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WORKING PARTY FOR THE PREPARATION OF THE FOURTH MULTILATERAL CONSULTATION OF PARTIES TO THE EUROPEAN CONVENTION FOR THE PROTECTION OF VERTEBRATE ANIMALS USED FOR EXPERIMENTAL AND OTHER SCIENTIFIC PURPOSES (ETS 123)

8th Meeting of the Working Party
Strasbourg, 22-24 September 2004

Species specific provisions for Amphibians

Background information for the proposals presented by the Group of Experts on Amphibians and Reptiles

PART B

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Background information

On the species-specific proposals for amphibians

Presented by the Expert Group on Amphibians and Reptiles

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Contents

Preamble

This document contains species-specific proposals for amendments to the Appendix A of the Council of Europe's Convention ETS 123 dealing with the protection of animals used or intended for use in any experimental or other scientific procedure which may cause pain, suffering, distress or lasting harm.

In 1997, the Council of Europe established working groups with the aim of advising the Council whether, how and to what extent the Appendix A of the Convention ETS 123 needed revision. The expert group appointed to deal with species-specific aspects of amphibians and reptiles was set by representatives of the following international organizations:

European Science Foundation, *ESF*
European Federation of Pharmaceutical Industries and Associations, *EFPIA*
European Federation of Animal Technologists, *EFAT*
Eurogroup for Animal Welfare, *EUROGROUP*
Federation of European Laboratory Animal Science Associations, *FELASA*
Canadian Council of Animal Care, *CCAC*

Representatives were:

Prof. Dr. Jörg-Peter Ewert (Coordinator)
ESF

Prof. John E. Cooper, DTVM, FRCPath, FIBiol, FRCVS
FELASA

Dr. Tom Langton
EUROGROUP

Prof. Dr. Gilbert Matz
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Dr. Helen Schwantje
CCAC

The Group was complete in September, 2000. Unlike expected, no representative from the *EFAT* participated in this Group. Yet Mr. Chambers of *EFAT* let the Council's Secretariat know that *EFAT* remain very interested in the work of the Group and will give comments and suggestions on the proposal to be made by the Group.

The general tasks of the Group were defined as follows:

a. listing, for the species concerned, the main questions to be answered with a view to revising Appendix A;
b. examining results already available and practical experience acquired which could possibly answer these questions;
c. identifying areas where further research would be needed;
d. preparing proposals for amendments to Appendix A, providing information in particular to the ethological and physiological needs of the animals. These proposals (Part A) should be supported by background information in an explanatory report (Part B), presenting scientific evidence and/or practical experience.

The Group was expected to send a first draft of the proposal for the revision of the species-specific parts of Appendix A by 15 January 2001.

By the middle of November, 2000, the General Coordinator Dr. Wim de Leeuw suggested that Prof. Dr. J.-P. Ewert be the Coordinator of the Expert Group on Amphibians and Reptiles. Since there were no objections, Ewert accepted to do this job, presented a preliminary draft of a proposal to the group members on December 12, 2000, and asked for suggestions for improvements. In this draft, the presentation of the consensus proposals made by the Group of Experts on Rodents and Rabbits (Strasbourg, 21 February 2000) and the Standard Format for Species Specific Sections was used formally as a basis. The Resolution on the Accommodation and Care of Laboratory Animals adopted by the Multilateral Consultation on 30 May, 1997, was taken into account where appropriate. Furthermore, the Guide to the Care and Use of Experimental Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council; National Academy Press, Washington, D.C., 1996) and the Guide of the Swedish Board of Agriculture (Department for Animal Production and Health, Animal Welfare Division) were considered. Since there were no suggestions for improvements on the draft proposal by the group members, the Coordinator sent the consensus proposal to the European Council's Drafting Group in due time by 14 January, 2001.

The original draft proposal for the revision of Appendix A concerning amphibians and reptiles presented by the Expert Group was sent to the members of the Drafting Group for consultation [see paragraphs 236 and 237 of the Summary Proceedings GT123(2000)39]. In agreement with the General Coordinator, the Chairman of the Working Party and the members of the Drafting Group, and in order to bring the document in line with the presentation previously adopted by other groups of experts, the document was divided into two parts: Part A containing paragraphs with proposals for Appendix A [doc. GT123(2001)1], and Part B providing detailed scientific background information supporting these proposals [doc. GT123(2001)23]. Paragraphs referring to Part A are in bold face italics, framed by boxes.

Part A. The species-specific proposals of Part A, concerning amphibians and reptiles, were revised based on suggestions and comments provided by the documents of the Drafting Group (*) and the representatives and observers at the meetings of the Working Party at Strasbourg from 2001 through 2003 [cf. also Summary Proceedings of the Working Party, GT123(2001)35, GT123(2002)41, GT123(2003)40, GT123(2003)41, GT123(2003)72, and of the Drafting Group GT123(2003)57rev].

Amphibians & Reptiles:
GT 123 (2001) 1)*
GT.123 (2001) 23)[A&B]*
GT 123 (2001) 30
GT 123 (2001) 31
GT 123 (2002) 5
GT 123 (2002) 10)*
GT 123 (2002) 25
GT 123 (2002) 40)*
GT 123 (2002) 56
GT 123 (2002) 59
GT 123 (2002) 62
GT 123 (2002) 64
GT 123 (2002) 69
GT 123 (2003) 15)*
GT 123 (2003) 27
GT 123 (2003) 33
GT 123 (2003) 35

Amphibians:
GT 123 (2001) 1E rev3)*
GT 123 (2003) 51)*
GT 123 (2003) 62
GT 123 (2003) 65
GT 123 (2001) 1
GT 123 (2004) 1E)*
[Rev. Appendix]

The revision of the proposals proceeded preferably via e-mail communication in the Expert Group on Amphibians and Reptiles. On September 10th and 11th 2001, a meeting of the Expert Group was organized by Kathryn Reilly at Harlow/Essex UK. This meeting was supported by the Merck Sharp & Dohme Company. Furthermore, the Coordinator participated in meetings at the *Bundesministerium für Verbraucherschutz, Ernährung und Landwirtschaft* organized by the representative of Germany of the Working Party at February 19th and November 6th ,2003.

At the 6th meeting of the Working Party in March 2003 it was decided to separate the proposals of Part A in two documents: *Species-specific Provisions for Amphibians* and *Species-specific Provisions for Reptiles.* Both documents were adopted and finalized at the 7th and 8th meeting of the Working Party in December 2003 and September 2004.

Part B. The present background information provides, where possible, scientific evidence for the *Species-specific Provisions for Amphibians*. Where this is not available, they take account of established good laboratory practice, based both on the experience of the members of the Expert Group and also on consultations with other experts. Additional comments and suggestions from members of the Working Party are considered and incorporated in Part B where appropriate. The revised Part B including Part A [GT123(2003)69] was submitted to the Council of Europe in advance of the 7th meeting of the Working Party in December, 2003.

In reply to the general tasks a-d:

a. *Listing, for amphibians, the main questions to be answered:*

(1) The peculiarities of the body skin make amphibians significant bio-indicators of the environmental health. In view of the world-wide declines of populations of amphibians and [cf. Sections 1.1 and 4.8] a selection of suitable species for the use in scientific procedures should be recommended [Section 1.2]. "Suitable" means that captive breeding programs for this species exist, and/or the population of this species is not in danger. Captive breeding programs should be promoted [Section 3.3].
(2) Recommendations for housing amphibians (minimum cage sizes, heights) under consideration of the natural biotope and the species-specific needs are required [Section 4.3].
(3) Amphibians are ectothermic and thus strongly adapted to their different biotopes. This requires species-specific considerations regarding temperature and humidity preferences, homeostatic capabilities, and seasonal activity patterns [Sections 2.1 to 2.4].
(4) Knowledge on diseases of amphibians and their treatments should be incorporated [Sections 1.1 and 3.4].

b. *Examining results already available and practical experience acquired which could answer these questions:*

CITES lists of protected amphibian species are cited in Section 1.1. Examples of amphibian species from the four main habitats (aquatic, semi-aquatic, semi-terrestrial and arboreal) frequently used in experimental and other scientific procedures are listed in Section 1.2. Furthermore, a selection of amphibian species is recommended and an example of a breeding program is elaborated. A reference list is provided. Information on caging amphibians (cage dimensions, temperature/humidity preferences) is provided in Section 4.3 and in an Appendix.

c. *Investigating what research is being carried out within the field and identifying areas where further research would be needed*:

An Internet MEDLINE search on research activities in amphibians among different science disciplines is provided in Section 1.2. See also reference list.

d. *Providing information in particular to the ethological and physiological needs of amphibians and reptiles:*

The standard format of the species-specific Sections 2-4 is provided with explanatory reports for the proposals and recommendations and these are supported by scientific evidence and practical experience.

Amphibians

I. Species-specific provisions for amphibians

1. Introduction

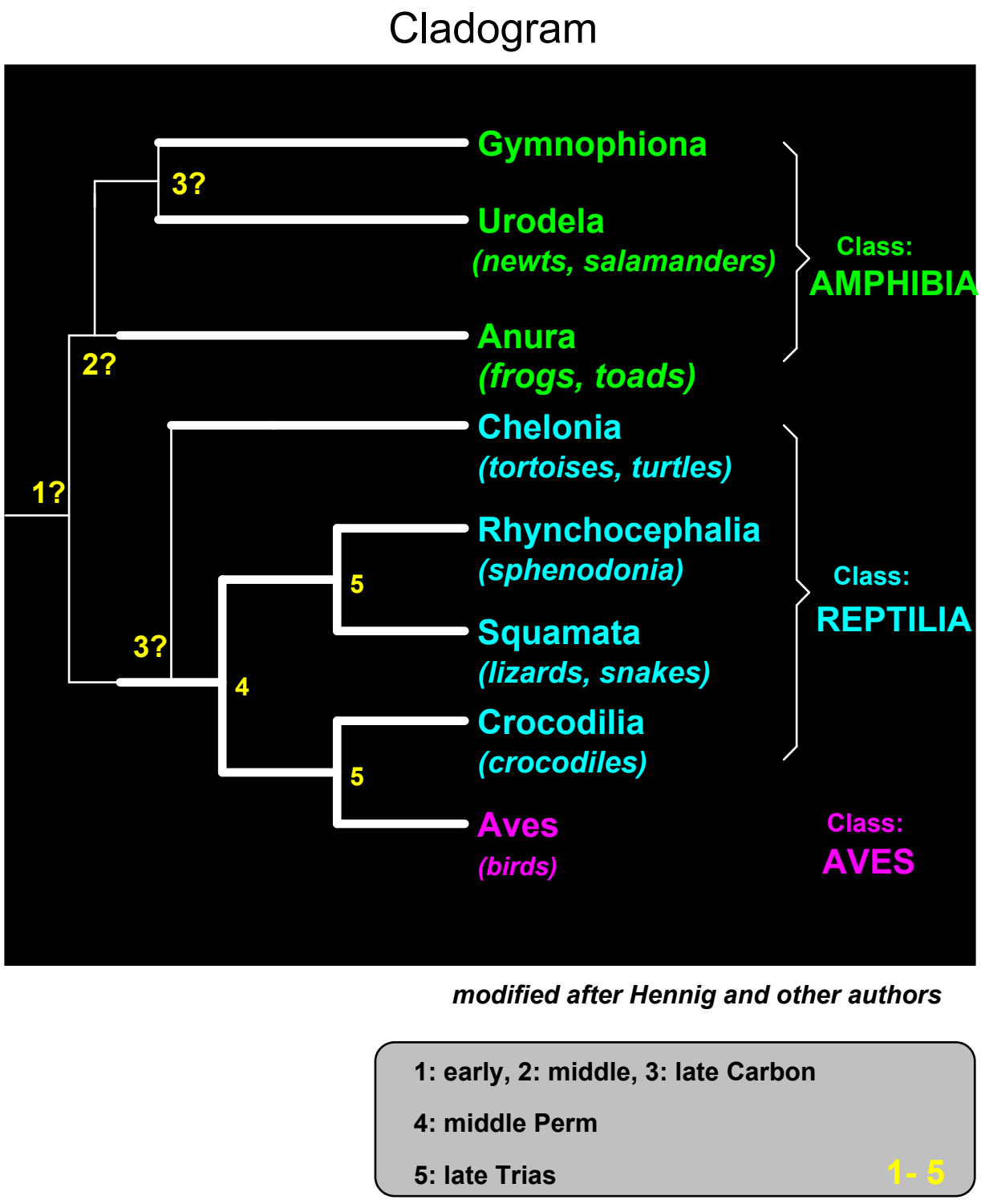

Fig. 1. Cladogram of amphibians and sauropsides (birds and reptiles).

According to systematics, amphibians involve three main orders: Urodela (Caudata), Gymnophiona (Apoda), and Anura (Ecaudata). The Anura belong to the super-order Salientia. For the present provisions, Urodela (salamanders, newts) and Anura (frogs, toads) are of interest. They differ greatly in their patterns of geographic distribution and in the diversity of living types, such as aquatic (for example, Xenopus laevis), semi-aquatic (for example, Rana temporaria), semi-terrestrial (for example, Bufo marinus) and arboreal (for example, Hyla cinerea). Amphibians occupy a wide range of habitat types from arid deserts to deep freshwater lakes. Some may spend most of their life underground or high in cloud forest canopy. Some are found north of the Arctic Circle and can tolerate freezing conditions, while others have evolved a range of adaptations to avoid desiccation in hot areas of the world.

In the cladogram shown in Fig. 1, amphibians and reptiles are traced back to a common ancestor living in early Carbon. Lizards and snakes, on the one hand, and crocodiles and birds, on the other hand, can be traced back to a common ancestor living in the middle of Perm. The common ancestor of crocodiles and birds probably lived in late Trias. Among the amphibians, the urodela (newts and salamanders) and the anura (frogs and toads) are of importance for the present proposals.

Amphibians and reptiles are different in many other aspects of which some will be mentioned. An ontogenetic developmental aspect points to the fact that reptiles – like birds and mammals – belong to the Amniota due to the existence of embryonic sheaths. Amphibians are Anamniota. The eggs of reptiles are mostly laid on land and develop into animals already adapted for land life. Amphibian eggs are mostly laid in the water and develop into tadpoles which after metamorphosis mature to the adults. Whereas almost all salamanders and gymnophiona (caecilians) are internal fertilizers, most anurans are external fertilizers.

