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Nutritional Status of Tadpole Populations of *Rana temporaria* in the Central Alps (Austria)

by

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Synopsis: The nutritional status of the tadpole populations of *Rana temporaria* in the Hohen Tauern, Austria was investigated. In only 5 out of 19 populations were large and well fed tadpoles found, whereas those of other populations displayed a small body size and atrophic livers with a depletion of storage products (lipids) and an accumulation of melanomacrophages. These symptoms correlated with tadpole densities, but not with water parameters (pH, conductivity, aluminium), altitude, pond area, or toxicants accumulated in tadpoles. Comparable symptoms were found in the laboratory tadpoles that were fed a low energy diet. This confirmed the assumption that high population densities in small mountain ponds lead to the malnutrition of tadpoles, delayed development, and a small size at metamorphosis. As the period between metamorphosis and the beginning of hibernation is only two months and sometimes even less, underfed froglets may not accumulate sufficient energy to survive the long winter period, which lasts 6-8 months. On the other hand, breeding ponds of *R. temporaria* at high altitudes are often surrounded by pasture land, which improves the feeding conditions of tadpoles due to the fertilisation of oligotrophic water bodies.

Key words: High mountains, tadpoles, malnutrition, body size, liver histology

1. Introduction:

At high altitudes of the Alps, *Rana temporaria* accepts all kinds of stagnating water bodies for reproduction, even those which are not suitable for successful development, such as small ephemeral sloughs filled with water for only a few days during snow melt. Even in persistent shallow habitats egg mortality is often extremely high as frogs spawn close to the shoreline, and due to the natural fluctuations of water levels the majority of the egg clutches fall dry. Thus, reproductive success primarily depends on the weather conditions during embryonic development (Hofer, unpublished observations). After hatching and passing the more or less immobile phase, most of the larvae can escape to deeper parts of the habitat. In contrast to the situation at lower altitudes, high mountain ponds contain less or even no predators resulting in a high rate of survival until metamorphosis. In years with favourable weather conditions this leads to high population densities. However, oligotrophic ponds may not offer sufficient food for the growing biomass of tadpoles. In the

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current study we investigated the nutritional status of *R. temporaria* tadpoles in their natural habitats of the National Park of the Hohen Tauern (Austria) and compared these results with those of laboratory experiments under controlled feeding conditions.

2. Material and Methods:

Tadpoles were collected in June and July 1999 and 2000 from 26 ponds in different regions of the National Park Hohe Tauern (Austria) at altitudes between 1240 and 2150 m a.s.l. The majority of ponds were small with a surface area between 3 and 25m^2, only 5 ponds were larger than 100 m^2. The surface areas of ponds were estimated and divided into 5 classes: <5m^2 (1), 6-10 m^2 (2), 11-25 m^2 (3), 26-50 m^2 (4), >100 m^2 (5).

Water was analyzed at the time of sampling the tadpoles: conductivity with a WTW conductivity meter (25°C) and pH with an Ingold electrode, recommended for water of low ionic strength. Dissolved reactive aluminium was analysed in filtered samples (DOUGAN & WILSON 1974). Only in four ponds was water analysed six times between May and July.

Sampling of tadpoles:

The population density of tadpoles was estimated and classified into 3 categories: 1-low, 2-middle, and 3-high density. The estimations refer to sunny conditions where tadpoles aggregated in the shallow water near the shoreline.

40-60 tadpoles of different developmental stages from each site were killed in MS222 and fixed in a solution of 35% ethanol and 4% formaldehyde, which prevented shrinkage of their bodies. The livers from 6-20 tadpoles (stage 35-36, GOSNER 1960) were fixed in 5% buffered formaldehyde for histology. At eleven sites the livers of 12-20 tadpoles were frozen in liquid nitrogen and stored at -80°C until further use (analysis of glutathione).

Laboratory experiments:

Three freshly fertilised egg clutches were collected from a mountain pond near Innsbruck, brought to the laboratory and stored in shallow containers with constant water flow and a temperature of approx. 21°C. From each clutch vital tadpoles of stage 23 (GOSNER 1960) were separated into four groups of 20 specimens and kept in 1 litre containers fed by a constant water flow of 5 ml.min^{-1} from a tank filled with filtered pond water. Tadpoles were fed twice a day with flakes for aquarium fish (Novo Bel / JBL flakes; 45% protein, 5% lipids, 39 % carbohydrates).

