigmentation on the dorsum and the cornea may increase (Worrest & Kimeldorf 1975, Grant & Licht 1995). Fite et al. (1998) induced various abnormalities in retinal pigment cells of adult *Lithobates pipiens* by exposure to high intensity fluorescent light that resembled the histopathological conditions detected in adult *Rana cascadae* collected from high altitudes in the wild. When UV-B was enhanced 4.8–23% over ambient levels at noon, *Pseudacris regilla* and *R. aurora* developed significantly more skin sores and opaque lenses than controls (Novales Flamarique et al. 2000). Most adult *Triturus carnifex* developed tumours after intensive UV-B irradiation (Zavanella & Losa 1981). In a variety of organisms, including humans, tumours may also develop at environmentally relevant doses (van der Leun & Gruijl 1993) but for amphibians such data are lacking.

The relevance of UV-B for anomalies in natural populations of amphibians has been controversial. Licht & Grant (1997) did not find any anomalies in embryos of *L. clamitans* and *L. sylvaticus* in treatments with environmentally relevant doses. They reviewed earlier studies on the effects of UV-B enhancement or shielding on amphibians and concluded that under natural conditions UV-B is unlikely to cause anomalies. In contrast, Blaustein et al. (1997) argued that natural levels of UV caused the torsion of tails, blisters and edema

in *Ambystoma macrodactylum*. Starnes et al. (2000) did not obtain significantly increased rates of anomalies in *L. sylvaticus* and *A. maculatum* but in *Dryophytes chrysoscelis* and *P. triseriata* (under current taxonomy presumably *P. feriarum*) (blisters, malformed tails, torsion of the spine and edema). Pahkala et al. (2001) observed significantly elevated rates of anomalies (ectromely, ectrodactyly, bent knees and the torsion of tail) in metamorphosed *R. temporaria* that were treated as embryos with enhanced UV-B still in the range of the natural variability. In contrast, Smith et al. (2000) did not obtain such a delayed effect in *L. blairi*.

These studies suggest species differences in sensitivity to UV-B and that the conditions under which the experiments were conducted also strongly influenced the results. However, in most studies the dose actually received by the treated animals was not measured and the conditions used were often unnatural. For example, all *Hyla arborea* tadpoles darkened when they were exposed to ambient levels of UV-B in the laboratory, whereas *T. cristatus, Bufo bufo, E. calamita* and *R. temporaria* did not show such an effect (Langhelle et al. 1999). Edema and the torsion of tails in *R. cascadae* and *P. regilla* treated with moderate levels of UV-A plus UV-B occurred at lower rates or were absent when only UV-A was applied or when tadpoles were kept under moderate compared to dim laboratory light conditions (Hays et al. 1996). Similarly, the filtration of UV-B plus UV-A was most effective in reducing the incidence of ectromely, ectrodactyly and anophthalmy in *L. pipiens* tadpoles (Ankley et al. 2002).

Moreover, the results of studies on the same species may be inconsistent. For example, Merilä et al. (2000) obtained asymmetric bodies and the coiling of tails in embryos of *R. temporaria*, whereas neither Langhelle et al. (1999) nor Pahkala et al. (2000) found significant UV-B effects at ambient UV-B levels. In a later study, UV-B was effective but only at low pH, causing edema and the torsion of tails (Pahkala et al. 2002). In contrast to their previous study, there were significant population effects. It should be noted that low pH can alone induce edema and the torsion of tails (see section 6.1.2.9). Genetic variability of individuals may further interact with the effects of UV (Weyrauch & Grubb 2006: edema, wavy tails and bent tails in *L. sylvaticus*) and may thus partially explain different results within the same species. Moreover, some early studies used cellulose acetate as a filter that was in contact with water. However, cellulose acetate is highly toxic to newly hatched tadpoles (Berrill & Lean 1998) and thus casts doubt on previous experiments that used these filters.