From an evolutionary point of view, basic research both in amphibians and reptiles is of fundamental interest in order to evaluate comparable functional principles in mammals and, thus, also in humans (e.g., see Ewert, 1998). Phylogenetically, reptiles and mammals can be traced back to a common ancestor from that amphibians are derived. Current research suggests that certain morphological and physiological basic principles, present in amphibians and reptiles, were conserved during evolution up to the primates, in an appropriate differentiation and specification.

Amphibians are very much adapted to the substrate on/in which they live. In this context, the body skin plays an important role in the transfer of water, soluble substances, including toxic substances and oxygen. Therefore it plays key roles in the survival of amphibians, their interaction with their environment, and their ability to exploit a wide range of habitats and ecological conditions. An amphibian's health depends on certain properties and peculiarities of its body skin, thus making amphibians significant bio-indicators of environmental health.

A variety of functions in amphibians is performed by the skin as a complex organ:

- Sensing of mechanical, thermal, and chemical stimuli [cf. Sections 1.1, 2.2 and 2.3].
- Exchange of respiratory gases [cf. Section 2.1] which requires a moist body surface [Section 4.5].
- Hormone-controlled uptake of Na^+, Cl^-, and water through the epithelium [cf. Section 2.3].
- Communication through odorous substances.
- Chemical defence by antimicrobial peptides and poisons secreting glands [cf. Section 4.8].
- Mechanical protection by a tough, flexible and slippery wrapping.
- Development of specialised mechanical devices, such as warts and claws.
- Protection by camouflage, i.e. change of colour and/or colour pattern.
- Protection (e.g., against parasites) by the regular shedding of the outermost cell tissue.

(Lindemann & Voûte, 1976). Amphibians shed their skin spontaneously irregularly, in response to certain chemical stimuli even suddenly.

1.1. Declining amphibian populations

Amphibians are an important part of the ecological balance of many habitats. Amphibians thus are environmental sentinels (Roy, 2002). In some habitats, the biomass of amphibians makes them significant prey items for, and predators of, other species. Amphibians are also important for ecological and biodiversity studies, giving information on the ecological impact of both local and global changes, sometimes with implication for humans (e.g., Heyer et al., 1994; Daszak et al., 2000; Daszak & Cunningham, 2003). Many populations of amphibians are declining on all six continents on which they occur (e.g., Kiesecker et al., 2001; Pounds, 2001). Some causes of declines – habitat destruction, application of xenobiotics, introduction of predators or competitors – are attributable to human activities. As pointed out by the Declining Amphibian Population Task Force (DAPTF), over the last 50 years, many species of amphibians (frogs, toads, salamanders, and newts) throughout the world have declined marked by in numbers. Some species have even become extinct. The reasons for the declines are, on the one hand, a direct response to the impact of human activities (habitat destruction, pollution) acting on local level. On the other hand, there may be several global factors that are adversely affecting amphibians. Strategies for assessing the implications of malformed frogs for environmental health are discussed by Burkhart et al. (2000). Possible causes of declining amphibian populations are, for example:

- Increase in ionising radiation resulting from ozone layer depletion (eggs of frogs and toads can be damaged by UV-B).
- Chemical contamination (e.g., oestrogenic effects of pesticides and precipitation, effects of fertilizers and herbicides).
- Contamination by natural fertilizers (e.g., leading to an increase in the population of parasites, such as the trematode worm *Ribeiroria ondatrae* whose larvae – developing in the snail *Planorbella* – affect tadpoles and disturb the normal development of the extremities during metamorphosis). For effects of trematode infection see also Johnson et al. (1999) [cf. also Section 3.4].
- Fungal infectious diseases, e.g., chytridiomycosis (Berger et al., 1998; see also Section 3.4).
- Introduction of exotic competitors and predators (e.g., fish introductions, aqua-cultural practices; see also Chivers et al., 2001).
- Pathogens (e.g., environmental stress reduces the ability of amphibians to resist diseases, such as those caused by poxvirus-like particles).

Results of long-term studies on fluctuations observed in natural amphibian populations are presented by Meyer et al. (1998). Several incidences where sentinel species have responded to effects of chronic exposure to ambient levels of environmental contaminants are discussed by LeBlanc & Bain (1997). The need for water quality criteria for frogs is pointed out by Bover & Grue (1995). Contaminated ground water, for example, poses a significant health hazard and may also impact wildlife, such as amphibians, when it surfaces (Bruner et al., 1998). Decreases in water quality may be associated with frog embryo mortality and malformations (Boyer & Grue, 1995). Wastewater effluent-irrigated ponds may affect amphibian populations by reducing the survival of amphibian eggs and larvae (Laposata & Dunson, 2000; Harris et al., 2000). Acidification of breeding ponds has been identified as a potential threat to the survival and health of North American amphibian populations. Exposure of embryonic or larval *Bufo americanus* to moderately acidic water (pH 6.0) disrupts the nitrogen balance by increasing nitrogen loss as ammonia, with no compensatory decrease in urea excretion (Tattersall & Wright, 1996; cf. also Hatch et al., 2001; Marco et al., 2001). Amphibian larvae are commonly exposed to low levels of pesticides during their development. Effects of the insecticide Carbaryl are different at different life stages. Any delay in metamorphosis or decrease in size at metamorphosis can impact the population, potentially leading to declines or local extinction (Bridges, 2000). For effects of pesticide exposure at various life stages of leopard frogs see Bridges (2000) and Harris et al. (2000; cf. also Kiesecker et al., 2001; Sparling et al., 2001). Effects of the herbicide Diuron on survival and growth of tree frogs are described by Schuytema & Nebeker (1998; cf. also Normile, 1999; Withgott, 1999; Renner, 2003). Antimicrobial peptide defences against pathogens, too, are associated with global amphibian declines (Rollins-Smith et al., 2002).

Another cause of amphibian decline may be crankcase oil that leaks from motor vehicles and washes into ponds. Ponds containing oil and silt produce salamanders of reduced size and weight. Silt

results in reduced growth, earlier metamorphosis and increased susceptibility to *Saprolegnia parasitica* (Lefcort et al., 1997). The impact of lead (Pb) was seldom considered in declines of amphibian populations due to man-made changes in the environment (Vogiatzisch & Loumbourdis, 1999). Under laboratory conditions, concentrations of boron and nitrate within the range measured in wastewater effluent (likely due to rainwater dilution) reduced the hatching process in *Bufo americanus* and produced deformed off-springs in *Rana sylvatica*, *Ambystoma jeffersonianum* and *A. maculatum* (Laposata & Dunson, 1998). It is suggested (Rouse, 1999) that nitrate concentrations in some watersheds in North America are high enough to cause death and developmental anomalies in amphibians and impact other animals (amphibian prey) in aquatic ecosystems (see also Laposata & Dunson, 1998; cf. also Nebeker & Schuytema, 2000; Johansson et al., 2001). For strategies for assessing the implications of malformed frogs for environmental health see Burkhart et al. (2000).

The mortality in amphibians is positively correlated with solar UV radiation (Bruner et al., 1998; see also Ankle et al., 2002; Diamond et al., 2002; Peterson et al., 2002). Differential sensitivity to UV-B radiation among species may be one factor contributing to population declines in amphibians (Phalli et al., 2003). In some species, ambient levels of UV-B cause embryonic mortality in nature or cause deformities in amphibian embryos (Blustering et al., 1997; cf. also Hays et al., 1996). The detrimental effect of UV-B alone or with other agents may ultimately affect amphibians at the population level. For example, synergistic effects between UV-B and a pathogenic fungus increase significantly the mortality of amphibian embryos (Diesinker & Blustering, 1995). The eggs, the most UV-B-sensitive stages, contain photolyase the important enzyme for repair of the major UV photoproducts. Hatching success correlates strongly with photolyase. Different species display (100-fold) different levels in photolayse. Low-egg-photolyase species decline. High-egg-photolyase species may be either robust or are showing declines rarely. The latter effect seen in some species may be due to low-skin-photolyase levels in the developing tadpoles (Hays et al., 1996; for defence against UV-B radiation see also Blaustein & Belden, 2003).

Besides the causes clearly attributable to human activities, infectious diseases appear to be a direct cause of mass amphibian die-offs in relatively undisturbed areas of the world (Daszak et al., 1999). In these cases, it is not yet clear whether these epizootics result from the natural evolution of new pathogens from environmental changes that promote the emergence of pathogenic forms and/or that weaken the immune defence of amphibians (Carey, 1999, 2000). With the amphibian metamorphosis, the immune system becomes reorganized. Most amphibians probably survive the temporary immune-suppression associated with metamorphosis with no deleterious effects (see also Flajnik, 1996). However, if environmental stressors result in the induction of metamorphosis at a less than optimal body size and state of immune maturation, the amphibians could be at a greater risk of infection and death (Rollins-Smith, 1998; see also Denver, 1997). Urodele amphibians show relatively weak and slow immune responses compared to anuran amphibians (Salvadori & Tournefier, 1996).

Future goals in this context should include the establishment of further activities of scientific research that cover all areas of the world where amphibians live, in order to discover which species are rare or declining and to investigate the reasons behind these declines. The DAPTF, established in 1991 by the Species Survival Commission (SSC) of the World Conservation Union (IUCN), can be reached:

http://www.open.ac.uk/daptf/about_daptf.htm - 5k -
On protected amphibian species, see:
http://www.CITES.org

Where possible, amphibians used for experimental or other scientific purposes should be bred and reared in captivity. Purpose-bred animals should be used in preference to animals taken from the wild.

A main problem to be addressed with a view to revising Appendix A to the Convention ETS 123 concerns the declining amphibian populations, on the one side, and the consumption of amphibians for the use in experimental or other scientific procedures, on the other side. One way to tackle this problem is a selection of species under the aspects of protection and breeding programs that maintain the population of amphibians in captivity.

1.2. Selection of species

1.2.1. Examples of species from the four main habitats

Table I.1 lists the four main habitats of amphibians and examples of species of each habitat frequently used for experimental and other scientific purposes. The following proposals provide details on the basic accommodation and care conditions to be covered for species of these habitats. Specific procedures may require the use of certain other species which do not fall into the four habitat categories. Further advice on requirements for these and other species (or if behavioural or breeding problems occur) should be sought from expert specialists and care staff to ensure that any particular species needs are adequately addressed. Additional background information on less commonly used species and habitats is available in the background information document elaborated by the Group of Experts (see paragraph 4, Introduction, General Section).

Table I.1: Main habitat categories and examples par habitat of species frequently used

Habitat	*Amphibian species*	*Size*	*Original geographic distribution/ Biotope*	*Optimal temperature*	*Relative humidity*	*Main period of activity*
Aquatic Urodeles	*Ambystoma mexicanum (Axolotl)*	*24-27 cm*	*Mexico / Channels of the former sea of Xochimilco*	*15-22°C*	*100%*	*Twilight*
Aquatic Anurans	*Xenopus laevis (Clawed frog)*	*6-12 cm*	*Central and South Africa/ ponds, ground water and spring-fed*	*18-22°C*	*100%*	*Twilight/ Night*
Semi-aquatic Anurans	*Rana temporaria (Common frog)*	*7-11 cm*	*Europe (middle and north) to Asia (without southern Balkan) / Near ponds, lakes, streams (shores, meadows)*	*10-15°C*	*50-80%*	*Day/night*
Semi-terrestrial Anurans	*Bufo marinus (Marine toad)*	*12-22 cm*	*Central and South America/ Mangrove, woods*	*23-27°C*	*80%*	*Night*
Arboreal Anurans	*Hyla cinerea (Green tree frog)*	*3-6 cm*	*Southeast USA / Open bushy borders of cypress swamps, flat country, forest*	*18-25°C*	*50-70%*	*Day/night*

1.2.2. Recommended species

The variety of adaptation and the diversity in environmental requirements among amphibian species and subspecies lead to restrictions in establishing proper general conditions for their long-term maintenance in captivity. This is one point suggesting a selection of species of amphibians used for experimental or other scientific procedures.

Another restrictive point concerns the experience that certain European ranid frogs will often not feed in captivity and are, therefore, not suitable for long-term maintenance. One reason is that they hardly accept artificial or semi-natural environments and do not tolerate the disturbances of human traffic, such as even slight vibrations caused by steps at long distances.

A further problem concerns the fact that – unlike fish, birds, and mammals – there are only a few strains of amphibians (genetically adapted to captive conditions) available commercially [for an instruction of breeding *Bombina orientalis* see Section 3.3]. Therefore, most amphibians suitable for holding under laboratory conditions must be collected from the wild. However, consumptive use of significant numbers of wild caught animals from a species or population, which is rare, endangers its extinction or local extirpation. This aspect together with ecological constraints in connection with the progressively endangered natural biotopes of amphibians strongly calls for species protection.
Weighing all these aspects together, it is advisable to consider amphibian species for experimental or other scientific purposes which can be bred and reared easily under laboratory conditions, such as

Ambystoma mexicanum	(Urodela neotenic larvae, aquatic)
Xenopus laevis	(Anura, aquatic)
Xenopus tropicalis	(Anura, aquatic)
Bombina orientalis	(Anura, semi-terrestrial),

and amphibian species whose populations grow progressively, such as

Bufo marinus	(Anura, semi-terrestrial)
Rana catesbeiana	(Anura, semi-aquatic).

Although there may be health concerns over obtaining *Xenopus* from the wild, views have been expressed that these animals obtained from the wild are in general more fecund than their lab-bred counterparts and as a result, scientists use fewer animals to obtain the amount of biological material that is required. *Xenopus laevis* is not an endangered species, and there is no indication that numbers of this species are decreasing in the wild. The same holds true for the European common frog *Rana temporaria* and the American green tree frog *Hyla cinerea*.

The use of certain foreign, not endangered species may become problematically. The UK government, for example, has put severe restrictions on the importation of certain species capable of breeding in the wild in the UK, including the bullfrog *Rana catesbeiana.* The UK government spent about €50,000 for removing a bullfrog population in 1999-2001. In Germany (Rhine area) bullfrogs propagate increasingly and in France the situation seems to be almost out of control. In Spain, on the other hand, bullfrogs belong to anuran species most frequently used in research.