Starting with stage 29 (total length approx. 24 mm) the diet changed to agar-bound flakes of four different concentrations, 2, 1, 0.5 and 0.5 %, respectively: Fine ground flakes were mixed into 100 ml of viscous agar solution (1% w/v, 35°C) and immediately transfused to an ice-cold petri-dish where the solution hardened within seconds and the powder was uniformly distributed. This artificial diet simulated the high inert portion of the natural diet of tadpoles and the different nutritional status of natural populations in high mountains. In the morning and evening, tadpoles were fed with fresh agar pieces, and food residues and faeces were removed.

The water pH was between 7.8 and 8.0 and unionized ammonia concentrations (MERCK no 1.0824.0001, semi-quantitative determination) were below 2 μg.l^{-1}. The containers were radiated 14 hours per day by Duro-Test, Philips LTD lamps 36W/33 resulting in a light intensity of 8 W.m^{-2}. Due to heat production by the lamps the water temperature varied from 20°C at night to 22°C during the day. The experimental temperature was monitored by temperature loggers or measured three times per day.

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After 14 days eight tadpoles were sampled from each container, killed in MS222, and the total length as well as developmental stage was determined. The liver was dissected and fixed in 5% buffered formaldehyde. After 10 days an additional sample was taken from the slower growing 0.2% group. At this time the animals of the other feeding groups had almost completed their metamorphosis.

Liver histology:

Fixed livers were gradually dehydrated in ethanol, embedded in methyl methacrylate, and 3μm thin sections were stained with May-Grünwald-Giemsa and haematoxyline-eosine (HE). Periodic Acid Schiff staining (PAS) was applied to identify glycogen inclusions. For the identification of lipid vacuoles, fixed livers of selected specimens were rinsed several hours in water, then transferred into 30% sucrose overnight and cut in a Frigocut (Reichert-Jung). The sections (10μm) were stained with Sudan IV and embedded in Geltol (ROMEIS 1968). In the first step the evaluation of Maywald-Giemsa- and HE-stained histological sections (n = 6-20 per site and 18 per feeding group) was performed qualitatively, followed by a semi quantitative evaluation of lipid accumulation on Maywald-Giemsa sections (empty vacuoles in hepatocytes, which corresponded to the lipid inclusions) and a quantitative evaluation of the density of melanomacrophages on HE sections using an Image Analysing System (Optimas). The degree of lipid storage was divided into 6 classes: livers with no lipid vacuoles (lipid-index 1) to those with maximum lipid accumulation (lipid-index 6).

Glutathione:

The frozen liver was homogenized in 0.2 ml of ice-cold methaphosphoric acid (10%) and centrifuged (10 min at 13.000 r.p.m.). The supernatant was used for the analysis of glutathione on an HPLC column (HOFER et al. 2001). The pellet was hydrolysed in 1 N NaOH and the protein determined photometrically (LOWRY 1951).

Statistics:

Mean total body length of tadpoles at stage 35 (GOSNER 1960) was calculated from the linear regression line obtained from larvae in the range of the stages 33-38. Spearman's rank correlations between variables were calculated using the Statistica software package (SPSS Inc.).

3. Results:

We investigated tadpole populations of *R. temporaria* from the northern, western, and southern parts of the Hohe Tauern. As a consequence of the crystalline catchments most of the ponds contained soft water with conductivities <50 μS, acidic water, and high concentrations of reactive aluminium that increased with the acidity of the water (Table 1). In four ponds the water parameters were measured 6 times between May and July: Due to the small water bodies the run-off water during snow melt and raining periods caused considerable fluctuations in aluminium concentrations (up to factor 40) and only modest in pH and conductivity (e.g. pH 4.8 -5.4 in pond no.13) . In four ponds (not considered further) all embryos died, probably due to acidic melt water, and in two other ponds (no. 11 and 17) only some egg clutches were affected.