Evaluation. Increased levels of UV radiation have been suggested as a potential cause for anomalies mainly for higher altitudes because of higher UV-B intensity but Roßwag is located at a low altitude and no similar anomalies are known from populations surveyed in the vicinity of Roßwag, especially not from the neighbouring quarry with similar UV-B conditions. Artificial sources of increased UV-B radiation did not exist in the Roßwag quarry. Although UV-B can cause a number of the anomalies observed in the Roßwag *B. viridis* population under laboratory conditions (Tab. 9), most of the anomalies recorded in Roßwag were never detected in experiments with ambient levels of UV radiation and UV-B is unlikely to be a major cause of limb anomalies in nature (see also Johnson et al. 2010). Thus, ambient level of UV-B radiation can be excluded as a cause of the anomalies.

6.1.3.6 Radioactivity (and other mutagenic factors). ***Evidence put forward for the cause.*** Field measurements of radioactivity (see section "radioactivity assessment results") have been put forward as evidence for this cause (Henle 1981, 1982, Henle et al. 2017b), though this evidence has been challenged (Landesregierung von Baden-Württemberg 1984). The similarity in the spectrum of anomalies induced by experimental irradiation of amphibian germ cells (Kawamura & Nishioka 1978, Nishioka 1978) to the one observed in Roßwag and demonstrated heritability of at least one type of anomaly were again regarded as supportive evidence (Puhlmann 1981a,b, Henle 1982). No evidence for any mutagenic factor other than radioactivity has been put forward.

Anomalies known to be caused by irradiation. In amphibian embryology the use of irradiation (X-rays, γ-rays, neutron rays, radioactive substances, high intensity UV-B) to study differentiation processes has a long history. Most of the earlier studies used lethal doses and only assessed individual development for a short period of time (e.g., Hertwig, O. 1911, Hertwig, G. 1913, Stachowitz 1914, Rugh 1950; reviewed among others by Rugh 1939, Brunst 1950, 1965, Peters 1960, Labrousse 1967, Ahmad 1976, Kawamura & Nishioka 1978). More recently, some researchers used lower doses and studied the effects on the descendants from irradiated eggs or sperm throughout most of the larval development (Blair 1960, Oerter 1985, Trux 1985) and even for one or several generation(s) (Nishioka 1977, 1978, Nishioka & Ueda 1985, Kawamura & Nishioka 1978, Kashiwagi 1980, Hart & Armstrong 1984, Nishioka & Ohtani 1986). Mutagenic chemicals have been used much less frequently (on the level of full organisms). Jones & Jackson (1974), Hemsworth & Wardhaugh (1978), McKinnell et al. (1979), Armstrong & Fletcher (1983) and Hart & Armstrong (1984) tested their effects but did not analyse offspring beyond hatching.

All studies showed a wide range of anomalies in individuals descending from treated sperm or eggs (see e.g. Tab. 7). In *Pelophylax nigromaculatus* alone, Kawamura & Nishioka (1978) and Nishioka (1978) reported 18 different types of anomalies, 13 of which appeared frequently in the Roßwag population. Oerter (1985) listed 12 types of anomalies in *B. bufo* tadpoles that were irradiated at an early stage (Tab. 7); Blair (1960) also mentioned 12 different types of anomalies in offspring of irradiated *Incilius valliceps* males and crosses of the

surviving offspring among each other and wild caught individuals. Among these were several individuals with the forelegs remaining covered by the opercular fold, white individuals that failed to metamorphose and with amely. In addition, all types of anomalies found in the Roßwag population have been reported from irradiation experiments except for giant tadpoles (Tab. 9) and rare anomalies that affected only a few individuals and that have never been specifically considered in experimental studies.

As expected for mutagenic factors, the types of anomalies differed among individuals and among experiments, but some unspecific anomalies, such as abnormal cleavage, exogastrula, abnormal neurula, edema, tumours and bent bodies or tails, are commonly observed in early developmental stages (e.g., Schinz & Fritz-Niggli 1953, Jones & Jackson 1974, Oerter 1985) and edema, malformed mouthparts, bent tails and nanism in tadpoles (e.g., Oerter 1985). Recently metamorphosed individuals usually showed a range of leg and colour anomalies (e.g., Schinz & Fritz-Niggli 1954, Nishioka 1977, Kawamura & Nishioka 1978, Oerter 1985, Nishioka & Ohtani 1986).