Suggested reading for:

Ambystoma mexicanum [Armstrong & Malacinski, 1989; Brandon, 1989; Smith, 1989]
Xenopus spp. [Kobel & Tinsley, 1996; Elepfand, 1996a,b; Elepfand et al., 2001]
Bombina orientalis [Zimmerman, 1986; Mattison, 1987; Plesner, 2002]
Bufo marinus [Alexander, 1964; Duellman Trueb, 1985; Zug, 1979; 1993; Wachowitz & Ewert, 1996]
Rana catesbeiana [Korschgen & Moyle, 1955; Hayes & Jennings, 1986; Stinner et al., 1994; Bee, 2002]
Rana temporaria [Savage, 1961; Aertsen et al., 1986; Miaud & Guyetant, 1998; Miaud et al., 1995, 1999]
Hyla cinerea [Capranica & Moffat, 1983; Gerhardt, 1981; Moulton et al., 1996; English & English, 2000]

Specific procedures may require the use of certain other species (except those listed in CITES). The permission to use such species for investigations will depend on the applicant's justification for the use and on the decision of the respective authorities of the country in which these investigations are conducted.

1.2.3. Current research

An internet MEDLINE search comparing the number of publications (original research papers) on amphibians in relation to those on reptiles and mammals (rhodents, such as rats and mice) shows that amphibians and reptiles are much less frequently used (Fig.2). Among amphibians, *Xenopus* spp. is the most frequently used genus. An ISI search for all publications on *Xenopus* spp. even shows some kind of exponential increase from 1980 to 2000. The frequent use of *Xenopus* spp. as research subject explains the dominance in the number of research papers on amphibians compared to those on reptiles.

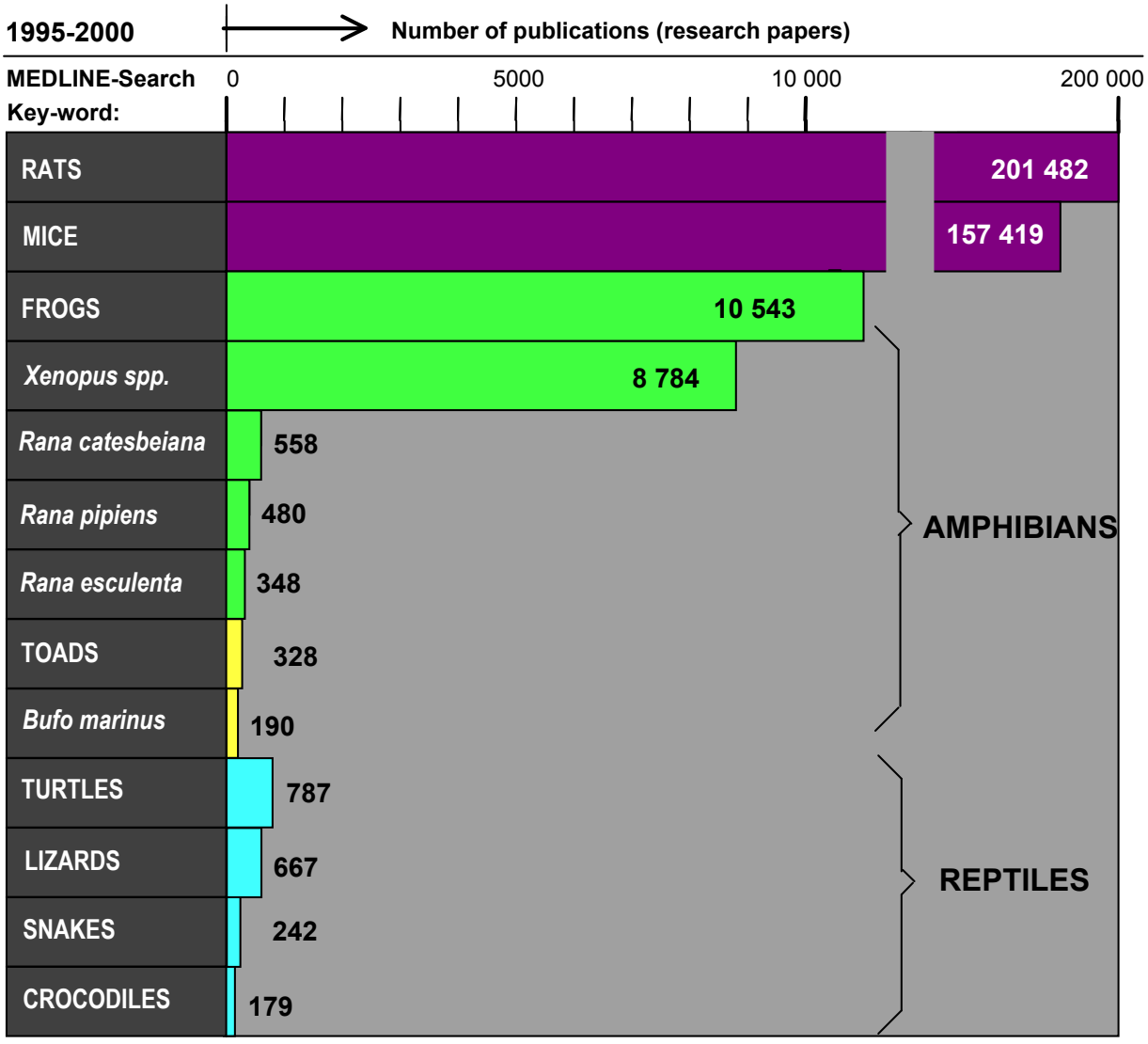

Fig. 2. Internet search for scientific publications related to rhodents, amphibians, and reptiles.

For the time segment 1995 to 2000, a MEDLINE search shows the following research activities in different scientific disciplines (key words: amphibia, amphibians):

Research Discipline 1995-2000	Publications (%) on amphibians
Cellbiology	**17.0**
Vegetative Physiology	**16.9**
Genetics	**14.7**
Morphology	**14.2**
Brain Research	**11.3**
Ecology	6.4
Sensory Physiology	6.1
Biochemistry	5.2
Parasitology	4.9
Pharmacology	2.0
Ethology	1.0
Housing	0.3

By setting a 10-% level, it is evident that studies in Cellbiology, Vegetative Physiology, Genetics, Morphology, and Brain Research preponderate. In functional Morphology, for example, basic research on regeneration capabilities is of medical interest. Urodele amphibians are unique among adult vertebrates in their ability to regenerate organs or parts of it. Regenerated limbs are often indistinguishable from the developmentally produced original (Christensen & Tassava, 2000; see also Crews, 1995; Tsonis et al., 1995; Brockes, 1997; Imokawa & Yoshizato, 1998; Carlson et al., 1998; Torok et al., 1998; Gardiner et al., 2002; Nye et al., 2003). Urodeles also regenerate, for example, the tail (Benraiss et al., 1996) and parts of the spinal cord (Chernoff, 1996; Echeverry & Tanaka, 2003; Chernoff et al., 2003; Zhang et al., 2003).

Obvious deficits in research emerge, for example, in studies on housing and captive breeding amphibians in view of declining populations [see also Section 1.2].

2. The environment and its control

First of all, experience from *good laboratory practice* (GLP) should be considered. In 1990 Greendale Laboratories UK became the first dedicated laboratory to achieve full GLP compliance for all its disciplines. The GLP scheme became administered by the Medicines Control Agency of the Department of Health. Having GLP means that every aspect of the laboratory from automatic analysers right down to the smallest piece of equipment has calibration, maintenance and test methodologies rigorously controlled and checked both prior and during their working lives. It is internationally recognized that GLP is one of the highest quality assurance accreditations that a laboratory can attain. GLP is concerned with the organisational processes and the conditions under which laboratory studies are planned, performed, monitored, recorded and reported. Adherence by laboratories to the principle of GLP ensures the proper planning of studies and the provision of adequate means to carry them out. It facilitates the proper conduct of studies, promotes their full and accurate reporting, and provides a means whereby the integrity of studies can be verified.

Cooper (1984) working on Exotic, Zoological and Wildlife species, is a Consultant Pathologist with Wildlife Health Services UK (see also Cunningham et al., 1996; Cooper & Cooper, 2003).

Persons carrying out scientific procedures on amphibians will maximise the overall benefit for animals as individuals and as a group. Laboratory amphibians must be provided with accommodation and care appropriate to their health and welfare, with consideration of their physiological and ethological needs (e.g., see Dickerson, 1931; Matz, 1983; Duellman & Trueb, 1985; Zimmerman, 1986; Mattison, 1987; Stebbins & Cohen, 1995; Conant & Collins, 1998; O'Rourke, 2002; Matz & Weber, 2002; Matz & Vanderhaege, 2003; Bolhuis & Giraldeau, 2004).

Laboratory amphibians are accommodated in a *vivarium* (e.g., terrarium, aqua-terrarium, tank). A vivarium is any room, building or other facility in which live, vertebrate animals are housed for periods of time exceeding 24 hours. The housing of animals should conform to the *NIH Guide for the Care and Use of Laboratory Animals in Research* (NIH publication No. 85-23, 1985 or succeeding editions). A new vivarium to be constructed should conform to the recommendations of the NIH Guide. Although desirable, it is impractical to require older vivaria to meet all of these standards. Remodels of older facilities, however, should attempt to bring the facilities more nearly in compliance with these standards. Animals may be held in laboratories or other locations outside vivaria for periods of time not to exceed 12 hours, after which time they should be returned to a vivarium. Exceptions to this policy need to be justified. Vivaria should be located in close proximity to the laboratories.

2.1. Ventilation

Enclosures for amphibians should be adequately ventilated. The water in enclosures of aquatic caged amphibians should be filtered, circulated, and aerated (see also Section 4.3.1).

Most amphibians cover reasonable amounts of their oxygen consumption by lung (or gill) and by skin respiration. About 80% of respiratory needs are met by pulmonary gas exchange (rather than cutaneous). With increasing body weight, lung respiration dominates. In order to guarantee sufficient fresh air and to keep down the level of noxious gases, the holding rooms must be adequately ventilated. Unventilated moist "frog cellars" are inadequate. It is generally considered adequate in practice for humidity and temperature control to change the air by means of a correctly engineered air-flow system. The water of the aquatic area in the terraria of terrestrial and semi-terrestrial amphibians should be renewed at least twice a week. In aquatic caged animals, there should be preventive measures to avoid larvae be trapped by filters. *Xenopus* spp. rarely inhabits highly oxygenated (flowing) water in the wild. Therefore, air stones are not necessary for *Xenopus.* If the purpose of air stones is to reduce the fouling of the water, then this should be tackled by more frequent water changes.

2.2. Temperature

Amphibians are ectothermic. Areas of different temperature and humidity are beneficial, to allow amphibians to seek their preferred micro-environment. Amphibians exposed to frequent fluctuations in temperature and humidity may be severely stressed and may be more prone to health problems. Room and water temperatures should be controlled.

Hibernation in amphibians may be induced or interrupted by regulating light-dark rhythm and room temperature. Before inducing hibernation in captivity, animals should be in good health and body condition. In animals used for breeding, a state of near winter torpor (for example dim light to darkness and 8-10°C room temperature) may be simulated where appropriate. Under these conditions, the animals can be kept without feeding for as long as four to five months. Restoration of pre-hibernation environmental conditions will induce activity and mating behaviour.

Prevention of hibernation in a laboratory environment will not cause major welfare problems.

Amphibians are ectotherms ("cold-blooded" animals); unlike endotherms ("warm-blooded"animals) their body temperature is dependent on the ambient environment. The advantage of ectothermy is that the resting metabolic rate and general energy requirements are less than those for mammals or birds of comparable size since no metabolic energy is spent on warming or cooling the body, and less energy is spent on food intake because less food is required to meet the body's low energy demands. The

disadvantage of ectothermy, however, is that the ambient temperature determines the animal's metabolic processes and behaviour. The animal must actively seek temperatures that will allow it to feed, digest food, hibernate, etc. Amphibians adapt their body temperatures by finding the appropriate thermal environment through burrowing, hiding under logs or leaves, or entering water.

In many respects ectothermic animals are more interactive with their environments than endotherms. At the same time, they tend to have greater problems adapting to changes in their species-typical environment. Therefore, the design of their artificial habitats demands special care, since research-biasing stress and distress responses to species-inadequate environmental conditions are to be avoided.

Depending on the conditions in the natural biotope, the optimal temperatures of amphibians vary markedly among the species and also within a species with the divergent functional states as feeding and digestion, on the one hand, and torpor on the other hand. In amphibians, body temperature varies with environmental temperature which in turn is associated with changes in metabolic activity (Lillywhite et al., 1999). With decreasing temperature, the processes of respiration and circulation gradually slacken speed. Changes in temperature also modify the transport of body fluids (Pelster, 2000). Effects of temperature on size and developmental time are reported by Gilloly et al. (2002).

Both tadpoles and adults of frogs acclimate their locomotor system to different temperature, i.e., maximum swimming performance of cold-acclimated animals is greater at cool temperatures and lowest at warm temperatures in comparison with the warm-acclimated animals (Wilson & Franklin, 2000; Wilson et al., 2000).

Hibernation in amphibians may be induced or interrupted by regulating light-dark rhythm and room temperature. Before inducing hibernation in captivity, animals should be examined and assessed to be in good health and body condition. In animals used for breeding, a state of near winter torpor (for example dim light to darkness and 8-10°C room temperature) should be simulated. Under these conditions, they can be held without feeding for as long as four to five months. Restoration of pre-hibernation environmental conditions will induce activity and the mating behaviour.

Amphibians can endure an astonishing amount of cold. Torpor like sleep is induced in specimens at varying temperatures below 10°C. In autumn, frogs or toads creep away into some protected place or burrow into the soil into a greater depth, depending on whether the place chosen is at the bottom of a pond (frogs), under logs or stones, or in the earth (common toad). This hibernation lasts until a return of higher temperatures beginning in spring. Many amphibians encounter conditions each winter when their body temperature is so low that normal activities are suspended and the animals enter into a state of torpor. In ice-covered ponds or lakes, oxygen levels may become limiting, thereby forcing animals to endure prolonged periods of severe hypoxia or anoxia. Certain frogs (e.g., *Rana temporaria*) can dramatically suppress their metabolism in anoxia but are not as tolerant to prolonged periods of complete O_2 lack as other facultative vertebrate anaerobes (e.g., turtle, goldfish). Many over-wintering amphibians do, however, tolerate prolonged bouts of severe hypoxia, relying exclusively on cutaneous gas exchange (Boutilier et al., 1997). Various amphibians, which survive at temperatures several degrees below the freezing point of their body fluids, apply adaptive mechanisms that promote freeze avoidance by: (1) colligative depression of the blood freezing point, (2) super-cooling of the body fluids, and (3) biosynthesis of unique antifreeze proteins that inhibit the propagation of ice within body fluids. Freeze tolerance can be achieved by biosynthesis of permeating carbohydrate cryo-protective substances and extensive dehydration of tissues and organs (Costanzo et al., 1995; cf. also Storey et al., 1996).