The mean total body length of tadpoles at stage 35, used as an indicator of the growth rate, varied considerably from 29.4 to 43.7 mm (Table 1). Fig. 1 shows the linear growth

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of a well-fed population (pond no. 17) and the stagnating growth of a crowded population (pond no. 8). The majority of ponds (74 %) contained poorly growing populations with mean body lengths <36mm at stage 35 (Fig. 2). Only in 5 out of 19 ponds were large and well fed tadpoles found. Growth correlated only with the estimated population density but not with other parameters such as altitude or the area of the ponds, and pH, conductivity, or the aluminium concentration of the water (Table 2).

Table 1: Characterisation of the ponds and their tadpole populations investigated in the Hohen Tauern. Mean total body length of tadpoles at stage 35 was calculated from the linear regression line obtained from larvae in the range of stage 33-38 (n = 40-60).
Estimated area of the ponds in five classes: <5m² (1), 6-10 m² (2), 11-25 m² (3), 26-50 m² (4), >100 m² . Population density: low (1), middle (2), and high (3) density.

Lake number	Altitude (m a.s.l.)	Pond area (classes)	Population Density (classes)	Mean total body length at stage 35 (mm)	Water pH	Water conductivity μS (25°C)	Water aluminium (μg/l)
1	1460	5	1	43.7	7.1	25	229
2	2010	5	2	33.8	6.6	8	448
3	1690	3	3	31.9	6.9	17	374
4	2150	3	2	38.9	5.5	5	240
5	1690	2	2	32.6	7.3	46	348
6	2080	3	3	34.9	6.6	43	861
7	2020	3	1	38.4	6.8	17	246
8	1770	2	3	29.4	5.0	11	1129
9	2110	3	1	42.1	5.9	11	1121
10	1760	4	3	30.7	7.1	32	103
11	1770	3	2	33.3	5.9	21	1488
12	1770	1	1	35.1	7.6	120	20
13	1800	1	3	33.7	4.9	12	329
14	1820	3	3	30.6	7.6	162	n.d.
15	1810	2	1	35.3	8.2	168	29
16	2020	4	3	31.6	6.7	30	61
17	2020	5	1	42.8	5.7	5	129
18	2100	2	3	32.2	5.5	7	197
19	2100	1	3	32.5	5.5	5	142

Table 2: Spearman's correlation coefficient (R) and significance (P) between the total body length of tadpoles from the Hohen Tauern and environmental parameters and the estimated population density.

	Altitude	Area	Pop. Density	pH	Conductivity	Aluminium
Body length: R	0.22	0.23	**- 0.84**	0.02	- 0.18	- 0.08
Body length: P	0.36	0.33	**<0.001**	0.91	0.47	0.75

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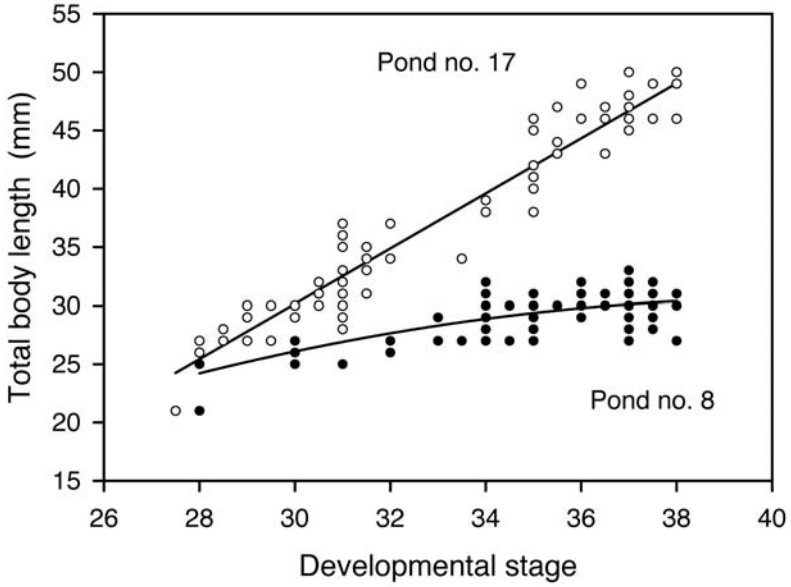

Fig. 1: The growth of tadpoles from a well-fed population (pond no. 17, n = 81) and a crowded population (pond no. 8, n = 85) of the Hohen Tauern (see Table 1). Data were obtained at two different times of the year.