After irradiation the incidence of anomalies decreases from generation to generation. In the fourth generation offspring of treated *P. nigromaculatus* and *Rana japonica,* the incidence had dropped to the level of the controls (Kawamura & Nishioka 1978, Nishioka 1978) but recessive colour mutations were retained for 10 years (Nishioka & Ohtani 1986, Nishioka et al. 1987).

Evaluation. The evidence from 1025 publications reporting anomalies in experimental studies, including 74 using irradiation (other than UV), shows that mutagenic factors are the only potential cause that can explain the range of anomalies observed in the Roßwag population except for a cocktail of teratogenic chemicals (see section 6.1.2.9) (Tab. 9). Chemicals and radioactivity are the only potent mutagenic factors that could have caused the range and frequency of anomalies under natural conditions. For a discussion of mutagenic chemicals as a potential cause, see section 6.1.2.9). Giant size of tadpoles is the only type of anomaly that was frequently observed in Roßwag but so far has not yet been reported in irradiation experiments. However, increased growth following radioactive contamination was observed, for example, in some plant species after the Chernobyl accident (Savchenko 1995). Furthermore, giant size in larval amphibians is caused by a disruption of the hypothalamus-pituitary-thyroid axis that may be evoked by genetic factors (reviewed by Borkin et al. 1982) and irradiation may interfere with the thyroidal adrenocorticoid hormonal balance (Ahmad 1976). Moreover, Uehlinger (1965) demonstrated that some recessive mutations lead to giant-sized tadpoles. Thus, a giant size is not incompatible with an explanation of the anomalies in Roßwag by radioactive contamination.

The laboratory studies undertaken clearly showed a genetic basis for at least one type of anomaly, orange colour patches, and made a genetic basis very likely for others, which again is in concordance with radioactivity as a cause. Genetic theory (Falconer 1989, Hedrick 1994) and experimental studies (Kawamura & Nishioka 1978, Nishioka 1978) demonstrate a rapid decrease in the range and severity of developmental anomalies with time after exposure to a mutagenic factor as observed in Roßwag from 1980 to 1984. Similar declines of anomalies were observed in barn swallow populations (*Hirundo rustica*) in areas contaminated by the Chernobyl accident (Møller et al. 2007). Thus, this decrease is also consistent with the assumption that toads were exposed to radioactive contamination within the quarry during the breeding period of 1980 and that radioactivity has either decayed and/or the source was covered by fill after 1980 (as happened).

In terms of the number of different types of anomalies, the laboratory studies of Nishioka (1977, 1978) and Kawamura & Nishioka (1978) on the effects of irradiation on the descendents of *R. japonica* and *P. nigromaculatus* (18 different types of anomalies, 13 of which were frequently observed in the Roßwag population) and the field study of Saveliev et al. (1996), Kuranova & Saveliev (1997) and Kuranova (2003) on *R. arvalis* and *Salamandrella keyserlingii* exposed to irradiation from a nuclear accident (at least 25 and 21 types of anomalies, respectively), come closest to the observations in Roßwag. It is worth noting that in barn swallows (*H. rustica*) from the Chernobyl and Fukushima area, in the butterfly *Zizeeria maha*, in the common lizard (*Zootoca vivipara*) from the East Uralian radioactive trace and in true bugs (Heteroptera) from the Chernobyl area and downwind from La Hague, a much higher frequency and wider range of different types of anomalies have been observed than it is known for these species from anywhere else (Semenov & Ivanova 1995, Ellegren et al. 1997, Møller et al. 2007, Hesse-Honegger & Wallimann 2008, Taira et al. 2014, Mousseau & Møller 2014). In areas contaminated with high levels of radioactivity caused by the accident in the Mayak plant, Ural, the frequency of morphological anomalies was also significantly elevated compared to control populations (Pyastolova et al. 1996). In addition, the irradiation of *B. bufo* embryos in the laboratory by Oerter (1985) resulted in more different types of anomalies – many shared with the Roßwag population – than any other experimental study known to us that did not use mutagenic factors. Finally, a wide range of anomalies and huge individual differences like those in Roßwag are expected, if eggs or sperm of the toads were exposed to radioactivity because of the random nature of mutations. These differences are still expected in the second and third year after irradiation because most of the offspring will still be from parents whose germ cells obtained mutations and not from surviving abnormal offspring.