Most amphibians can endure a greater degree of cold than of heat. In water, death may occur at about 40°C. Certain species of tree frogs – sitting in the sun – can endure temperatures up to 45°C because of the moisture secreted by their skins, in combination with surface cooling by evaporation. In South America, in savannah-like vegetation, during the dry season amphibians burrow into the mud or soil, and either form a cocoon or increase the osmotic concentration of body fluids to reduce evaporative water loss. Some tree frogs coat their body surface with skin secretion and excrete uric acid to minimize water loss. Tadpoles of desert amphibians evolved traits which allow successful development in unpredictable environments. As their pond dries, this environmental stress (involving the corticotropin releasing hormone) sets the cue to accelerate metamorphosis, thus escaping mortality in larval habitat (Denver, 1997). Estivating reed frogs (*Hyperolius viridiflavus*) are extraordinarily resistant to adverse climate conditions in their African savannah habitats during the dry season: air temperatures up to 45°C; solar radiation load up to 1,000 W/m²; no water replenishment possible for up to 3 months. To avoid lethal heat stress, solar heat load is reduced by an extraordinarily high skin reflectivity for solar radiation. Furthermore the half cylindrical body shape allows the animal to expose only a small surface area to

direct solar radiation (Kobelt & Linsenmair, 1995). For estivation in South American amphibians see also Abe (1995).

Different species of urodele and anuran amphibians living in different biotopes are listed in the Appendix.

2.3. Humidity

Amphibians do not drink but absorb moisture through their skin. Water loss is an especially critical problem in captive terrestrial and semi-terrestrial amphibians, as a properly hydrated integument is essential to the normal function of the amphibian skin. Areas of different humidity within the enclosure are beneficial. Even desert-adapted amphibians should have access to a humid environment.

Amphibians have developed mechanisms to minimize cutaneous dessication (Castillo & Orce, 1997; Schmuck & Linsenmair, 1997). They absorb water osmotically across their skins and rely on chemosensory information from the skin to assess the suitability of hydration sources. Chemosensory transfer through the skin involves both trans-cellular and para-cellular pathways (Sullivan et al., 2000). Terrestrial amphibians take up water by abducting the hind limbs and pressing a specialized portion of the ventral skin to a moist surface; this behaviour is called the "water absorption response" (Hillyard, 1998, 1999). It can be influenced by barometric pressure and is avoided on hyper-osmotic substrates (Hillyard et al., 1998). Toads are able to detect hyper-osmotic salt solutions in their environment due to chemosensory function of the ventral skin (Nagai et al., 1999). Some amphibians tolerate high ambient salinities (e.g., *Bufo calamita*). In response to high salinities and water shortage, these species accumulate urea (osmo-regulatory urea synthesis) (Jorgensen, 1997a,b; cf. also Pozzi et al., 2002). The hormone angiotensin II – an inducer of thirst-related behaviour in many vertebrate species – increases the water absorption response behaviour in amphibians, such as the spade foot toad, *Scaphiopus couchii* (Propper et al., 1995; see also Slivkoff & Warburton, 2001; Viborg & Rosenkilde, 2001). Whereas the osmo-regulatory system in the tadpole comprises three organs – gut, kidney and gills – the adults involve gut, kidney, urinary bladder and skin. Amphibians may use the urinary bladder as a reservoir from which water is reabsorbed on land (Jorgensen, 1997a,b). Aside from vasotocin anti-diuretic activity in the nephron, the substance hydrin-2 (most common in amphibians except hydrin-1 in *Xenopus*) acts on water channel recruitment (re-hydration) mechanisms in the body skin and urinary bladder (Acher et al., 1997). Frogs have distinct hydro-osmotic receptors for vasotocin and hydrins (Rouille et al., 1995). For hormonal effects on the osmotic, electrolyte and nitrogen balance in terrestrial amphibians see also Warburg (1995).

In the laboratory, the humidity is adjusted by appropriate levels of moisture and temperature. The water area of a terrarium will allow animals to submerge. However, if a toad or frog has escaped from its terrarium, it is advisable to put a wet towel on the floor in the corner of the holding room. Over night, the animal will detect it and approach it and can thus be carefully caught and transferred back to its cage.

Different species of urodele and anuran amphibians living in different biotopes are listed in the Appendix.

2.4. Lighting

Photoperiods reflecting the natural cycle from where the animals originate should be used. Light levels in the enclosures should be consistent with that expected to be encountered under natural conditions. Both semi-terrestrial and aquatic caged animals should have the opportunity to withdraw to shaded areas within the enclosure.

Regarding photoperiods, the recommended laboratory lighting for *Xenopus,* for example, is 14h light and 10h dark, which corresponds roughly to nature. Different anuran species may exhibit preferences for light or dark. *Rana pipiens*, for example, is photopositive while *Leptodactylus pentadactylus* is photonegative (Kicliter & Goytia, 1995). Forest mediated light regime is linked to amphibian distribution and

performance (Halverson et al., 2003). Comparing the energy spectra in woods of moderate and tropic climates, it is interesting to note that the UV proportion is about the same [8.5%:8.0%, 320-500 nm]. Differences exist in the green proportion and [15.5%:22.0%, 470-590 nm] particularly in the red [22.0%:45.0%, 600-710 nm]. Studies on spectral and polarization sensitivity of amphibian photoreceptors in the visible and ultraviolet are provided by Palacios et al. (1998).

2.5. Noise

Amphibians are very sensitive to noise (airborne stimuli) and vibration (substrate-borne stimuli) and are disturbed by any new, unexpected stimulus. Therefore, such extraneous disturbances should be minimised.

In neuroethological experiments, recordings from neurones of the auditory centres of the midbrains of various amphibian species have shown sensitivities to specific sound spectra, signalling different events, such as rival, mate, and threat (Feng et al., 1976; Wilczynski & Capranica, 1984; Mudry & Capranica, 1987a,b; Walkowiak et al., 1999; Murphy & Gerhardt, 2000; Bee & Gerhardt, 2002; Hobel & Gerhardt, 2003). Within and close to these brain regions, uni- and multimodal neurones were recorded which are monitoring stimuli of different modality, such as acoustic, vibratory, cutaneous, and visual. Vibration sensive neurones, for example, respond to steps on a floor from a distance of 10 m and even more. In the field, this allows frogs to detect potential predators when they are far out of vision and, eventually, to jump early enough into a pond. The visual receptive field of multimodal neurones may encompass the entire field of vision of both eyes. This massive convergence of different sensory channels toward single neurones allows multi-event detection (Ewert, 1984). Such neuronal "alarm systems" operate rapidly and highly sensitively, because information via one sensory channel (e.g., vibration) lowers the response threshold to information obtained via another sensory channel (e.g., vision), and vice versa.

Xenopus spp., being fully aquatic, lives permanently in a noisy and vibrating environment, – water (cf. Elepfand 1996a,b; Elepfand et al., 2001). Nevertheless, it was observed that populations of laboratory-bred *Xenopus* remaine completely “unmoved” by sudden sharp loud noise. When kept in clear wall tanks, they habituate to researchers moving frequently in the room and are also not necessarily startled by a human appearing overhead.

2.6. Alarm systems (See paragraph 2.6. of the General section)

Adequate alarm systems are recommended if circulation systems are used and/or aeration is required.

It is recommended that the housing facility is equipped with devices to detect fires and the intrusion of unauthorised persons and to maintain support systems such as current and water supplies. For general considerations in the care of captive amphibians see Freye (1977).

3. Health (See paragraph 4.1. of the General section)

Any effort should be made to keep animals under optimal conditions. High quality of scientific results obtained in experimental or other procedures depends largely on the physiological state of an organism. Therefore, animals should be routinely inspected by the authority responsible for the housing facility.

The physiological state of amphibians and, along with this, their behavioural activities rely largely on the time of the day depending on the animal's activity period, day or twilight/night. It could be shown that in the main activity period, heart rates, respiratory ventilations, and neuronal responses to sensory stimuli are higher than in the resting period. The season of the year, too, strongly influences physiological states and behavioural activity patterns. Even in laboratory amphibians whose winter torpor is prevented by constant light/darkness and temperature conditions, these changes are manifest due to an internal rhythm, at a lower level, so that in laboratory jargon names like "summer-frogs" and "winter-frogs" are used occasionally [cf. also Section 2.2]. Under constant laboratory conditions, food consumption is low during winter and spring, and relatively high during summer and autumn. During spring – in the mating

season – the cutaneous, auditory, and visual sensitivities are addressed to the sexual partner [cf. Section 4.1].

3.1. Animal supply

In the appropriate season of the year, amphibians should be ordered from commercial dealers or institutions, so that records about the number of individuals are available for book keeping [see also Section 4.10]. New amphibians need to be acclimated to the novel environment. Cages should be set up with all necessary accessoires and enclosures in advance. Amphibians will be often first stressed and will not eat. The temperature and humidity should be checked to be sure the range is right and there is adequate water.

3.2. Animal transportation

Amphibians should be obtained from those commercial institutions which follow the recommendations of adequate animal transportation: the *European Convention on the Protection of Animals during International Transport*, the *International Air Transport Association, and the Animal Air Transport Association*. Semi-terrestrial amphibians should be singly packed in boxes of adequate size and provided sufficiently with air and moisture. Transport of tropic species – depending on the local climate – requires an accommodation with appropriate heating devices. After arrival, animals must be unpacked without delay. Animals which arrived in a sick condition, and which do not have a chance to recover, should be sacrificed at once by a human method [Section 4.9]. The commercial sender should be informed.

3.3. Reproduction

Most amphibians appear to lack morphologically distinguishable sex chromosomes. Nevertheless, amphibians employ a genetic mechanism of sex determination (Hayes, 1998; Schmidt & Steinlein, 2001). Cases of spontaneous and experimental sex reversal (induced by temperature, hormones or surgery) are reviewed by Wallace et al. (1999). The few studies suggesting that temperature influences sex determination – as observed among reptiles – have been conducted at temperatures outside the range normally experienced by the species under study (Hayes, 1998).

Amphibian eggs are mostly laid in the water and develop into tadpoles which after metamorphosis mature to the adults. Unlike most salamanders and gymnophiona, most anurans are external fertilizers. Sperm storage in the oviduct of internal fertilizing frogs is reported by Sever et al. (2001; cf. also Sever, 2002).The developmental biology of amphibians after Hans Spemann is reviewed by Grunz (2001).

In most amphibians, laboratory bred strains are lacking for two reasons: (i) difficulties in establishing suitable environmental conditions to elicit sexual maturity and mating behaviour in captivity; (ii) difficulties in rearing amphibians successfully through their early larval and transformation stages (Nace, 1974). With metamorphosis, the immune system becomes reorganised involving a temporary immune-suppression (Flajnik, 1996). If the laboratory conditions result in the induction of metamorphosis at a less than optimal body size and state of the immune system, the animal runs a risk of infection and death (Rollins-Smith, 1998; cf. also Denver, 1997) [see also Sections 1.1 and 3.4].

Temperatures close to torpor (hibernation) during the winter period and re-warming in spring are often preconditions of successful breeding.

Xenopus laevis shows no seasonal changes in its physiological behaviour. Mating can be easily triggered in adult animals through a temporary UV radiation applied to the tank [aquatic caging see Section 4.3.1]. Alternatively, the gonadotrophic hormone chorionic gonadotrophin may be injected intralymphatically. Three to eight hours thereafter, animals are ready for mating which is indicated by the croaking advertisement calls produced by the males. The male, when ready for mating, also shows a brown velvet-type pigment on the front legs. Females have a fuller developed abdomen and are often twice as big as males. Copulation and spawning should take place in tanks with gravel and artificial water plants on the ground. Copulation lasts for about 24 hours and eggs are laid singly, often fastened on the plants. In most amphibians – especially anurans – eggs are fertilized outside of the female reproductive tract. Female sperm storage in amphibians is reported by Sever et al. (2001) and Sever (2002).

After spawning, animals should be removed from the tank. The hatching tadpoles are filter feeders and can be maintained on nettle powder (dried leaves of *Herba urticae*), egg-powder and dried yeast (the latter two in small amounts) suspended into the water. Under optimal conditions, maturity of the animals may be reached in the first year (Müller, 1976) [feeding adult amphibians see Section 4.4].

Great progress is being made in developing large scale breeding programmes for *Xenopus tropicalis* for genetic studies. The fact that *Bombina orientalis* can be easily maintained and bred in the laboratory under long-term conditions is a relatively new experience. The following list provides a short breeding instruction elaborated in the Department of Neurobiology, Faculty of Natural Sciences, at the University of Kassel, Germany.

Instruction for breeding *Bombina orientalis*

Adult *Bombina orientalis*

Males size: ca. 5.5 cm
Females size: ca. 4.5 cm
Colour: body green in shadings, belly carroty to red depending on vitamin A containing food.

Preparation for hibernation
November
↓
Vivarium: 95x60x30 cm³ in size arranged like the one described in Section 4.3.2, in addition green artificial plants that allow animals to climb up or to shelter
Watering: temperature of 8 to 10°C, renewing carefully once a week
Lighting: reduction of photoperiod and light intensity like outside, without artificial light
Feeding: mini-crickets, small pale mealworms (*Tenebrio molitor*) fruit flies (*Drosophila vestigial*), fly larvae (*Lucilia caesar*), red mosquito larvae (*Chironomus spec*), twice a week until the middle of December
Noise: the room must be absolutely quite, careful renewing the water
↓
Hibernation
December to February
↓
March
Watering: simulating rain by spraying warm water of ca. 18-20°C, renewal 4-5 times a weak
Lighting: elongation of photoperiod and light intensity like outside, without artificial light
Feeding: see above
↓
Mating and spawning
April to August:
↓
Release of eggs
↓ ca. 3 days
Tadpoles
To be separated from adults immediately

about 30 Tadpoles
Tank: 30x60x30 cm³
Watering: temperature 18-20° C, renewal 3-4 times a weak
Lighting: like outside
Feeding: toy fish food, dried mosquito larvae, daily
↓ 4-5 weeks
Beginning of metamorphosis
↓
Vivarium: 95x60x30 cm³ in size arranged like the one above, including stones that will allow juveniles to approach the land area; tadpoles in pool area
Watering: temperature 18-20°C, renewed 4-5 times a week; water level must be adjusted, so that juvenile animals can approach the land area of the vivarium; after metamorphosis, water renewed 3 times a week
Feeding: daily after metamorphosis with leaf-lice, fruit-fly larvae, mini-crickets, mosquito larvae, for about 6 month; later food diet like for adults
↓ ca. 2 years
Adult *Bombina orientalis*

3.4 Diseases

Comprehensive investigations on diseases in amphibians are relatively scarce (e.g., Reichenbach-Klinke & Elkan, 1965; Cosgrove, 1977; Siegmund, 1979; Marcus, 1981; Cunnigham et al., 1996; Mutschmann, 1998; Nichols, 2000; Davies & Johnston, 2000; Williams et al., 2002) in comparison to reptiles (Marcus, 1977; Bush et al., 1980; Murphy & Collins, 1980; Davies, 1981; Jackson & Cooper, 1981; Holt, 1981; Cooper, 1985; Miller, 1996; Mermin et al., 1997 Beynon & Cooper, 1994, 1999; etc). Ippen & Zwart (1996) discuss infections and parasitic diseases of captive amphibians with special emphasis of husbandry practices which prevent or promote diseases (cf. also Cunnigham et al., 1996).