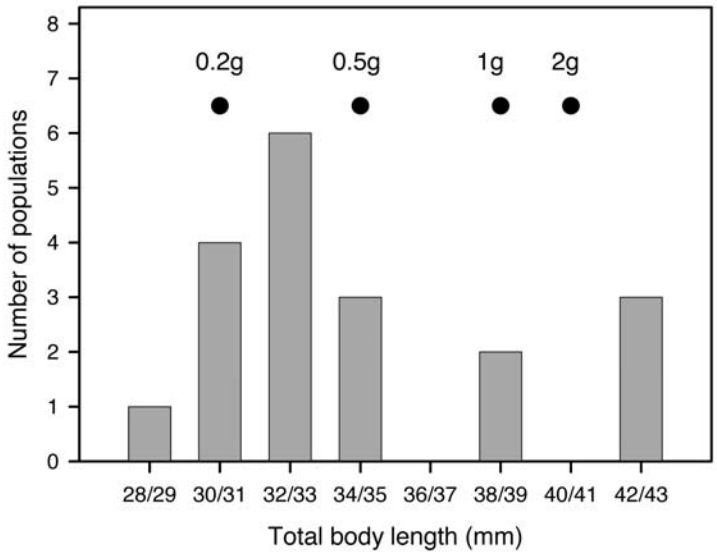

Fig. 2: Number of tadpole populations with different mean body length classes at stage 35 (columns). The dots represent the mean body lengths of experimental tadpoles at stage 35 (n = 20) fed with agar pieces of different concentrations of flakes (g flakes in 100 ml agar).

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The size of the tadpoles at stage 35 correlated positively with the lipid contents (Fig. 3) and negatively with the density of melano-macrophages in the liver (Fig. 4). The hepatocytes of well growing tadpoles contained large vacuoles containing lipids (Sudan IV-positive) and the cytoplasm was attached along the cell walls, around the nuclei, and bile canaliculi (Fig. 5A). The hepatocytes of the smallest tadpoles, however, were atrophic with a dark basophilic cytoplasm with few if any small scattered lipid droplets (Fig. 5B). Single melano-macrophages, components of the reticulo-histiocytic system of the liver localised predominately in the sinusoid space, were observed in all of the individuals. With declining body length, however, their number increased forming distinct melano-macrophage centres. The increase of melano-macrophages in small tadpoles is only partly explained by the shrinking process in atrophic hepatocytes: The reduction of the area in liver slides due to the depletion of lipids estimated from the correlation between body length and liver weight in Fig. 6 explains only a fourfold difference in the number of melano-macrophages between extremes. However, melano-macrophage numbers of the smallest tadpoles are 40 times higher than those of the largest populations (Fig. 4).

In addition to the decrease in lipids and the accumulation of melano-macrophages we have found high numbers of unidentified "granular cells" in the liver sinusoids of two poorly growing populations (Fig. 7; ponds 3 and 8). Small and therefore less important focal inflammations were seen in single specimens of nearly all of the populations without any correlation to other parameters obtained.

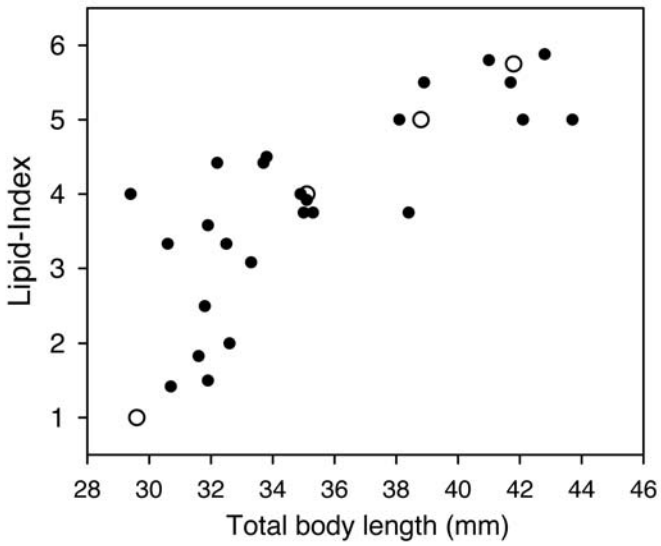

Fig. 3: Relationship between total body length of tadpoles at stage 35 and the lipid index of the liver. Index 1: atrophic hepatocytes without visible lipid vacuoles; index 6: enlarged hepatocytes extremely packed with lipids. See also Fig. 5. Filled circles: field (n = 6-20), open circles: laboratory (n = 18).