The official enquiry regarded all non-official radioactivity measurements as unreliable and as artefacts (Landesregierung von Baden-Württemberg

1984). However, all official and non-official measurements that can be compared are consistent with one another. All of these showed that the level of radioactivity across the earthen deposit was elevated by a factor of 2–3 compared to the surrounding area. Locations, where measurements resulting in very high values of radioactivity were taken by KH, were not assessed in the official survey. Therefore, the official measurements cannot provide any evidence to confirm or reject them. To reject them, first an explanation for similar artefacts by two technically very different instruments and in repeated measurements with the same instrument is required. Secondly, an explanation is needed as to why these instruments never produced artefacts whenever measurements were made that could be compared with the official measurements. Thirdly, one needs to explain why both instruments produced artefacts only at the same locations (large cracks opening to the interior of the fill) but never when tested at other places and by pressing the instruments against material from the fill, nor in other operational tests for the functioning of one of the instruments. Such explanations have never been given and are exceedingly difficult to construct. Finally, the official enquiry insisted that the results of the official measurements excluded the presence of radioactive contamination in the quarry with absolute certainty (Landesregierung von Baden-Württemberg 1984). In an interview given to sbs Television, Australia, in 2006, Prof. Schreiber, a key physicist involved in the controvery (Henle et al. 2017b), denied such a possibility for the official measurements and his own ones, if the source was covered by fill. Already a cover of more than 20 cm of deposit material makes it impossible to detect any α or β radiation with measurements made 1 m above the surface. In this case, only measurements made close to the opening of cracks that only partially shielded the source or that allowed leakage of radioactive gases could have detected a strongly elevated activity. A necessary corollary is that measurements would need to exponentially decline with the distance from the opening. These predictions are met qualitatively by the non-official measurements, which the official enquiry rejected as artefacts (see Table 5a for measurement results).

6.2 Conclusion

The mass anomalies observed for *B. viridis* in the Roßwag quarry score among the four most severe ones in terms of the number of individuals affected and it is by far the most extreme case regarding the different types of anomalies involved. A normal natural phenomenon can clearly be ruled out.

Most types of anomalies can have different causes (Tab. 9). As a consequence, it is usually impossible to infer the cause from a single type of anomaly as attempted by Flindt (1985a) for some anomalies of the Roßwag population. However, not all factors are able to induce all types of anomalies. For example, with the exception of bleaching, darkening and haemorrhage, colour anomalies caused by non-genetic factors are extremely rare.

Most factors cause few anomalies and usually all affected individuals exhibit the same types of anomalies. More than ten different types of anomalies in larvae and/or juveniles/adults have only been reported in four experiments involving radioactivity or endocrine substances. Most notable is the study on the effects of irradiation of eggs or sperm in *P. nigromaculatus*, in which 18 different types of anomalies were reported in tadpoles, many similar to those observed in Roßwag (Nishioka 1977, Nishioka & Ueda 1977, Kawamura & Nishioka 1978, Nishioka & Ohtani 1986). In a parallel study on *R. japonica,* ten different types were mentioned but several types of anomalies were combined in a single category without providing details (Nishioka 1978). Oerter (1985) listed 13 different types of anomalies in *B. bufo* tadpoles that were irradiated as embryos, many of which were shared by the Roßwag population of *B. viridis*. Feeding *X. laevis* with thymus oil and various antithyreoidal plant seed oils induced 11–13 different types of anomalies, among them phocomely, oligodactyly, torsion of legs, stiff legs, front legs covered by a fold of skin, mandibular hypoplasia, giant larvae and edema but neither colour anomalies nor polymely (Pohland 1962, Wurmbach et al. 1964, Wink & Wurmbach 1967).

