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Studies on the mechanisms that affect the growth and development rates of *Leptodactylus melanonotus* tadpoles (Anura: Leptodactylidae) at different population densities

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Abstract. The influence of population density on the growth and development of *Leptodactylus melanonotus* tadpoles was studied in field and laboratory experiments with ad libitum feeding. The differences in growth and development rates were far greater between the different density groups than between the groups of equal density but at different water quality. An increasing pollution of the water with nitrogen compounds corresponds to higher development rates, but the nitrogen content did not influence tadpole growth rate up to the moment when the first tadpole reached metamorphosis stage. Generally, in freshwater as well as in the flowing polluted water treatment, the ammonium content was low, while the nitrite and nitrate contents increased. In contrast, the two nitrogen acid radicals remained low in stagnant polluted water treatments with low oxygen content. When all tadpole groups were exposed to identical water in flowing-water experiments, the individually housed tadpoles reached growth rates that were more than three times higher than in all the tadpoles in groups. Therefore, such remarkable growth delay in groups must be caused by other factors than natural pollution of the water by tadpole faeces, and natural pollution may be ruled out as the dominant factor for explaining the growth and development patterns. Between siblings that were reared together in the beginning, but separated into groups of larger and smaller tadpoles later, the first ones doubled their total mass within 19 days of observation while the smaller ones increased their mass by a factor of six and produced about four times more nitrate and nitrite. Tadpoles that were kept individually in small mirror-walled containers responded to the visual stimuli of their own mirror images with more movement than those that were reared in non-mirroring, sand-coated containers. In confined conditions, visual stimuli of virtual tadpoles seen in mirrors tend to have similar effects as physically present tadpoles. The generally weak response to their mirror images in larger containers can be regarded as tolerance to conspecifics in tadpoles of schooling species like *L. melanonotus*. Nevertheless, their motoric activity increases much more by the physical presence of other tadpoles than by their virtual mirror images due to the real mutual disturbances. Individually housed tadpoles in sand-coated containers spent much more time resting on the bottom and grew and developed best. Disturbance reduces the time available for resting and digestion, and increases energy expenditure for movement. With the latter being directly linked to population density, this could be the dominant influence on tadpole development.

Key words. Amphibia, Anura, *Leptodactylus melanonotus*, larvae, growth rate, development rate, visual stimuli, mirror experiments, tactile stimuli, ammonium, nitrate, nitrite.

Introduction

Population density is one of the most important factors influencing the larval development in amphibians. Overall, a low tadpole density results in higher growth rate and faster development; contrarily, high population density causes delayed growth and increased development time, even with ad libitum feeding. In addition to food availability, which directly controls tadpole development and survival, other factors may skew the development rates within a population. With the presented experiments I try to examine more closely some of these influential factors. Various authors have offered hypotheses about how density affects tadpole growth and development. Wilbur & Collins pointed out as early as in 1973 that the tadpole populations with the highest initial densities had the most skewed distribution of body size, and that the standard deviation of body mass increased with population density. Subsequently, the different reactions of tadpoles to conspecific and interspecific competition as well as stress factors arising from the presence of predators were studied (e.g., Crump 1981, 1984, Alford & Wilbur 1985, Alford 1986a, 1986b, 1999, Semlitsch & Reyer 1992). Comprehensive synopses on this topic are given in Alford (1999) and Wells (2007).

A decline in water quality is another complex factor linked to developmental delay in tadpoles living under crowded conditions. High amounts of tadpole faeces lead

to the deterioration of water quality, especially in small closed habitats or laboratory conditions. Tadpoles excrete nitrogen mainly as ammoniac (Tattersall & Wright 1996), but the insoluble constituents of faeces are additionally metabolised by bacteria to simple nitrogen compounds like nitrate and nitrite. Accumulated non-ionic ammoniac is the most toxic form of nitrogen in high-pH water (Jofre & Karasov 1999, Little et al. 2002). However, the nitrate concentration is mostly non-injurious, as Rouse et al. (1999) documented based on large samples collected around the Great Lakes. Nevertheless, differences in tolerance to these substances occur even between closely related species (Smith et al. 2005). Nitrite, on the other hand, is always harmful in that it disturbs vital physiological processes in aquatic animals even at very low concentrations (Jensen 1995, 2003, Marco & Blaustein 1999, Griffis-Kyle 2005, Hoffmann 2010).