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A positive correlation was found between liver lipids and the glutathione (GSH) concentration in the liver of tadpoles (Fig. 8).

The body mass of froglets from a well growing population (pond 17; mean body length of larvae 42.8 mm) immediately after metamorphosis was 341 ± 21 mg (n=6), whereas that of larvae with 29.4 mm mean body length was 3.5 times smaller (97 ± 4 mg, n=7; pond 8).

The laboratory experiments under controlled feeding conditions confirmed the findings from the field. The size of tadpoles at stage 35 fed with a diet containing only 0.2g flakes per 100g (only data of the second sampling were used) was comparable to that of the smallest populations in the field (Fig. 2). The size increased with increasing concentrations of flakes, and laboratory tadpoles fed with 1 and 2g flakes/100ml attained similar values as those of the best growing populations in the mountains. No mortality was observed during the experiments.

Corresponding results between the field and laboratory were also obtained in respect to lipid storage (Fig. 3) and macrophage accumulation (Fig.4) which confirms the hypothesis that the observations in the field are symptoms of malnutrition. "Granular cells" in liver sinusoids, however, could not be observed in laboratory tadpoles.

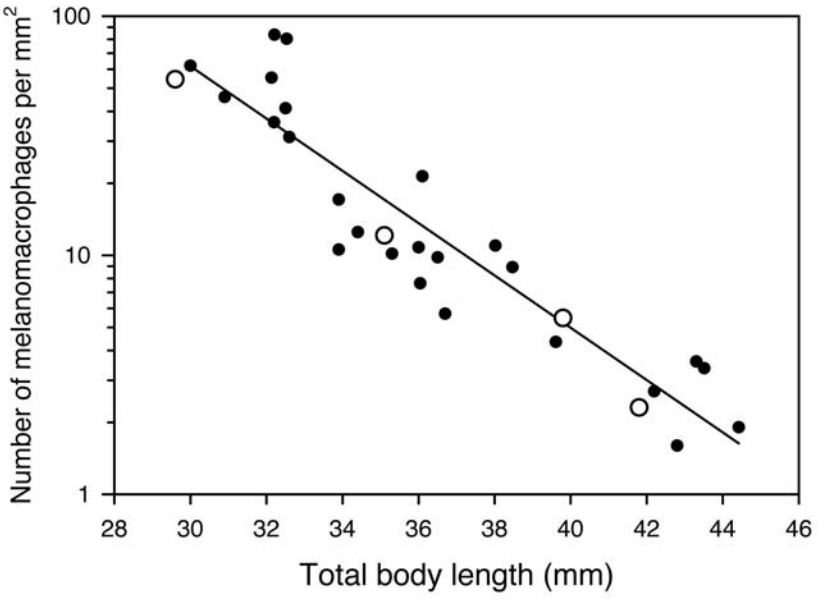

Fig. 4: Relationship between total body length of tadpoles at stage 35 and the number of melanomacrophages per mm² liver section. See also Fig. 5. Filled circles: field (n = 6-20), open circles: laboratory (n = 18).