Fungal infections play a prominent role in amphibians. An overview on fungal diseases in amphibians is provided by Pare (2003). Food toxicity associated with the presence of uneaten, partially decomposed food is often caused by growing fungal hyphae. Therefore, it is strongly recommended that overfeeding is avoided and that after each feeding session uneaten food particles are removed soon.

Superficial fungus *(Saprolegnia)* infections, especially among larval salamanders, appear as an opaque, usually fuzzy, white area of skin on an extremity, fin or external gill. Various treatments in segregated infected animals are recommended, such as:

- Solution of 2-3% calcium propionate for 1 min and immediately returned to fresh water, once daily;
- topical painting of infected areas with 2% mercurochrome solution, followed in a few minutes by washing in flowing water;
- dips at potassium permanganate at 1 : 5,000 for 5 min;
- solution of Trypoflavin for three weeks, changed every fourth day.

A newly discovered emerging fungal disease, chytridiomycosis, was found to be associated with amphibian mass mortality events and population declines in Panama and Australia. Since then, the disease has been reported also in North America, South America, Africa, and Europe (see Berger et al., 1998; Daszak et al., 1999; Boyle et al., 2003; cf. also Daszak et al., 2002, 2003; cf. Section 1.1). Various hypotheses are currently tested on what factors drive its emergence and how it causes death in amphibians. Recently, Daszak and co-workers have provided evidence that "pathogen pollution", the anthropogenic introduction of non-native hosts or parasites to new locations, is a major factor contributing to this disease's emergence. The bullfrog, *Rana catesbeiana*, is globally traded as a food item and appears to be relatively resistant to chytridiomycosis. It may serve as an efficient carrier host.

Zygomycosis refers to an angiotropic (blood vessel-invading) fungal infection produced by the various Zygomycetes. This disease is also sometimes referred to as mucormycosis, but the term zygomycosis is preferred. For a review on zygomycosis due to *Basidiobolus ranarum* see Gugnani (2000). Myositis associated with fungal infection by *Ichthyophonus*-like organisms was diagnosed in 35 of 260 wild amphibians (such as *Rana clamitans, R. sylvatica, R. palustris, R. catesbeiana*) collected in Quebeck Canada (Mikaelian et al., 2000). Ichthyophonosis is enzootic in amphibians from Quebeck. Infections with the fungus *Ichthyophonus hoferi* as well as the Gram negative bacterium *Aeromonas hydrophila* were diagnosed in some of the samples. For infections by iridoviruses see Hyatt et al. (2000).

Aeromonas hydrophila is a species of bacterium that is present in all freshwater environments and in brackish water. Some strains of *A. hydrophila* are capable of causing illness in amphibians as well as in humans who may acquire infections through open wounds or by ingestion of a sufficient number of the organisms in food or water. In captive toads and frogs the *Aeromonas* infection (red leg) is the most common disease. Signs of red leg include cutaneous ulcers and characteristic pinpoint hemmorrhages over the abdomen, leg, and tongue, with lethargy and emaciation. This external bacterial infection begins with mechanically caused abrasions or wounds to the skin. Toads and frogs must therefore be inspected for such signs, any diseased animal removed immediately, kept in segregation and treated with antibiotics such as Tetracycline. For amphibian skin diseases see also Reavill (2001).

Amphibians are hosts of nematodes (Goldberg et al., 1996; Moravec & Skorikova, 1998; Barton, 1999). Most nematode species are not host-specific. About 40 families infect amphibians and reptiles; 68 genera infect anurans, of which only 22 are specific to frogs, 4 are shared with salamanders, 11 with fish, 6 with mammals or birds and 37 are shared with reptiles. Systemic infection of frogs with the trematode *Alaria* (Fernandes et al., 1976) may be fatal also to humans handling those frogs (Freeman et al., 1976) [see also Section 4.5]. Alarm over increasing reports of deformed amphibians has intensified since the early 1990s. Over the last decade, abnormalities have been reported in 36 species of amphibians in the USA. The trematode *Ribeiroia* was isolated from deformed frogs in the field, was employed at realistic

concentrations in experimental exposures, and produced the same range and frequency of amphibian limb abnormalities as observed at field sites [cf. also Section 1.1]. Ectoparasites in amphibians include leeches (Hirudinea) and helminths. For regulation of worm burdens in amphibians see Tinsley (1995).

Xenopus laevis in captivity is sometimes found turning around its sagittal body axis while swimming, a behaviour that usually "recovers" after two to three days without any therapy. However, if such an animal is swimming with uncoordinated hind legs, it should be isolated, since in a few hours it will become bloated and the lymphatic sacks appear to be extremely filled. This unknown disease is lethal in a few days.

4. Housing, enrichment and care

4.1. Social behaviour

In most amphibians, social behaviour is mainly restricted to the mating season. However, group-housing of amphibians is advisable, for instance to improve feeding and reduce fear responses. For example, in Xenopus spp. group feeding promotes feeding frenzies inducing all animals to feed. At very low stocking densities such frenzies do not occur and food is frequently not eaten.

To avoid cannibalism in certain species (particularly among larval Ambystoma spp. and Scaphiopus spp.), these animals should be maintained in small groups. Cannibalism in groups can be reduced by size grading.

Due to tigmotaxic effects, (semi-)terrestrial and (semi-)aquatic amphibians get their skin in touch with the surface of the hiding place. Terraria should make some allowance for that. Especially in captivity, *Bufo* spp. and *Xenopus* spp. are searching for body contact to conspecifics. This may be also a response to lack of refuges in most terraria where animals attempt to hide under another because of allround illumination. When maintained under natural conditions, *Xenopus* do not live in contact with each other. Nevertheless, group housing is recommended for the stimuli (sight, sound or smell) that conspecifics create within a group [cf. Section 4.3.1]. Group housing is also required for procedures which investigate breeding behaviour or are concerned with captive breeding programmes that maintain the population of laboratory animals.

Mating plays a prominent role in social behaviour of amphibians. The mating season is introduced with the migration over long distances towards the pond in which the animal was born. It is suggested that toads (*Bufo japonicus*) use a local map to orient to the breeding pond. Probably, the local map was memorized by the newly metamorphosed toads at their terrestrial trip from the pond. Since blinded toads – but rarely anosmic toads – could reach the pond, olfaction seems to be an important cue in this context (Ishii et al., 1995). Sexual and seasonal differences in the vomeronasal epithelium were observed in the red-backed salamander (Dawley & Crowder, 1995).

In spring, the males of terrestrial and semi-terrestrial anurans attract their females by species-specific mating calls (e.g., Wilczynski & Capranica, 1984; Mudry & Capranica, 1987a,b; Walkowiak et al., 1999; Murphy & Gerhardt, 2000; Bee & Gerhardt, 2002; Hobel & Gerhardt, 2003). Vocalization behaviours of anuran amphibians are universally sexually dimorphic. Usually, only males give an advertisement call, while female frog calls are limited to a soft and simple release call which is specifically suppressed during mating. In a very few species, however, female frogs also give mating vocalizations (Emerson & Boyd, 1999). Certain visual and/or tactile stimuli release in the males clasping behaviour that is addressed to conspecific females. Most anurans are external fertilizers, whereas most salamanders are internal fertilizers. Among the species *Ambystoma*, there are bisexual and parthenogenetic subspecies.

4.2. Environmental enrichment

The terrestrial habitat of amphibians should be structured, including, for example, branches, leaves, pieces of bark and stones. Amphibians benefit from such environmental enrichment in different ways: for example, such inclusions allow animals to hide, and provide labels for visual and spatial orientation. The side walls of the terraria should be textured to provide a structured surface.

The provision of hiding places/shelters that are appropriate to the amphibian's needs is recommended, because they can reduce stress on captive amphibians. For example, in Xenopus spp. a tube of ceramic or plastic may be provided. Refuges should be inspected regularly for sick or injured animals. A dark floor to the tank may enhance the sense of security in the animals.

Materials used for enrichment devices should not be detrimental to the health of the amphibians. Enclosures and enrichment structures should have smooth surfaces and rounded edges to minimise the risk of injury to the amphibian's skin.

Behavioural studies under laboratory conditions have shown that the presence of background textures is necessary for the accuracy of visual functions (Ewert, 2004a). These capabilities require an evaluation of the visual target in relation to its background. The impact of this experience on the holding conditions is important, particularly in captive terrestrial and semi-terrestrial amphibians. The opaque side walls of the terraria should be textured. Frogs and toads become adapted to their environment based on textural patterns and configurations. Transferred into a different environment (such as an optical homogeneous cage), the animals are scared and will often neglect prey.

Terrestrial amphibians feel more comfortable if a hiding place is available that allows them to get their skin in touch with the shelter's surface [see Section 2.1]. The provision of shelter places for *Xenopus* is advisable, but should not be prescribed. Observation of a large number of laboratory environments has shown that laboratory-bred *X. laevis* and *X. tropicalis* are rarely found to use shelters placed in their tanks. They appear to prefer more lighted environments, "light twilight" to bright light.

4.3. Enclosures – dimensions and flooring

The most practical way of providing suitable holding conditions for amphibians within an animal facility is, first, to establish a set of general environmental conditions for the rooms as a whole and, secondly, for each tank or terrarium to be established as an environmental chamber for one or several individuals. This involves structuring the cage space to allow an individual's activity-related use as well as introducing appropriate stimuli and materials.

Xenopus laevis [Photo: W. Leonard]

4.3.1. Enclosures for aquatic amphibians

Aquatic amphibians, such as Xenopus laevis, or amphibian larvae are housed in tanks and aquaria. These may be equipped with a gentle flow-through water system for the circulation of uncontaminated (for example, dechlorinated) water, a heating device to maintain suitable temperatures, and a compressed air supply and air stones for aeration. Care is needed to ensure that aeration does not cause injury to the animals. Unless a proper flow system is in place, the water in the enclosures should be renewed with water of an appropriate quality about twice a week.

For Xenopus spp., systems with regular changes of water (fill-and-dump systems) are sufficient for maintaining appropriate water quality (such as minimising levels in ammonia). Airstones are not required for Xenopus.

Furthermore, too long, narrow enclosures should be avoided since they may restrict locomotor activity and social behaviour such as feeding frenzies.

Most tadpoles are herbivores or/and detritivores, so that cannibalism will not be a problem. Due to growth inhibition in tadpoles, the population density of the larvae must be kept relatively low (Rose & Rose, 1961). Tadpoles sort out species-specific factors into the water which inhibit growth (Aikin, 1966). This problem can be reduced or eliminated if the water is changed 4 to 5 times a week. Water temperatures for larvae are in the range of 10-12°C (salamanders) or 18-22°C (most species of tadpoles, including the neotenic larvae of *Ambystoma mexicanum)*. Temperatures of 10-25°C are tolerable, whereas 1-10°C lead to torpor.

From the species *Ambystoma mexicanum*, individuals are accommodated in tanks of the recommended size at a dim place. The aerated, circulating filtered, water should have a temperature of 10-25°C; pH=8.5, and 6-16° dH. The bottom of the tanks contains tubes of clay, gravel and stones, so that individuals can select their own territory. *Ambystoma mexicanum* stays for the whole life (up to 13 years) in larval stage (neotenic) due to a dysfunction of its thyroid gland. The larvae reach sexual maturity with an age of one year. Each releases up to 500 eggs. Juvenile larvae (ca. 1 cm in size) are hatching after about four weeks.

Table I.2. Aquatic urodeles,* e.g., *Ambystoma spp: Minimum enclosure dimensions and space allowances

Body length*) ***(cm)***	***Minimum water surface area*** ***(cm^2)***	***Additional minimum water surface area for each animal in group holding*** ***(cm^2)***	***Minimum water depth*** ***(cm)***
≤ 10	***262.5***	***50***	***13***
11 – 15	***525***	***110***	***13***
16 – 20	***875***	***200***	***15***
21 – 30	***1837,5***	***440***	***15***
31 – 40	***3150***	***800***	***20***

****) measured from snout to tail***

The recommendations for minimum space requirements for aquatic urodeles, e.g. *Ambystoma* spp. differ from the guide of the Swedish Board of Agriculture (Department for Animal Production and Health, Animal Welfare Division) that suggests more space. The Axolotl is a native of permanent lakes near Mexico City where extinction is imminent due to loss of original habitat. Virtually all information on life

history of the Axolotl has been obtained from laboratory colonies as it has been reported that no ecological study of a wild population has been conducted (Armstrong & Malacinski, 1989). This lack of basic information from natural populations contributes to current uncertainties about how best to maintain animals within the laboratory situation. Therefore the proposed recommendations have taken into consideration the wealth of practical experience (good laboratory practice, GLP) gathered from the maintenance of Axolotl colonies in the laboratory situation within the UK, Europe and the United States. The Indiana University having received the first Axolotl's in 1956 from Robert Briggs who pioneered the study of amphibian tumours and nuclear transplantation (Armstrong & Malacinski, 1989). This practical expertise has achieved excellent captive breeding results from Axolotl colonies, ensuring the survival of the species in captivity. Moreover, it has enabled the supply of fertilised embryo's essential for ongoing research programmes that include investigations into development of germ cells and blood cells (Johnson et al., 2001, 2003a, 2003b). The proposed minimal space requirements enable the Axolotl's freedom of movement and importantly satisfy the physiological and behavioural needs as follows:

1. The behavioural repertoire of Axolotl's appears relatively limited. It is clear from numerous observations that the animals have a sedentary lifestyle when maintained under optimum environmental conditions. The animals move in a slow and gentle manner unless startled. If stimulated, they are able to perform tight twists and turns with ease. Therefore tank sizes that are around 5cm wider and 1.75 times longer than body length for single housed animals (see Table below), enable the Axolotl's to move relatively freely within the tanks and without need to resort to tight twists and turns. Increasing tank size does not appear to alter movement significantly. It is recognised that further more detailed studies are required, but that in the absence of evidence to the contrary, tank sizes based upon current successful housing strategies are recommended.