Lannoo (1999) referred to mechanoreceptor mechanisms as a possible cue for specific tadpole development. Rot-Nikcevic et al. (2005) took up these suggestions and studied, beside visual cues, the influence of tactile cues on the growth and development of the tadpoles of three species. They documented both elevated corticosterone levels in hyper-stimulated tadpoles and species-specific reactions to enhanced environment cues. Their data suggest that stress levels increased by visual and mechanical stimuli alter tadpole growth.

In the present study, I used these studies as a foundation to examine more closely some of the environmental factors linked to tadpole development, focusing on *Leptodactylus melanonotus*. This species is a common frog in the Atlantic and Pacific lowlands of Costa Rica, with its tadpoles forming high-density schools. It inhabits ponds and temporary eutrophic soil depressions in wet pasture areas. Female *L. melanonotus* afford their tadpole cohorts intensive parental care in the form of leading and monitoring them as described by Hoffmann (2006). Although parental care is favourable to tadpole development, it is not obligatory, and larger tadpoles may leave their schools to live solitarily. The development of *L. melanonotus* larvae takes about one to one and half months. Field and laboratory experiments were performed to identify the roles of potential stimuli on the density-dependent responses of tadpoles.

Material and methods

Generalities

All field and most of the laboratory observations were made between 2002 and 2007. The field experiments were performed on or near the 'Ombú' INASAG-Experimental Farm in the Caribbean lowlands of northeastern Costa Rica (Province Limón) at 70 m above sea level (10°16' N, 83°43' W). The annual rainfall at this site ranges from about 3,500 to 4,500 mm. The laboratory experiments were performed in the installations of the INASAG Costa Rican Tadpole Research Center located in the Highlands of the province of Heredia at 1630 m above sea level (10°04.8' N, 84°07.9' W). Sizes and other specifications of the experimental containers were modified to address specific study objectives in the different laboratory experiments. In these glass containers, the tadpoles were fed ad libitum with withered leaves of the root beer plant (*Piper auritum*). The tadpoles used in the experiments detailed here were extracted from foam nests found in natural and experimental outdoor ponds at the Ombú Experimental Farm and in adjacent wetlands. Unless otherwise stated, the experiments were each started with hatchlings. If older tadpoles were used, they were sorted into groups of similarly sized tadpoles prior to the commencement of the experiment.

For tadpole growth and development assessment, the tadpoles were staged following Gosner (1960). The growth rate of the tadpoles was determined for all density groups by averaging the results of individual weighing of the tadpole mass at the moment the first tadpole of the entire experiment arrived at the climax of metamorphosis (stage 42). At this point of time all the other tadpoles were also staged, weighed, and their body and tail lengths were measured. The development rate of a group can only be determined when the last tadpole of a specific group has arrived at the climax of metamorphosis. The development rate is then the average of the number of days that all tadpoles of a group needed to enter metamorphosis.

Equipment, parameters and procedures

Tadpole mass was weighed with an analytic scale (OHAUS GT 210) to the closest milligram to facilitate the subsequent determination of average development rates for each tadpole group. For this procedure, adherent water was gently removed from the tadpoles with filter paper. After weighing, the tadpoles were returned to their respective containers. Nearly all tadpoles survived this manipulation. For water quality assessment, the oxygen content of the water was measured with the Dissolved Oxygen Meter YSI '55–50 FT' (test-accuracy ± 0.3 mg/L = ± 2%), acidity or alkalinity were assessed with the pH-meter HANNA 'pHep' (accuracy ± pH 0.05), and the colorimeter HACH 'DR/850' (accuracy NO_3-N ± 0.03 mg/l, NO_2-N ± 0.003 mg/l, NH_3-N ± 0.02 mg/l) assessed the amount of the three most important waste nitrogen compounds in the water.

Special notes on the performance of field experiments

Four field experiments were conducted at the Ombú Experimental Farm between 2002 and 2003. In the first exploring experiment, the tadpoles were reared in open-ended cylinders of 1 m in diameter that were set up in the habitats in which the tadpoles naturally occurred. The individuals of the groups of 2 and 50 were each weighed 37 days later. The three sequel field experiments were then performed in bottomless pails with three and five replicates, respectively. Depending on the experiment, one to 200 hatchlings were

released in each pail, respectively. These experiments are synoptically summarized in the chapter "Results" below.