Summing across more than 1000 publications on experimentally applied teratogenic factors, more than ten different types of anomalies are known only for three of them: chemicals, irradiation and mutations revealed by breeding experiments (Tab. 9). If experiments are included, in which an anomaly was only occasionally observed and no statistical tests were made, then hybridization also surpasses ten different types of anomalies. Even in combination, all the remaining potential causes could explain at most approximately 50 % of the different types of anomalies observed in Roßwag (Tab. 9). Except for irradiation (Nishioka 1977, Nishioka & Ueda 1977, Kashiwagi 1980, Nishioka & Ohtani 1986), we know of no experimental study, which induced different types of colour anomalies in the same population. Thus, their co-occurrence at elevated rates within a natural population, such as in Roßwag, is an extremely strong indication for a genetic cause. Because the majority of colour anomalies are recessive and impose a reduced, often low viability (e.g., Nishioka 1977, Nishioka & Ohtani 1986; own observations of albinistic tadpoles in Roßwag), the simultaneous co-occurrence of many individuals carrying different types of colour anomalies can only be explained by the recent presence of a strongly mutagenic factor.

Inheritance could be demonstrated for the orange colour spot by the appearance of the same anomaly in the offspring of an affected individual and is supported for other anomalies by their appearance in tadpoles and eggs transferred to the laboratory. The individual with a squashed head aside, there is no convincing evidence

that any non-genetic factor contributed to the anomalies. While the non-mutagenic effects of chemicals could theoretically explain the spectrum of anomalies observed, this would require the assumption that many different substances contaminated the pond and that the tadpoles were exposed to different subsets of these substances, which is highly improbable given the homogeneous nature of the pond. Although there is a possibility that pesticides sprayed in the neighbouring vineyards might have contributed to the appearance of tumours, bent tails, edema and ectromely, the evidence for this hypothesis is weak and evidence against it is strong – and the anomalies can be explained without such an assumption.

Hybridization, inbreeding, mutagenic chemicals, UV-B and radioactivity can result in the appearance of genetically-based anomalies. Genetic and morphological studies attempting to demonstrate hybridization were all negative: anomalies developed from a clutch of a pair that was genetically *B. viridis* and no population of other toad species existed in the near vicinity of the affected population. Supporting hybridization as an explanation requires several biologically extremely unlikely and impossible assumptions (see above for details).

Similarly, inbreeding can also be excluded with certainty on theoretical and empirical grounds. The Roßwag population belongs to the largest populations in southern Germany and it is inconsistent to assume that the population was closed to conspecific immigrants from a nearby population but hybridized intensively with other toads from far more distant populations. Most importantly, however, inbreeding would lead to the same types of anomalies in all affected individuals, not to the huge variability observed. Explaining the anomalies by inbreeding thus requires nothing less than assuming that a fundamental biological law did not apply to the Roßwag toads.

In the Roßwag quarry, no artificial source of UV-B existed and ambient levels of UV-B do not induce a similar spectrum of anomalies as observed in the Roßwag population. Moreover, it should have caused the same mass anomalies in *B. viridis* breeding in the structurally very similar neighbouring quarry. In conclusion, only chemical mutagenes or radioactivity are compatible with the biological observations made in Roßwag and the huge body of experimental literature on amphibian anomalies.

Biologically, it is impossible to differentiate between different mutagenic factors based on externally observed anomalies. However, none of the chemical analyses did provide any kind of indication of chemical pollution of the breeding site. While the chemicals sprayed in the neighbouring vineyards by helicopters presumably contaminated the quarry pond, they are widely used in viniculture and are not known to be potent mutagenic substances (see section 6.1.2.9). Moreover, the declining range of anomalies observed from 1980 to 1984 would require a considerably reduced application of chemicals or manual spraying in the later years as opposed to helicopter spraying in the neighbouring vineyards, especially since the water bodies were much smaller in the years 1981-1984 compared to 1980. Although we cannot exclude with absolute certainty that some mutagenic chemicals contaminated the fill in 1980, there is no indication that any such substance was present in the quarry in sufficient quantities to affect a considerable part of the population.