Body length * (cm)	Minimum water surface area(cm^2)	Minimum tank width (cm) 5cm wider than axolotl	Minimum tank length (cm) 1.75* longer than axolotl	Minimum additional water surface area for each additional animal	Minimum water depth (cm)
<10 single	262.5	15	17.5	50	13
3 animals	362.5	15	24.2		
11-15 single	525	20	26.3	110	13
3 animals	745	20	37.3		
16-20 single	875	25	35	200	15
3 animals	1275	25	51		
21-30 single	1837.5	35	52.5	440	15
3 animals	2717.5	35	77.6		
31-40 single	3150	45	70	800	20
3 animals	4750	45	105.6		

2. The proposed tank sizes also allow the introduction of environmental enrichment materials such as plastic tubing and artificial weed. The use of which is being evaluated in the UK.

3. Females appear gregarious, and do not actively seek to maintain a distance between themselve. When housed together, even in larger tanks, they frequently position so that individual limbs are in contact with each other while resting on the floor of the tank. Only limited additional surface area is required when animals are group housed. The minimum additional surface area for each animal (group holding) has therefore been calculated to give enough extra space to allow the animal to lie diagonally across the additional surface area provided for it. The proposed tank sizes allow animal's space to achieve this.

4. When anticipating being fed, the animals raise their snouts to the surface but maintain contact with the floor of the tank using their hind-limbs and tail. The proposed water depth will achieve this. Increasing water depth to 20cm for animal's of 16 cm body length would not allow this behaviour to occur.

5. The proposed tank sizes should not lead to increased stress for animals housed. Measurable stress factors are: health status, high quality ovulatory response, and successful metamorphosis and development of juveniles. All of which are achieved using the proposed tank sizes. For example at the University of Nottingham the colony of 22 males and 34 females produced 27 successful matings in 13 months with an average embryo yield two to three hundred per month. 50 young axolotls are currently being reared with a mortality level of 3.8% to date. Note: in respect to the development of juveniles, a surface area of around 20 square cm and a depth of around 7cm has been used successfully to raise young animals to around 5cm body length. Given that around 100 offspring are produced from a single spawning, it is highly likely that high densities of juvenile offspring would occur in the wild. However as no information appears to be available about the situation in the wild, and that the density used above proves successful, then until further detailed studies can be undertaken, successful breeding and rearing strategies should be used to maintain this endangered species.

6. "Footprint" area and stocking denstities. Axolotl's like *Xenopus* live in a three-dimensional environment. For *Xenopus* (see below) it has been argued successfully that the minimum water surface area of the animal should be a constant function of the "footprint" area of the animal within a given species. To the first approximation, the animal footprint area is related to the square of any linear dimension such as length. Thus if one plots a graph of body length against the ratio {tank surface area}:{body length squared}, then one should expect to see a flat straight line parallel to the x-axis. Such a plot would indicate that on a proportional basis, one is not discriminating against either larger or smaller animals, in terms of the amount of space allocated to them. The proposed tank sizes do indeed give such a plot indicating minimal discrimination against larger or smaller animals. Nevertheless it is recognised that lack of scientific data makes it difficult to make categoric recommendations. The proposals are therefore based wholly on practical experience in successful husbandry methods in rearing Axolotl's. It is therefore appropriate that interim tank sizes are adopted until scientific data can be obtained to allow informed decisions on tank sizes to be made.

Table I.3. Aquatic anurans,* e.g., *Xenopus spp: Minimum enclosure dimensions and space allowances.*

Body length (cm)	***Minimum water surface area*** (cm^2)	***Minimum water surface area for each additional animal in group-holding*** (cm^2)	***Minimum water depth*** (cm)
< 6	***160***	***40***	***6***
6 - 9	***300***	***75***	***8***
10 - 12	***600***	***150***	***10***
13 - 15	***920***	***230***	***12.5***

** these recommendations apply to holding (i.e., husbandry) tanks but not to those tanks used for natural mating and super-ovulation for reasons of efficiency, as the latter procedures require smaller individual tanks. Space requirements determined for adults in the indicated size categories; juveniles and tadpoles should either be excluded, or dimensions altered according to the scaling principle*

*** measured from snout to vent*

All species of clawed frogs *Xenopus* are so-to-speak "technically" aquatic amphibians [quality of water see Section 4.5]. However, they live completely aquatic and die very quickly out of water. In the laboratory, *Xenopus laevis* are accommodated in small groups in a tank of the recommended size, covered by a perforated metal plate. All walls, except the front, should be painted grey or black with visual

textures. It is advisable that water depth should not be so great throughout the holding area as to preclude the opportunity to surface breathe with hind toes in contact with the base.

The regularly changed water should have room temperatures up to 22°C. In the wild, *Xenopus* spp flourishes in stagnant ponds. Thus, aeration in the tank may be even detrimental to the health, for example, egg production can be reduced. At the bottom of the tank, stones and tubes of clay provide the individuals shelter. However, there is no scientific evidence that such shelters reduce stress in *Xenopus* spp. Furthermore, it is not advisable in these species to enrich the environment with natural or artificial plants, since – in the wild – they live in plantless ponds.

The recommendations for minimum space requirements for aquatic anurans, e.g. *Xenopus* spp. (Table 3), too deviate from the Guide of the Swedish Board of Agriculture [Department for Animal Production and Health, Animal Welfare Division] that suggests more space. The present recommendations consider the ample experimental experience based on good laboratory practice, GLP, by the users, such as the British Xenopus Group. This group is based in about 20 institutions across the UK, has published over 1,000 papers to date and has considerable independent GLP experience in the use of *Xenopus* – ranging from up to 30 years, altogether 500-man years.

In their natural environment, *Xenopus* spp. lay large numbers of offspring and have a relatively fast developmental programme. This results in huge numbers of individuals and densities in ponds in the wild. It is reasonable to expect that *Xenopus* are well adapted to this existence, and there is considerable evidence to suggest that this is indeed the case [cf. "The Biology of *Xenopus*", Kobel & Tinsley, 1996, Oxford Science Publications]. Furthermore, it is known that the behavioural repertoire of *Xenopus* is relatively limited. It can be largely, if not completely, recapitulated using the recommended conditions. Group housing has a variety of advantages in the welfare of *Xenopus*:

(i) Body contact reduces stress. *Xenopus* live in very high densities in small pools in the wild. Irrespective of density and illumination frogs periodically lie on each other, swim into and out of groups and frequently collide with each other. Experience with raising *Xenopus tropicalis* in the laboratory over several generations shows that animals kept at low densities grow more slowly than those kept more densely and are subject to additional stress up to the point of lethality. Furthermore, it can be shown that absence of body contact by conspecifics is an important stress factor. Measurable stress factors are: health status, high quality ovulatory response, and successful metamorphosis and development of the juveniles.

(ii) Feeding frenzies. Another aspect of holding *Xenopus* in groups concerns feeding. Efficient group feeding normally involves feeding frenzies, whereby the feeding activity is initiated by one animal inducing similar behaviour in other animals in the close vicinity. At low densities, feeding frenzies do not occur and food is frequently not eaten.

(iii) Reduced protective arousal. In the wild, group-behaviour minimises the risk of predation by reducing the likelihood that any one individual will be taken by the predator. Consistent with this notion, it was demonstrated that *Xenopus* accommodated in densities according to the recommendations shown in Table 3 show reduced protective arousal responses.

(iv) "Footprint" area and stocking densities. Since *Xenopus* lives and operates in a three-dimensional environment with highly varied dynamics, understanding and optimising *Xenopus* in captivity is therefore dependent on four-dimensional analysis, with dynamics being the fourth dimension. In view of differing depth of water in which *Xenopus* are held, it has become accepted to consider "footprint" as well as quantities of water.

The minimum water surface area per animal should be a constant function of the "footprint" area of the animal, at least within a given species. To the first approximation, the animal footprint area is related to the square of any linear dimension such as length. Thus if one plots a graph of body length against the ratio {tank surface area}:{body length squared}, then one should expect to see a flat straight line parallel to the x-axis. Such a plot would indicate that on a proportional basis, one is not discriminating against either larger or smaller animals, in terms of the amount of space allocated to them.

4.3.2. Enclosures for semi-aquatic and semi-terrestrial amphibians

Semi-aquatic and semi-terrestrial amphibians are kept in enclosures consisting of a terrestrial part and an aquatic part. The water area of the terrarium should allow animals to submerge. Unless a flow-through system is used, water should be renewed at least twice a week.

Each terrarium should be covered to prevent escape. It is advisable to paint or otherwise cover the outside of transparent walls to minimise damage to the animal. Additions to the interior design can include: soft-foamed plastic material on the floor near the pool area, stones, pieces of artificial bark material, artificial branches and leaves, and shelves. Fine sawdust and any other related small-particle substrate should be avoided, as it affects the sensitive body skin, harbours pathogens and is difficult to clean and re-use.

Table I.4. Semi-aquatic anurans, e.g., Rana temporaria: Minimum enclosure dimensions and space allowances

*Body length** (cm)	*Minimum enclosure area*** (cm^2)	*Minimum area for each additional animal in group holding* (cm^2)	*Minimum enclosure height**** (cm)	*Minimum water depth* (cm)
= 5.0	*1500*	*200*	*20*	*10*
5.5 - 7.5	*3500*	*500*	*30*	*10*
> 7.5	*4000*	*700*	*30*	*15*

** measured from snout to vent*
*** one third land division, two thirds water division sufficient for animals to submerge*
**** measured from the surface of the land division up to the inner part of the top of the terrarium; furthermore, the height of the enclosures should be adapted to the interior design*

Table I.5. Semi-terrestrial anurans, e.g., Bufo marinus: Minimum enclosure dimensions and space allowances

*Body length** (cm)	*Minimum enclosure area*** (cm^2)	*Minimum area for each additional animal in group-holding* (cm^2)	*Minimum enclosure height**** (cm)	*Minimum water depth* (cm)
= 5.0	*1500*	*200*	*20*	*10*
5.5 - 7.5	*3500*	*500*	*30*	*10*
> 7.5	*4000*	*700*	*30*	*15*

** measured from snout to vent*
*** two-thirds land division, one-third water division sufficient for animals to submerge*
**** measured from the surface of the land division up to the inner part of the top of the terrarium; furthermore, the height of the enclosures should be adapted to the interior design*

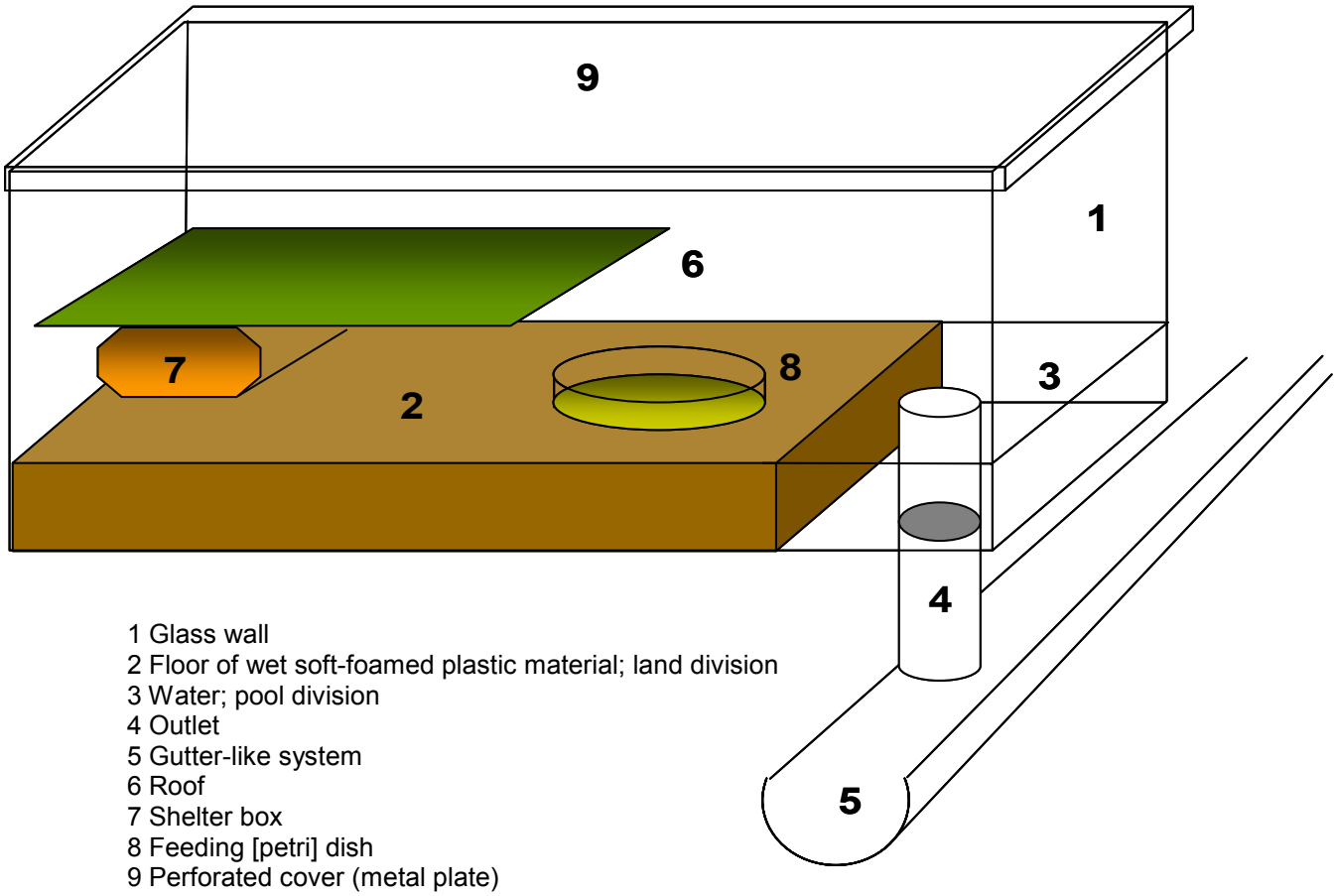

Fig.3. Examples of enclosures of a cage for housing semi-terrestrial anurans. (Department of Neurobiology, Faculty of Natural Sciences, University of Kassel, Germany).