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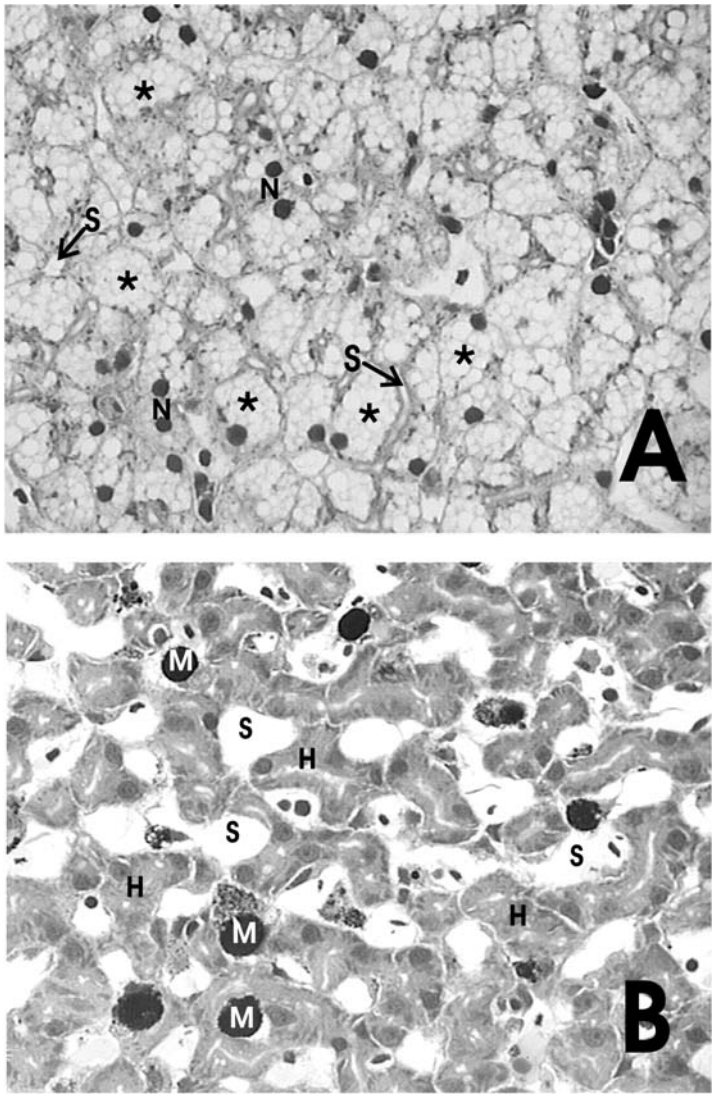

Fig. 5: Histological sections of the liver of tadpoles stained with May-Grünwald-Giemsa.
H: hepatocytes; asterisks: lipid vacuoles of hepatocytes; N: nuclei of hepatocytes; S: sinusoids; M: melano-macrophages. Magnification: 400.
A: The liver of well fed tadpoles contained only a few melano-macrophages (not present in this figure). Hepatocytes are extremely packed with lipid vacuoles (Sudan IV-positive, unstained areas in May-Grünwald-Giemsa sections; lipid index 6). The basophilic (= dark) cytoplasm is attached mainly along cell walls and around nuclei and bile canaliculi.
B: The liver of a tadpole from a crowed population with small body sizes at stage 35. Hepatocytes are atrophic: dense basophilic cytoplasm without distinct lipid vacuoles (lipid index 1). The liver contains large numbers of melano-macrophages, located mainly in the sinusoids of the liver, sometimes accumulated to small centres.

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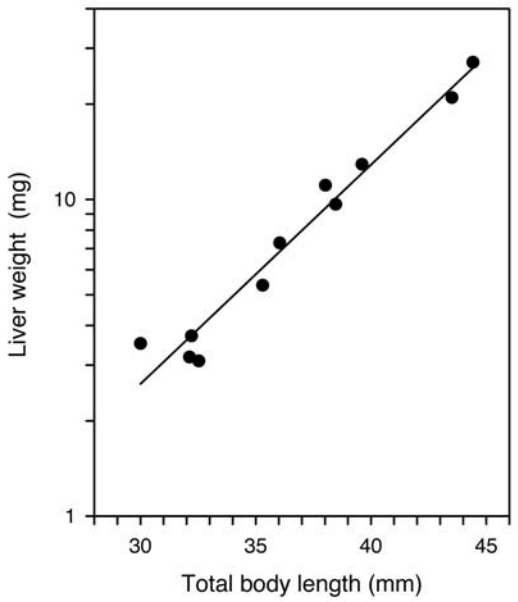

Fig. 6: Relationship between total body length of tadpoles and the liver weight. Each point represents the mean of 20 specimens of a specific population. Data were obtained from ethanol-formaldehyde fixed animals without shrinking symptoms.