Radioactivity is another mutagenic factor and measurements were made that indicated the presence of very high unnatural levels, although these measurements were disputed. Casting aside for a moment the disputed measurements and the notion that it is difficult to grasp that humans could have illegally deposited radioactive material in the quarry – though theft and illegal deposition of plutonium in the environment was discovered in 2000 in Baden-Württemberg at a location less than 100 km distant from Roßwag (Struktur- und Genehmigungsdirektion Süd Rheinland-Pfalz 2002) – what would be the predicted observations and measurements, what would contradict and what would support such an explanation?

Firstly, there were large cracks in the fill, which were ideal hiding places for adult toads, especially as few other hiding places were available in the immediate vicinity of the pond and thus it is very likely that a considerable number of toads found a retreat in these hiding places. They would be exposed to elevated radioactivity and contract mutations in their germ cells. As known from the experiments of Kawamura & Nishioka (1978), Nishioka (1978), Oerter (1985) and other studies summarized in Table 9, this would lead to a very wide range and high incidence of anomalies both in tadpoles and in recently metamorphosed individuals, many of which would be lethal. Notably, most of the frequent anomalies in the Roßwag population were also frequently observed in the experimental studies on the decendants of irradiated eggs and sperm of amphibians. The only exception, giant tadpoles, is caused by a disturbance of the thyroid axis, which is tightly regulated genetically and thus can also be caused by mutations as observed in other organisms (Savchenko 1995). Secondly, because of the genetic basis, anomalies would also occur when eggs or tadpoles were transferred to the laboratory as well as in their offspring – all predictions that are met by the observations for the Roßwag population. Finally, since the source was inaccessible to toads after 1980 because of several meters of additional fill, theory (Falconer 1989, Hedrick, 1994) predicts and the experiments of Kawamura & Nishioka (1978) and Nishioka (1978) demonstrated empirically, that the spectrum and frequency of the anomalies would rapidly decline with time as was the case in the Roßwag population (Tables 1 & 2). Moreover, toads spawning in neighbouring water bodies in the following years because of the destruction of the original breeding ponds would bring their anomalies to these water bodies, each differing in the spectrum of anomalies as observed by Flindt (1985a,b).

The single adult individual with an anomaly found in 1981 does not contradict these concluding remarks. Firstly, it is much more parsimonious to explain this single individual with an injury (or another cause) and the rest with a single cause than to use many different causes as explanation and still not being able to explain the majority of the anomalies. Moreover, *B. viridis* grows rapidly and is able to reach adult size in the year after metamorphosis (Flindt & Hemmer 1970b, Günther & Podloucky 1996). In 1980, flood-light was frequently used in the quarry, which attracts insects and *B. viridis* that hunt for insects under the light (Baumgart 2003). Thus, unusually good growth conditions existed and the individual may well have metamorphosed in 1980.

What are the predictions about radioactivity measurements? Already a cover of more than 20 cm with deposit material would make it impossible to detect any α or β radiation with measurements taken 50 cm or 1 m above the deposit of earth. Neither the measurements of IFEU, nor those of LfU or Schreiber would have detected it (confirmed by Schreiber in an interview to sbs television Australia). The same applies to those measurements of Henle that were made 1 m above the ground. All these measurements were consistent with those that were not disputed. Measurements made close to the opening of cracks that only partially shielded the source or allowed radioactive gases to leak would have provided different results: they would have been considerably elevated (depending on the radiation intensity and shielding), exponentially declining with the distance from the opening. Again, these predictions are met qualitatively by the contested measurements (Tab. 5a). There are no contradictions concerning the measurements taken, only in their interpretation.

It is not the cause of the anomalies [highly mutagenic material] that remains a mystery, the open question is rather: exactly which material was deposited, where did it come from, who deposited it in the quarry and why? Since any mutagenic material poses hazards not only to wildlife but also to humans, if they are exposed to it (Savchenko 1995, Lannoo 2008, Mousseau & Møller 2014, Taira et al. 2014), the most important questions that remain are: How long will the material remain active? Is there still a long-term hazard to toads, other wildlife or humans? And was it a single case or has it happened elsewhere as well (Henle et al. 2017b)?

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