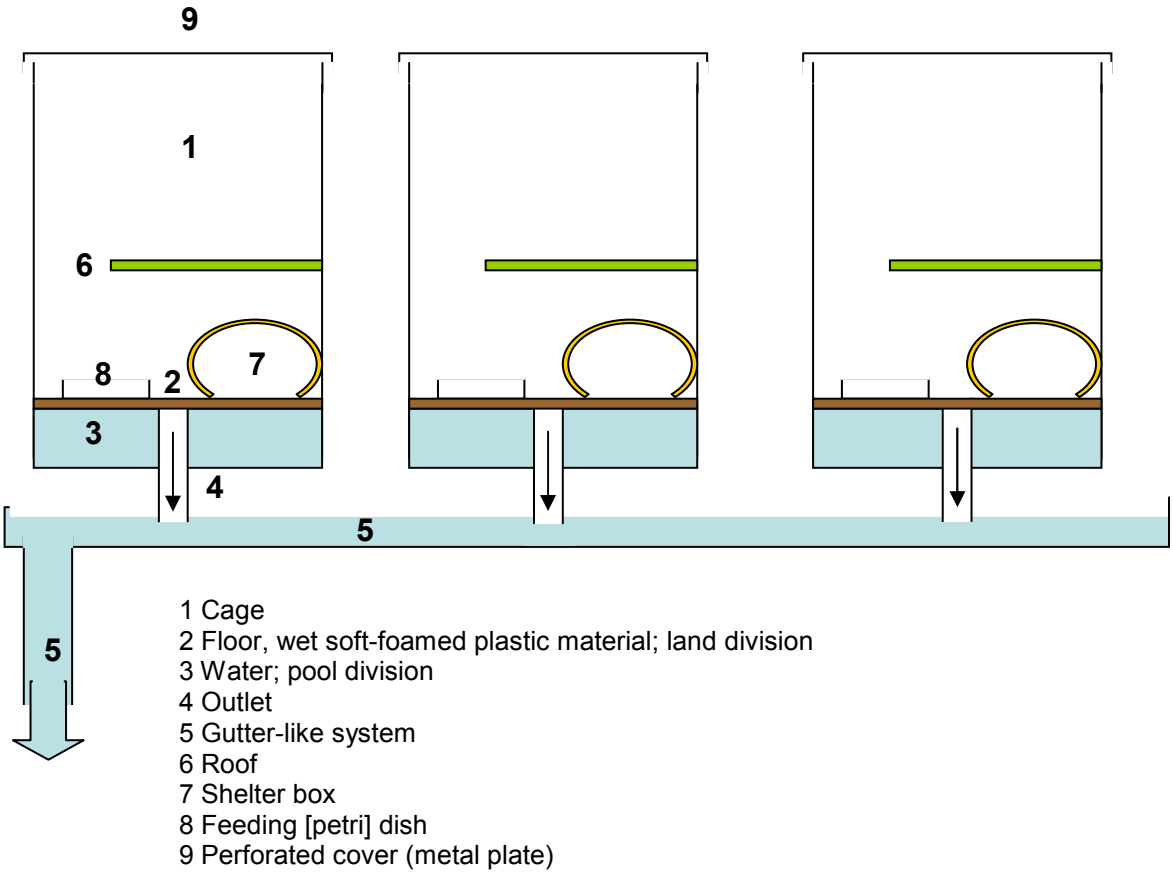

Fig.4. Example of an arrangement of several cages (cf. Fig.3) for housing semi-terrestrial amphibians.

Unlike many "show terraria", laboratory terraria – which accommodate reasonable numbers of individuals – should not be equipped in a manner similar to natural conditions, that is with fine gravel, earth, sand, peat, and living plants. GLP experience has shown that such substrates are contra-indicated for laboratories since it is difficult to remove dirt, excrements, and uneaten partially decomposed food particles to the extent required for scientific standards. As a result, mortality in such-semi-natural environments can be very high due to infections after mechanical injury or food toxicity [see Section 3.4].

Based on GLP experience and local initiatives for improving the housing of toads in captivity, the following standard terraria provide holding conditions that keep the mortality rate very low (Ewert & Ewert, 1981). Experimental animals, e.g., *Bufo marinus*, are kept in an animal facility (amphibian room). Terraria are made of glass. Each one is covered by a removable perforated metal plate that prevents toads from climbing out. Except of the front side, the remaining glass walls are painted dark green with textures. Behavioural studies under laboratory conditions have shown that the presence of visual background textures is necessary for the accuracy of visual functions, such as depth estimation, judging absolute object sizes, and spatial orientation (Ewert, 2004). A portion of grey structured soft-foamed plastic material covers about two thirds of the terrarium floor.

Fig. 5. Arrangement of several cages for housing semi-terrestrial amphibians (cf. Figs.3 and 4) [Source: Department of Neurobiology, Faculty of Natural Sciences at the University of Kassel].

Fig. 6. View into a terrarium (cf. Fig. 5) showing the land division of wet soft-foamed plastic material (right) and the pool division with a bathing cane toad, *Bufo marinus* (left). Examples of enclosures: artificial bark; a feeding dish with mealworms; a half piece of plastic tube; stones; artificial branches with leaves; a piece of pottery.

Artificial bark and tubes of clay or plastic material offer shelter. The remaining part of the terrarium is filled with water and serves as a pool. The bottom has an outlet that is closed by a plug. A gutter-like system underneath the terrarium collects the outlets of a row of several terraria. In this arrangement, the toad's skin is kept moist by the wet foam material, thus allowing the "water absorption response" behaviour [cf. Section 2.3]. Furthermore, these terraria are easy to clean. After feeding toads once or twice a week, uncontaminated (dechlorinated) water is supplied to the terrarium by a hose. This cleans the foam from excrements and food particles that pass the pool through the outlet into the gutter system.

Bombina orientalis can be accommodated in a comparable manner. Artificial branches and leaves, as well as smaller tubes and stones will allow the animals to climb or to take shelter, respectively.

4.3.3. Enclosures for arboreal amphibians

Having regard for the behaviour of different arboreal species, every effort should be made to allow for this by the provision of appropriate structures for climbing and resting by arboreal species (see section 4.3.2). In addition, it is necessary to provide water in which they can submerge themselves or seek greater humidity. If water dishes are used, they should be arranged in such a way that they are easy for the amphibians to enter or to leave.

Table I.6. Minimum space requirements for arboreal anurans, e.g., Hyla cinerea

Body length* ***(cm)***	***Minimum enclosure area*** ***(cm²)***	***Minimum area for each additional animal in group-holding (cm²)***	***Minimum enclosure height**** ***(cm)***
= 3.0	***900***	***100***	***30***
> 3.0 - 6.0	***1500***	***200***	***30***

**** measured from snout to vent***
***** two-thirds land division, one-third pool division sufficient for animals to submerge***
****** measured from the surface of the land division up to the inner part of the top of the terrarium; furthermore, the height of the enclosures should be adapted to the interior design including, e.g., shelves, large artificial branches, and structures for climbing***

4.4. Feeding

The majority of amphibians are carnivores with food preferences for living small invertebrates (such as larvae, insects and worms). Captive animals should be maintained on their natural foods or on foodstuffs approximating those of their natural diets. However, captive aquatic amphibians can successfully be maintained on pieces of fish fillet or scrapings from frozen liver and heart. The feeding frequency should be related to environmental conditions, such as temperature and light intensity. Daily feeding is not advisable for adult animals, but once to three times weekly to satiation at each feeding is recommended.

4.4.1. Food suitable for aquatic amphibians

It has been shown experimentally that in aquatic amphibians configurational aspects of the shape of prey do not play a role for prey-catching, since the water turbulences do not allow animals to define an object as prey, e.g., by its orientation to its direction of movement. Observation has shown that laboratory-held *Xenopus* have no difficulty in finding their food, as these aquatic frogs do not use their sight primarily, rather often use their limbs, sense of touch and their lateral-line-system to localize and gather food (Elepfand, 1996a,b; Elepfand et al., 2001).

Aquatic adult newts will feed on various aquatic invertebrates, such as snails, crustaceans, aquatic insect larvae and *Tubifex*. *Ambystoma mexicanum* can be fed with *Daphnia*, earthworms, mosquito larvae, and scrapings from frozen heart. Captive aquatic amphibians can be also fed with frozen shrimps, frozen whole minnows, pieces of fish fillet. Aquatic frogs, such as *Xenopus laevis*, will readily take scrapings from frozen liver and heart.

Special food diet is recommended for breeding programs, such as the one proposed for *Bombina orientalis* in Section 3.3.

4.4.2. Ethological studies on prey-catching releasers in terrestrial amphibians

The prey-catching behaviour of toads and frogs (Ewert, 1984, 1997, 2003, 2004a) and salamanders (Roth, 1987) is well investigated in experiments using prey dummies [see also movie on *Image Processing in the Visual System of the Common Toad – Behaviour, Brain Function, Artificial Neuronal Net.* IWF-Film C1805, Video or CD-ROM; IWF Wissen und Medien gGmbH, Institut für den Wissenschaftlichen Film, Nonnenstieg 72, D-37075 Göttingen, Germany, vertrieb@iwf.de].

These amphibians respond preferably to living prey; thus, movement is an essential component of the prey sign stimulus (Ewert, 2004a,b). Jerky movements make prey especially attractive. In common

toads, *Bufo bufo*, the speed of prey dummies eliciting maximal prey-catching activity is 30-60 deg. of visual angle per sec [°/sec]. Prey stimuli moving less than 3°/sec are still detected and responded. The minimal detectable speed for movement specific ganglion cells of the retina is even less than 0.1°/sec. Another important component of the prey stimulus is size. In response to square or disc-shaped prey dummies, contrasting with the background, those of an edge length of 5 to 10 mm are optimal prey-catching releasers. Moving small objects of 0.3 mm will still elicit prey-capture. Investigations on different anuran species have shown that the preferred prey size s [mm] correlates with the width w [mm] of the animal's jaw, s=0.43w. Moving big objects elicit avoidance behaviour, especially those traversing the animal's dorsal visual field, thus simulating a bird of prey. Configurational aspects of prey, too, play an important role for the release of prey-catching behaviour. Experiments using small moving bars of card board of different length and different orientation, either parallel or across the direction of their movement, have shown: elongation of the edge of a bar parallel to the direction of movement ("worm" configuration) increases the similarity to prey, whereas elongation of the edge across the direction of movement ("anti-worm" configuration) strongly reduces the prey value. From the toad's midbrain roof, responses of neurones were recorded which show preferences to prey-like stimuli. In fact, natural prey objects of the toad's biotope move in direction of their longitudinal body axes, such as earthworms, millipedes, wood lice, and slugs (Ewert, 1974, 1998).

The "anti-worm" stimulus resembles configurational components of moving snakes, the arch-enemies of toads. Certain snakes (e.g., hognose snakes *Heterodon* spp.) are primarily toad predators in nature. Hognose snakes are opisthoglyphous (having fangs at the back of the mouth) and they use this feature to "deflate" toads which may puff themselves up with air to unswallowable proportions. If, under captive conditions, a hognose snake will not accept frogs (which are easier to obtain than toads) they may be "scented" by rubbing with fresh toad skin before feeding. If a hungry hognose refuses mice, rubbing a mouse against a toad, so that the mouse smells like a toad, will often "trick" them into eating mice.

4.4.3. Laboratory food

The preference to worm-like moving objects justifies feeding toads on mealworms (larvae of *Tenebrio molitor),* which can be obtained from mealworm farms. Laboratory experience has shown that for large numbers of toads mealworms are a suitable long-term diet. In this case, however, it is advisable to maintain mealworms on mouse diet pellets which contain the essential nutrients and are enriched with vitamins and trace elements. In this food chain, toads develop no nutritional deficiencies. Nevertheless, the diet of anurans and salamanders should be enriched by earthworms, crickets, collemboles, beetles, woodlice, millipedes, fly larvae, etc.

In many frog species, the preference for worm-shaped prey animals is much less developed than in common toads. Water frogs, especially *Rana esculenta*, prefer various flying insects, attracted by their irregular movements. These frogs are difficult to maintain in captivity. Their terraria must be covered with fine screens to insure that the insects offered as food do not escape. Very large frog and toad species, such as *Rana catesbeiana* or *Bufo marinus* need large amounts of food. They take even live young mice. By conditioning, they can be induced also to take dead food items like liver, heart, cray fish, or small fish. *Bufo marinus* can be conditioned by smell to take even dog food.

4.4.4. Food conditioning and consequences of hand-feeding

During feeding, laboratory toads and frogs quickly associate the prey odour with prey. As a result, in the presence of familiar prey odour, the toad's prey-catching motivation increases to such an extent that non-prey items are included in their prey schema, such as a moving conspecific, or even the moving hand of the experimenter. In the absence of familiar prey odour the prey-selective behaviour is normal. The neurobiological basis of this phenomenon was investigated quantitatively (Ewert et al., 2001). Since in the presence of familiar prey odour, the toad's prey-selecting behaviour is strongly reduced, it is possible to feed toads with mealworms that are filled in a dish. Attracted by the prey odour, the toads approach the dish and – sitting around it – will catch the worms. This arrangement has the advantage that prey items are not scattered over the terrarium. Efficient group feeding involves feeding frenzies, whereby the feeding activity is initiated by one animal inducing similar behaviour in other animals in the close vicinity [cf. also Section 4.3.1].

Toads can be conditioned also in another way, namely by hand-feeding. If a prey object is presented to the toad with the experimenter's hand, for several days once, the toad associates the moving hand (previously a threatening stimulus) with prey. Consequently, the toad treats the hand like a prey and snaps towards it. This effect is generalized to such an extent that almost every moving object (in the absence of prey odour!) releases prey-catching behaviour. Here, too, the neurobiological basis of this type of conditioning was studied in detail (Ewert et al., 2001). It should be emphasized that hand-feeding is unsuitable for procedures in which conditioning would have a negative impact on the experimental results.

Most of the salamander species, too, not only possess a highly efficient visual system, but can orient themselves almost as effectively by means of olfaction or vibration sense. Furthermore, it has been shown that at least part of their behaviour – especially that concerned with feeding – is influenced by individual experience (e.g., see Roth, 1987).

4.5. Water quality

For aquatic and semi-aquatic amphibians water quality, including the concentration of ammonia and the pH level in water, should be regularly monitored.