Special notes on the performance of laboratory experiments

Studies on density-dependent development: A preliminary laboratory experiment was designed to identify approximately the critical population density that affects markedly the development of the tadpoles. The experiment started with 22 day-old tadpoles. They were split up in groups of 5 and 10 individuals each; additionally, 5 tadpoles were housed individually. Their initial individual masses averaged 82.0 mg for the individually housed tadpoles and 101.1 and 70.4 mg for the groups with higher population densities. This experiment was continued by starting with one day-old hatchlings weighing approximately 10 mg. All tadpoles were reared in aquaria that were divided into 4 compartments of 18.5 × 13 × 10 cm each. They had a sandy bottom and were filled with 1.5 litres of water.

Experiment with differently sized siblings: The tadpoles used in this experiment came from a single foam nest, but began to vary in size after approximately 8 days. In order to start the experiment with nearly uniformly sized larvae, two groups were selected from the cohort, one group of small tadpoles (average mass 40 mg) and another group with medium-sized tadpoles (average 142 mg).

Fresh and polluted water experiments: To assess the effects of fresh and polluted water on tadpoles, stagnant fresh and polluted water were compared to flowing water of equal water quality. In the 'fresh-water treatments', 75% of the water was exchanged for well water twice weekly; while in the 'stagnant wastewater treatments', the water remained unchanged during the course of the experiment. For this purpose, a special aquarium facility was developed, which allowed maintaining homogeneous water qualities throughout all compartments, independent of the numbers of tadpoles in each. It consisted of a linear row of aquaria with 6 rigid interconnected compartments. Each compartment measured 10 × 20 cm in floor space and 15 cm in height. They were filled with water to a level of 10 cm. The partitions between the compartments were coated with sand that prevented visual contact between the compartments, with mesh-covered windows in the centres of the partitions allowing water to flow from one compartment to the next. Only the four central compartments were used as experimental chambers; a water pump in one of the two unoccupied end chambers moved the water via a tube to the opposite end chamber from where it would flow back through all compartments, providing all experimental chambers with the same water. In the stagnant water treatments with 1.2 litres of water, ordinary aquaria with sand-coated glass walls served to study the behaviour of the tadpoles in both isolated conditions and groups. One, five, ten, and 25 tadpoles were tested with the two described water qualities. While in the flowing water systems, water conditions were the same for all individuals, independent of the population density in their specific compartments, the concentration of waste matter in the stagnant water treatments depended solely on the number of individuals housed in their common tank. To quantify their growth rates, the tadpoles were weighed 23 days after the start of the experiment. The development rate is reported as an average of days the tadpoles needed to reach metamorphic stages 42–43. The experiments were performed at the Ombú Experimental Farm on shelves in open-air conditions. The shelves were roofed over to avoid the uncontrolled addition of rainwater and overheating due to insolation. Simultaneous to the assessments on the tadpoles described above, the water quality was monitored with the above-mentioned equipment. These experiments were replicated four times.

Influence of optical and tactile signals on tadpole development: To study the effects of optical and tactile signals as stimulants or retardants on growth and development, two experiments with four replicates each were conducted on the tadpoles of *Leptodactylus melanonotus*. The two experiments were initiated with less than one day-old (Experiment #1) and exactly one day-old hatchlings (Experiment #2); they had an average body mass of 8.3 and 10.6 mg, respectively. In the "mirror treatments", the tadpoles were kept in containers completely surrounded by mirrors; in the other treatments, they were reared in containers with sand-coated walls to even prevent reflection effects on the glass. In the first experiment (#1), the dimensions of the containers were 18.5 × 12.5 × 8 cm = 1850 cm^3 and in the second experiment (#2), the "tight-space experiment", 6 × 6 × 7.5 cm = 270 cm^3. They were filled with 1250 ml and 250 ml of well water, respectively. The tank bottoms were covered with washed river sand to a height of about 0.5 cm. Growth and development of the individually reared tadpoles were compared between containers with mirror and opaque walls. Considering that individually housed tadpoles were enabled to see their likeness four times in a container with facing pairs of mirrors all around, comparative treatments were set up with five tadpoles in sand-coated containers and five times greater water volumes (6.25 litres of water). In both experiments, the amount of tadpole movement was recorded based on daily observations. Experiment #2 was performed with the aim to produce an even more stressful situation using extremely confined containers. The water temperatures in Experiments #1 and 2 were taken daily; they were similar in both experiments at 22.8 ± 0.9 versus 23.1 ± 1.0°C.