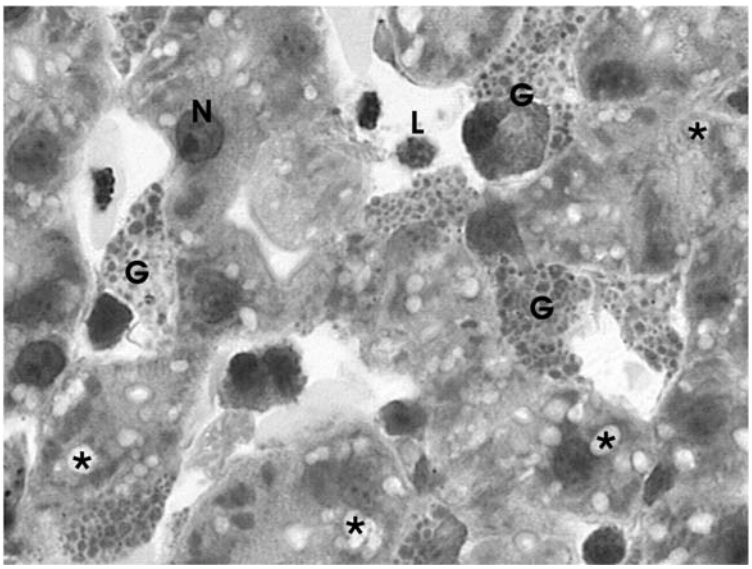

Fig. 7: Histological section (May-Grünwald-Giemsa staining) of a typical liver from two populations (ponds no. 3 and 8). The liver sinusiods (S) are filled with "granular cells" (G) which could not be identified. N: Nucleus of a hepatocyte; asterisks: lipid vacuoles in hepatocytes (lipid index 3); G: not identified "granular cells". Magnification: 1000.

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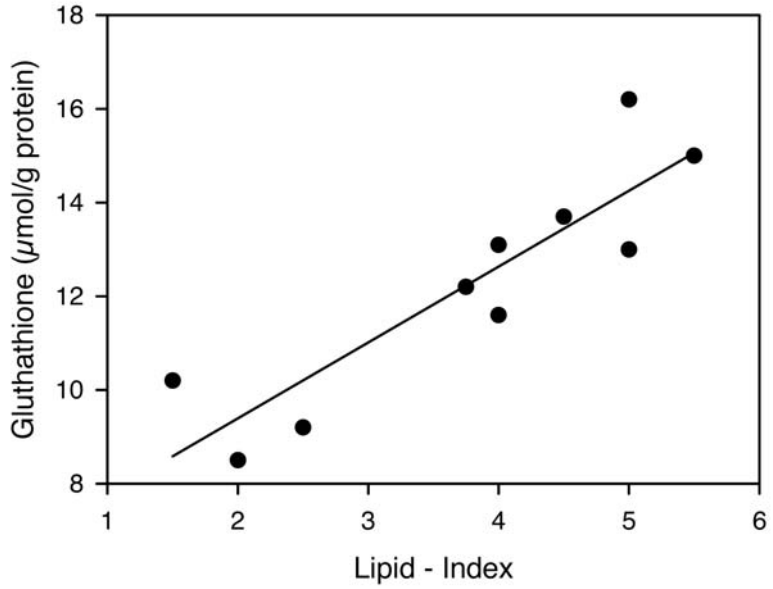

Fig. 8: Relationship between the lipid index and the gluthathione concentration in the liver of tadpoles from the field (n = 12-20).

4. Discussion:

High mountain habitats of *R. temporaria* tadpoles were heterogeneous in respect to size and water quality. Nevertheless, the growth rate did not correlate directly with one of these parameters obtained, although some of them were extreme, such as the pH or aluminium concentration. Negative effects of water parameters, probably episodic acidification, could be observed only during the embryonic stage leading to complete mortality in some ponds with extremely low conductivity in which acidic melt water obviously dropped the pH below the critical level of pH 4.0-4.5 for a limited period (BÖHMER & RAHMANN 1990). In un-buffered ponds this run-off water may acidify the water even to pH 2.3 (FABER 2000). Hatched larvae are less sensitive but at pH <5 they display reduced growth and delayed metamorphosis (CUMMINS 1986). Such effects could not be observed in the current study and were probably overlaid by other factors.