In order to avoid diseases, the land and pool areas in the terraria must be carefully cleaned from dirt, excrements and food particles [cf. Section 3.4]. Good hygiene improves the health of the laboratory animals.

For aquatic species it is advisable to apply re-circulated biologically filtered water re-use systems, because such systems permit most easily the controlling of water quality. Consistent water quality is of paramount importance, because it reduces stress and contributes to animal health, which in turn permits the user to perform successful science.

It is obviously important to keep *Xenopus laevis* in clean water, least of all to prevent disease. *Xenopus* are adapted to slow-moving, turbid water. However, there is no substantive evidence that circulating flowing systems are more healthful for this particular species of frog in the long run. In the UK, about 70% of institutional facilities operate so-called “fill and dump” systems. Many of these have been in place for 10 years or more and none report any persistent, detrimental welfare problems.

A key issue with fully aquatic species is the volume of water available to them and the frequency with which it is changed. The reason for this is that conditions leading to poor health (and presumably suffering) are those in which the ammonia level rises too high. Ammonia is excreted by amphibians, and its concentration will depend on the water handling regime. A level of 5 mg/lit would be cause for concern, although the degree of potential harm increases steeply with the pH of the water, as there is more ammonia and less $NH4^{+}$ at higher pH. All of the fill and dump facilities in the UK utilise a minimum of 2.5 litres of water per *Xenopus laevis* (1 lit for *Xenopus tropicalis*), and water changes occur at least two times per week with no apparent ill effects. Therefore, a system with regular water changes achieves the goal of maintaining appropriate water quality very successfully. Given the success which individuals have achieved with these systems, they should therefore be considered as well as flow-through water systems. In fact, it is clearly worthwhile to develop good re-circulating systems which utilise continuous purification regimes, since these can generate extremely low levels of ammonia and mimic the condition of an infinite water supply for each animal.

4.6. Substrate, litter, bedding and nesting material (See paragraph 4.8 of the General Section)

4.7. Cleaning

In order to avoid diseases, the terrestrial and aquatic areas in the terraria shall be carefully cleaned to remove dirt, excrement and food particles.

In order to avoid diseases, the land and pool areas of the terraria must be carefully cleaned to remove dirt, excrements, and food particles. The same holds for the tanks of aquatic animals; in these tanks the water

should circulate. It should be avoided to use aggressive detergents. Amphibians do explore their cages and will choose a favourite place (e.g., a stone or piece of bark) drinking from and sleeping on. They become used to the enclosures of a cage. After cleaning the cage, therefore, it is best to place each stone and all other enclosures in the cage in the same positions they were before cleaning [see also Section 4.8].

4.8. Handling

The skin of amphibians can be easily damaged. Care is required during handling, which should be kept to a minimum.

When handled roughly, most toads and frogs squirt out from the urinary bladder a transparent odourless and relatively harmless fluid. For studies on stress and adrenocortical modulation in amphibians see Moore & Jessop (2003). Due to various kinds of secretions by the skin of amphibians, persons handling these animals should always wear suitable gloves. There is some dispute over the requirement to use plastic gloves. Although there may be circumstances where the use of gloves is warranted, there are reports of some types of gloves causing severe skin reactions, for example, in *Xenopus*.

Persons dissecting freshly killed frogs should note that these animals may be infected with the trematode *Alaria* which may infect humans too [cf. Section 3.4]. For *Salmonella* infection in amphibians see Pfleger et al. (2003). Appropriate personal protective equipment should be used to protect the handler.

A peculiarity of many amphibian species – especially members of the Salientia – concerns the character of skin secretions (e.g., Summers & Cough, 2001; Chen et al., 2003). In threatened toads, a secretion is given out in minute quantities from glands of the body skin. The poison varies in amount and intensity with the species. Skin glands may be small, so that the skin appearing smooth (frogs). They are large at the warts and parotoid glands of many toads and at the lateral fields of some frogs. Slime glands assist in the processes of respiration by the skin and its alkaloid simultaneously acts protective as a narcotic. The secretion of the parotoids is milky, acid, and thought to act protective as a convulsive to the heart and central nervous system of an aggressor. For example, the poison secreting glands of the common toad *Bufo bufo* contain digitalis. The poison bufotenin is related chemically to the hallucinogenic psilocin of the Mexican fungus *Psilocybe mexicana*. The tolerance to these drugs of certain species of snakes which prefer toads as food [Section 4.3.2] is relatively high. More specifically, four categories of compounds are found in the granular or poison glands: (1) biogenic amines, (2) bufodienolides (bufogenins), (3) alkaloids and steroids, (4) peptides and proteins (Clarke, 1997). Over 300 alkaloids have been identified in the amphibian skin, many of which have unique profiles of pharmacological activity and therapeutic potential (Daly, 1995a). Interestingly, amphibian skin secretions also contain peptide antibiotics (Gallo & Huttner, 1998). Antimicrobial peptides are considered the effector molecules of innate immune acting as a first line of defence against bacterial infections (Simmaco et al., 1998; cf. also Duda et al., 2002; Rinaldi, 2002). Two groups of South African frogs have skin secretions that are potentially lethal to humans and animals (Pantanowitz et al., 1998). For the chemistry of poisons in the amphibian skin see also Daly (1995b). Amphibian skin collagens are identified by Sai & Babu (2001). Doyle et al. (2003) studied anticancer activities of the amphibian skin peptide citropin 1.1.

4.9. Anaesthesia and humane killing

Invasive, potentially painful procedures should be accompanied both by analgesia and anaesthesia. As amphibians' skin accounts for a significant portion of normal gaseous exchanges, in anaesthetised animals, in which lung respiration is reduced or interrupted, the body skin should always be kept moist, for example with a wet tissue.

Pain perception in amphibians is likely analogous to that in mammals. Since response thresholds to chemical (topical acetic acid), thermal, and mechanical stimuli are significantly elevated after spinal opioid administration (effects were abolished by prior systemic administration of the opioid antagonist naltrexone), these sensory modalities are measures of nociceptive activity in amphibians (Willenbring & Stevens, 1997). Amphibians possess neural systems for transmitting pain from peripheral receptors to the

brain and anti-nociceptive mechanisms to modulate pain (Downes et al., 1999; Machin, 1999). Invasive, potentially painful procedures should, therefore, be accompanied both by analgesia and anaesthesia.

Whereas opioids in mammals act by interactions with three distinct types of receptors (mu, delta, kappa), in amphibians mu, delta, and kappa opioids mediate anti-nociception via a single type of opioid receptor, the uni-receptor in the spinal route (Stevens & Rothe, 1997; Stevens & Newman, 1999; Newman et al., 2002). Furthermore, analgesia is mediated in frogs by an alpha2-adrenoceptor mechanism, since it was shown that epinephrine and norepinephrine – e.g., released by stress – have analgetic potencies (Stevens et al., 1995; Willenbring & Stevens, 1996). For analgesia in amphibians see also Wright (2000) and Machin (2001).

4.9.1. Anaesthesia

Suitable drugs for local analgesia are, e.g. Novocain®, Meaverin®, or Xylocain®. Suitable anaesthetics commonly used are Tricain (ethyl-2-aminobenzoat), also called MS 222®. This drug should be used as a solution (e.g., 150 mg/l) in combination with sodium-bicarbonate (300 mg) for neutralizing in order to avoid skin irritability. It is important to consider that the tolerance to drugs may depend on the season. Contrasting effects of anaesthetics in tadpole bioassays are reported by Downes & Courogen (1996).

4.9.2. Humane killing

Various methods are suitable for killing amphibians after experiments have been completed. Methods which provide least stress and no pain to the animal are an overdose of an appropriate anaesthetic.

Neuronal activity, and thus pain, depends on metabolic activity which depends on temperature. Some anaesthetic agents, such as ether or halothane, may affect the animal's sensitive skin, release strong secretions of the skin glands, and produce initial violent movements by the animal which can be interpreted as signs of distress. The use of such agents should be avoided.

All methods used must be in conformity with the principles set by the EC guidelines on the human killing of laboratory animals: *European Commission's Publication Euthanasia of Experimental Animals*.

4.10. Records (See paragraph 4.12. of the General Section)

The person responsible for an animal facility should keep a diary in which all events and activities are noticed: feeding, watering, cleaning, actual count of animals per tank or terrarium; admissions, loss by death; cases of disease; unusual disturbances; identification and marking of experimental animals.

4.11. Identification

Where animals need to be identified individually, there are a number of suitable methods such as transponders; tank labels for individually housed animals; monitoring pigment or wart configurations; small labels by coloured thread. Chemical markings should not be used, since substances are absorbed through the skin, possibly causing toxic effects. Toe clipping is deleterious and should not be carried out.

5. Transport

During transport, amphibians should provided with sufficient air and moisture and, if necessary, appropriate devices to maintain the required temperature and humidity.

In the appropriate season of the year, amphibians should be ordered from dealers which follow the recommendations of: the *European Convention on the Protection of Animals during International Transport*, the *International Air Transport Association*, and the *Animal Air Transport Association*. Semi-

terrestrial amphibians should be singly packed in boxes of adequate size and provided sufficiently with air and moisture. Transportation of tropic species – depending on the local climate – requires an accommodation with appropriate heating devices. After arrival, animals must be unpacked without delay. Animals which arrived in a sick condition, and which do not have a chance to recover, should be sacrificed at once by a human method [Section 4.9]. The commercial sender should be informed.

Appendix: Examples of amphibians and their biotopes*)

*) Source: Swedish Board of Agriculture, Department of Animal Production and Health, Animal Welfare Division

Biotope	**1** – trees (arboreal) **2** – ground (terrestrial/semi-terrestrial) **3** – half in water (semi-aquatic) **4** – fully in water (aquatic)

Species	
Agalychnis callidryas	1
Agalychnis spurelli	1
Alytes obstetricans	2-3
Ambystoma annulatum	2
Ambystoma cingulatum	2
Ambystoma gracile	2
Ambystoma jeffersonianum	2
Ambystoma laterale	2
Ambystoma macrodactylum	2
Ambystoma maculatum	2
Ambystoma mexicanum (metam.)	2
Ambystoma mexicanum (neoten)	4
Ambystoma opacum	2
Ambystoma talpoideum	2
Ambystoma tigrinum	2
Amphiuma means	4
Aneides ferreus	2
Anotheca spinosa	1
Atelopus varius	2
Batrachoseps attenuatus	2
Bolitoglossa altamazonica	1-2
Bolitoglossa mexicana	1
Bombina bombina	2-3
Bombina orientalis	2-3
Bombina variegata	3
Bufo alvarius	2
Bufo americanus	2
Bufo blombergi	2
Bufo boreas	2
Bufo bufo	2
Bufo carens	2
Bufo marinus	2
Bufo mauretanicus	2
Bufo melanostictus	2
Bufo paracnemis	2
Bufo quercicus	2
Bufo viridis	2
Caecilian thompsoni	2
Ceratophrys aurita	2
Ceratophrys cornuta	2
Ceratophrys cranwelii	2
Ceratophrys ornata	2
Chioglossa lusitanica	2-3
Chiromantis xerampelina	1
Colostethus trinitatus	1-2
Cryptobranchus alleganiensis	4
Cynops ensicauda	3
Cynops orientalis	3
Cynops pyrrhogaster	3
Dendrobates spp.	1-2
Desmognathus auriculatus	2-3
Desmognathus fuscus	2
Desmognathus ochrophaeus	2-3
Desmognathus quadramaculatus	2-3
Desmognathus wrighti	2
Discoglossus pictus	2-3
Dyscophus antongilii	2
Eleutherodactylus spp.	2
Ensatina eschscholtzii	2
Epipedobates tricolor	2
Euproctus plarycephalus	3
Eurycea bislineata	2-3
Eurycea longicauda	2-3
Gastrotheca spp.	1
Hyla arborea	1
Hyla cinerea	1
Hyla ebraccata	1
Hyla gratiosa	1
Hyla vasta	1
Hyla versicolor	1
Hymenochirus boettgeri	4
Hynobius spp.	2-3
Hyperolius marmoratus	1
Ichthyophis glutinosus	2
Ichthyophis kohtaoensis	2
Kaloula pulchra	2
Kassina senegalensis	1-2
Kassina weali	1-2
Lepidobatrachus laevis	2
Leptopelis macrotis	1-2
Litoria caerulea	1
Litoria infrafrenata	1
Manculus quadridigitatus	2-3
Mantella aurantiaca	2
Mantella betselio	2
Mantella cowani	2
Mantella madagascariensis	2
Megophrys spp.	2
Mertensiella luschani	2-3
Necturus maculosus	4
Neurergus spp.	3
Notophthalmus meridionalis	3
Notophthalmus perstriatus	3
Notophthalmus viridescens	3
Occidozyga lima	3

Osteopilus septentrionalis	1
Pachytriton brevipes	3
Paramesotriton caudopunctatus	3
Paramesotriton chinensis	3
Paramesotriton hongkongensis	3
Pedostibes hosi	1-2
Pelobates spp.	2
Pelodytes punctatus	2
Peurodeles waltlii	3-4
Phrynomerus microps	1-2
Phyllobates spp.	2
Phyllomedusa lemur	1
Pipa parva	4
Pipa pipa	4
Plethodon cinereus	2
Plethodon glutinosus	2
Plethodon jordani	2
Plethodon vehiculum	2
Polypedates leucomystax	1
Pseudacris crucifer	1
Pseudacris regilla	1,3
Pseudis paradoxus	3-4
Pseudotriton ruber	2
Pyxicephalus adspersus	3
Rana catesbeiana	3
Rana clamitans	3
Rana iberica	3
Rana pipiens	3
Xenopus muelleri	4
Rana ridibunda	3
Rana temporaria	2-3
Rhacophorus reinwardtii	1
Salamandra atra	2
Salamandra salamandra	2
Salamandrella keyserlingii	2-3
Salamandrina terdigitata	2-3
Scaphiopus spp.	2
Siren intermedia	4
Taricha granulosa	2-3
Taricha torosa	2-3
Triturus alpestris	2-3
Triturus boscai	2-3
Triturus carnifex	2-3
Triturus cristatus	2-3
Triturus dobrogicus	2-3
Triturus helveticus	2-3
Triturus italicus	2-3
Triturus karelini	2-3
Triturus marmoratus	2-3
Triturus montandoni	2-3
Triturus vittatus	2-3
Triturus vulgaris	2-3
Tylototriton spp.	2-3
Typhlonectes natans	4
Xenopus tropicalis	4
Xenopus laevis	4

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