For statistical analysis, DUNCAN multiple range tests were applied at 95% probability level ($\alpha = 0.05$). Tabular data are given as averages and standard deviations in Tables 1, 2, 4 and 6.

Results

Field experiments

The first field experiment was only exploring in character and involved two and 50 tadpoles, respectively. All tadpoles were weighed individually 37 days after the com-

Table 1. Assessment of weights, measurements, and staging of 25 (26) day-old *Leptodactylus melanonotus* tadpoles reared at different population densities in field experiments #2 and 3 (averages and standard deviations in the same column with the same letter are not statistically different; Duncan-Test, α = 0.05).

Number of tadpoles after 25 days (Experiment #2)	Tadpole mass [mg]	Tadpole length [mm]	GOSNER-stages
1	325.0 ± 26.9 a	34.5 ± 1.47 a	42.4 ± 2.07 a
3	260.5 ± 52.1 b	31.7 ± 4.24 a	36.0 ± 3.30 b
5	156.9 ± 29.8 c	26.6 ± 0.85 b	28.9 ± 0.99 c
8-24 (Ø 12.6)	100.3 ± 23.8 d	22.6 ± 1.86 b	25.7 ± 0.63 cd
41	34.4 ± 6.7 e	15.2 ± 0.86 c	25.0 ± 0.00 d
Number of tadpoles after 25 days (Experiment #3)	Tadpole mass [mg]	Tadpole length [mm]	GOSNER-stages
1	482.0 ± 72.1 a	39.0 ± 1.41 a	41.8 ± 1.64 a
2	419.3 ± 42.4 c	38.8 ± 2.17 a	40.8 ± 1.33 ab
3	461.3 ± 105.8 ab	38.7 ± 3.72 a	40.3 ±1.03 abc
5	487.4 ± 133.0 a	39.4 ± 5.09 a	39.8 ± 1.30 bcd
6	361.5 ± 19.4 d	35.2 ± 0.58 a	39.0 ± 0.00 cd
10	442.9 ± 176.0 bc	37.7 ± 4.24 a	37.3 ± 4.50 e
23	335.8 ± 46.2 d	35.1 ±1.68 a	38.7 ± 2.06 de
29	227.9 ± 41.7 e	30.7 ± 1.91 b	33.9 ± 2.45 g
154	184.4 ± 31.9 f	28.1 ± 2.00 b	35.8 ± 3.17 f
174	129.5 ± 22.2 g	25.1 ± 1.59 b	30.6 ± 2.05 h

mencement of the experiment. At this stage, the average individual body mass of the tadpoles was approximately 9 times higher in the groups of two tadpoles than in the crowded group (281.5 vs. 31.8 mg).

Considering these extremely high differences, field experiments #2 and 3 were conducted with the synoptically results shown in Table 1 and Figure 1. Again, but with greater differentiation, the density-dependent development was evident, even between tadpoles reared individually vs. in small groups. The differences shift to spectacular levels in high-density groups, though, with the potential of even causing stunting in growth and development.

Laboratory experiments

Studies on density-dependent development: In a preliminary laboratory experiment, the development of individually housed tadpoles was compared with those reared in groups of five and 10 individuals. Their corresponding individual average masses were 70.4 mg for the individually housed tadpoles, 82.0 mg for the groups of five, and 101.1 mg for the groups of 10 tadpoles. Although the group with 10 tadpoles initially had a head start due to their greater mass, 27 days later, they had been overtaken in growth by the initially smaller tadpoles in the smaller groups. The average tadpole mass decreased in a linear manner from individually reared tadpoles to five and 10 tadpoles per group, but the correlation of the development stages was skewed (Fig. 2). As expected, the tadpoles of the group of 10 were the most retarded, but the individually housed tadpoles and the tadpoles housed in groups of five individuals reached similar Gosner-stages. Due to the similarity of the results of the laboratory and outdoor experiments on tadpole development, further experiments were limited to the laboratory and designed to find out the causes for density-dependent development.