The run-off water does not only introduce acids from atmospheric deposition into the ponds but also aluminium leached from the catchment, resulting in considerable fluctuations with peaks up to 1 mg/l reactive aluminium and higher (Table 1). However, tadpoles are much less sensitive to aluminium than fish, and the toxicity depends on the water pH and dissolved organic matter (HOWELLS et al. 1990). Laboratory experiments with concentrations between 0.8 and 1.6 mg/l aluminium revealed reduced growth and delayed metamorphosis (CUMMINS 1986), disturbed feeding behaviour and morphological malformations (OLSSON et al. 1987). As aluminium peaks occurred only for a limited time such effec-

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ts could not be seen in tadpoles of the Hohen Tauern. Furthermore, toxicants (cadmium, lead, organochlorines) that accumulated in the body of tadpoles (HOFER et al. 2005) did not correlate with either growth rate or liver histology. The only correlation was found with the relative density of tadpole populations in their habitats: High density led to reduced growth and atrophic livers. Approximately 70% of the populations suffered more or less from overcrowding and malnutrition caused by a shortage of their diet directly or by the crowding stress resulting in a decreased food uptake. Several experiments confirm our findings that food availability and larval density have significant effects on growth rate, the duration of the larval period, and body size at metamorphosis (WEST 1960, BROCKELMAN 1969, WILBUR 1977, TRAVIS 1984, MALLORY & RICHARDSON 2005). STEINWASCHER (1978) made a protein, released from larger tadpoles, responsible for the growth depression in less competitive members of the population. More recent investigations correlated the growth depression with the accumulation of unpigmented heterotrophic algae, *Anuropheca richardsi*, in the intestinal tract of slowly growing tadpoles (WONG et al. 1994; BRADSLEY & BEEBEE 2000). Growth depression is compensated when sufficient food is available but the mechanism is not understood (BEEBEE 2000). However, in tadpoles from the Hohen Tauern we could identify only chlorophyll containing algae in their intestines. Some populations showed variable individual growth rates but this did not correlate with the nutritional status of the population. These observations were not quantified, as delayed spawning in some of the ponds could not be excluded.

Tadpoles of the majority of populations showed more or less clear symptoms of malnutrition which were comparable with those obtained in laboratory experiments with a low energy diet. Their liver contained less lipids and the number of melano-macrophages was increased, much more than it could be explained by the atrophy of hepatocytes. Similar conclusions have also been made for starving fish (AGIUS & ROBERTS 1981). Melano-macrophages occur in several organs of heterothermic vertebrates (AGIUS & ROBERTS 2003). Due to the phagocytic activity of these cells, and the property of their melanin to absorb toxicants and to neutralise free radicals (ZUASTI et al. 1998), they are generally involved in the recycling processes of endogenous and exogenous materials (VOGELBEIN et al. 1987). Their number and activity increase with age, environmental changes, during pathological and inflammatory processes, tissue breakdown, and starvation (AGIUS & ROBERTS 1981 and 2003; BARNI et al. 2002).

What are the consequences of a small body size and reduced energy resources after metamorphosis?

At low altitude, metamorphosis is completed in June, almost at the same time when the last high mountain populations of *R. temporaria* spawn. Although eggs from high altitude populations develop faster than those from the lowland (MIAUD et al. 1999) and tadpoles on high mountains always aggregate at shallow parts of the ponds where the temperature increases up to 28°C (Hofer, unpublished observations), which accelerates their growth, the larval development lasts until July or August, and even in September tadpoles can

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be found. As the winter usually starts in mid-October the froglets only have a few weeks for growth and to gain sufficient energy reserves for surviving the long winter period, which lasts 6-8 months. During the first weeks after metamorphosis, smaller froglets of different species show higher mortality rates, reduced growth, and are more susceptible to stress parameters than larger individuals (BERVEN 1990, GOATER 1994, BECK & CONGDON 1999 and 2000). Only those of the small specimens that survive this critical period have compensated for this lack of resistance. However, these observations were obtained from populations in moderate habitats and not in *R. temporaria* at high altitudes where the time between metamorphosis and hibernation is much shorter than at low altitudes.

Breeding ponds of *R. temporaria* at high altitudes are often surrounded by pastures. Although some of the ponds are used as watering places, in which the animals frequently devastate these ponds, they also fertilise them with their manure and thus contribute to better growth for tadpoles.

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