Mutual influences of differently sized members of a cohort on individual growth: I proceeded from the assumption that by separation of the smaller tadpoles, they would no longer be inhibited in their development by larger siblings. Indeed, when the growth and development of the two groups were assessed 19 days after separation, the initially medium-sized tadpoles had only doubled their corporal mass, while the initially smaller siblings had increased their mass by more than six times. Apparently the formerly smaller tadpoles had recovered over this period the gains in body mass that they could not add before due to competition with their larger siblings. Nevertheless, they only recovered with regard to their body mass while their development still remained decelerated. They could not compensate their initial losses in development, as is indicated by their Gosner-stages (Tab. 2, Fig. 3).

Influence of water quality: The assessment of water pollution by soluble nitrogen compounds, such as ammoniac, nitrate, and nitrite, was found to be correlated to the total tadpole mass in the above reported experiment. The group with the initially smaller but faster-growing tadpoles had a

lower ammoniac content in the water, but produced about 4 times more of the two nitrogen acid ions (Tab. 3). To investigate this further, experiments with stagnant and flowing water at two pollution levels were conducted. They were intended to assess the effects of water polluted with nitrogen compounds on the development of tadpoles reared at different population densities. The results are:

1. In the stagnant fresh and stagnant polluted water treatments, the body mass of the tadpoles increased inversely proportional to the number of tadpoles and showed about the same larval growth rate patterns (Fig. 4). However, the development rate accelerated in stagnant polluted water with the increasing waste content during the period of exposure. In contrast, in stagnant freshwater, the developmental rate was nearly equal for the tadpoles in groups of up to 10 individuals, with only tadpoles in groups with 25 individuals taking more time for development (Fig. 4, Tab. 4).

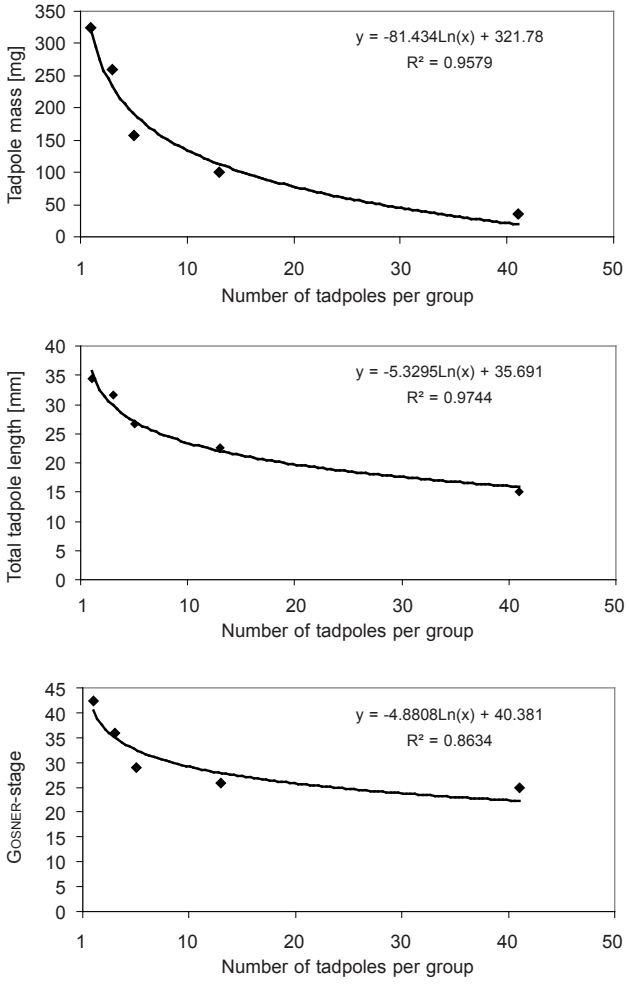

Figure 1. Correlation between the number of *Leptodactylus melanonotus* tadpoles per group and growth rate (as tadpole mass in mg), tadpole length and development rate (Gosner-stage) in the field density experiment #2 with bottomless pails.

2. With the water flowing through all compartments, possible differences of water chemistry between the compartments were eliminated; nevertheless, the growth rate patterns were again similar in both water qualities, with marked advantages for the individually housed tadpoles and a strong growth inhibition in the two crowded groups of 10 and 25 tadpoles (Fig. 5, Tab. 4). As in the stagnant water treatment, the development rates of tadpoles reared in flowing polluted water tended to be more extended than those of tadpoles living in flowing fresh water (Fig. 5, Tab. 4). In the polluted water treatment, the total nitrogen content increased drastically from one to five tadpoles per container, but when the tadpole density was increased even more, the nitrogen content rose only comparatively slowly, because the increase in the individual body mass, as well as the total body mass of the whole group, was delayed. Consequently, the total nitrogen content of the polluted water is only correlated strongly with the total tadpole biomass in the corresponding compartments (Fig. 6, Tab. 5). As expected, this relation between the nitrogen content in the water and the tadpole mass was not seen in the 'freshwater treatments' where nitrogenic waste was regularly removed or at least diluted.

3. A strong decline of the dissolved oxygen content (DO) occurred in stagnant polluted water when more than one tadpole was reared per container. Low oxygen content in the water coincided with high ammonium (NH_3-N) and

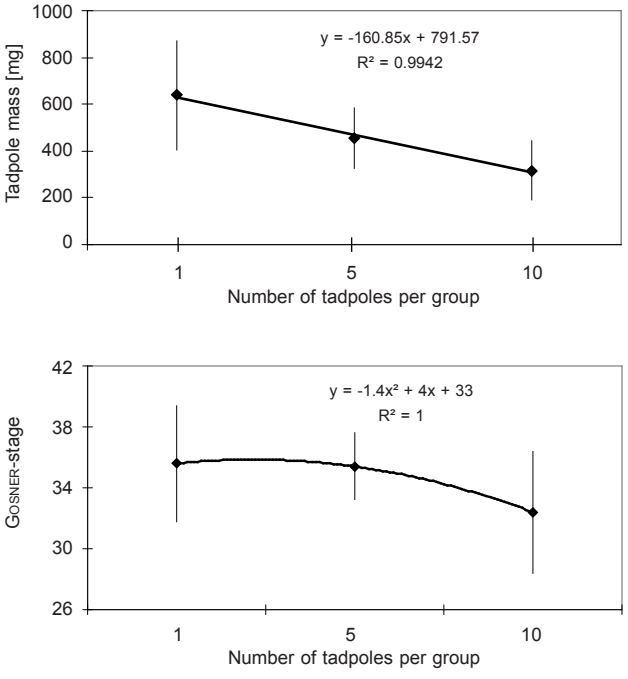

Figure 2. Tadpole mass of *Leptodactylus melanonotus* in mg (above) and development rates, expressed as Gosner-stages (below) when reared in groups with different numbers of individuals in the preliminary laboratory experiment (the vertical lines indicate standard deviations).

Table 2. Assessment of weights, measurements, and staging of initially differently sized *Leptodactylus melanonotus* tadpoles (average weights at experiment start: 40 mg = small vs. 142 mg = medium) reared in groups with different numbers of individuals at 19 days after start of trial.

Tadpole number per group	Initial tadpole mass (small vs. medium)	Body mass in mg 19 days later	Body length	Tail length	Total tadpole length	Gosner-stage
1	small	474	1.25	2.60	3.85	30.0
1	medium	376	1.30	2.40	3.70	33.0
2	small	385 ± 149	1.35 ± 0.07	2.45 ± 0.21	3.80 ± 0.28	31.5 ± 2.1
2	medium	391 ± 118	1.35 ± 0.00	2.45 ± 0.21	3.80 ± 0.21	37.5 ± 6.4
4	small	208 ± 179	1.00 ± 0.24	1.96 ± 0.48	2.96 ± 0.71	28.3 ± 3.9
4	medium	315 ± 105	1.21 ± 0.11	2.15 ± 0.16	3.36 ± 0.26	32.3 ± 3.0
8	small	216 ± 141	1.01 ± 0.13	1.80 ± 0.17	2.81 ± 0.24	27.9 ± 2.5
8	medium	213 ± 62	1.06 ± 0.14	1.95 ± 0.19	3.01 ± 0.29	28.6 ± 2.8

Table 3. Assessment of the nitrogen pollution in the aquarium water of the initially differently sized tadpoles in Table 2 of *Leptodactylus melanonotus* and their total tadpole mass at 19 days after the start of the experiment.

Initial tadpole size	pH	DO ppm	NO_3-N	NO_2-N	NH_3-N	N-total	Total tadpole mass in mg
small	7.54	5.33	0.56	0.378	2.63	2.29	3458
medium	7.82	5.43	0.12	0.102	4.00	3.17	3903

low nitrate (NO_3-N) and nitrite (NO_2-N) contents (Table 5).

Influence of optical and tactile signals on tadpole development (mirror experiments): When individually housed tadpoles were exposed to their own images in mirrors, they needed a slightly more extended development period than those in sand-coated containers. However, tadpoles housed in groups of five responded with an even more extended development period than the individually housed tadpoles in containers with mirrored walls (Tab. 6, Fig. 7). Under the confined conditions in the sand-coated containers of Experiment #2, the individually housed tadpoles spent more time resting quietly on the bottom. The tadpoles in the mirror cells as well as those in the containers with five individuals responded similarly with increased activity (Tab. 6, Fig. 8).

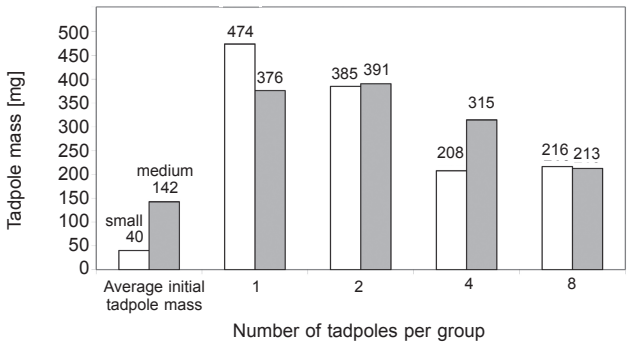

Figure 3. Correlation between population density and tadpole mass in mg (growth rate) of initially small- and medium-sized *Leptodactylus melanonotus* tadpoles, 19 days after the start of the trial. □ Individual mass of 1 to 8 initial small tadpoles assessed 19 days later; ▩ Individual mass of groups of 1 to 8 initial medium sized tadpoles assessed 19 days later.

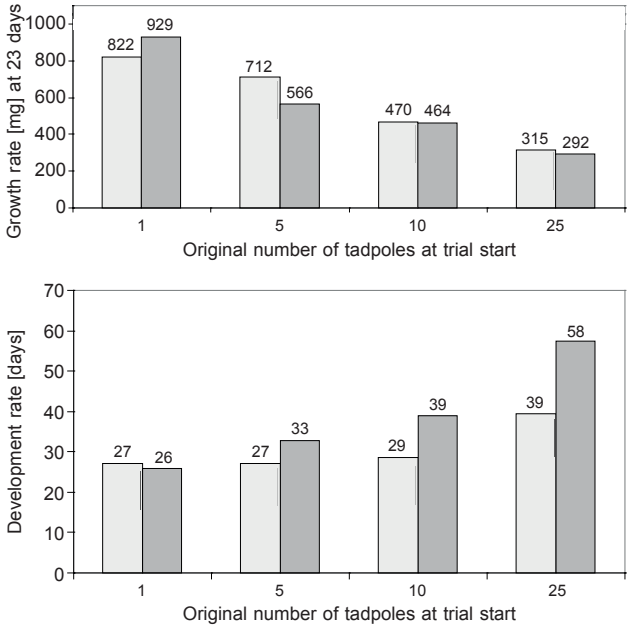

Figure 4. Growth rate at 23 days following the start of the experiment (above) and development rate (below) of *Leptodactylus melanonotus* tadpoles reared in stagnant fresh and polluted water. □ Stagnant fresh water; ▩ stagnant polluted water.

Table 4. Tadpole mass (growth rate), lengths and stages of *Leptodactylus melanonotus* tadpoles reared during 23 days in water with different degrees of pollution; the column 'Development rate' shows the number of days the tadpoles required to enter metamorphosis stage (Gosner-stage 42); (values in the same column with the same letter are not statistically different; Duncan-Test, $\alpha = 0.05$).

Nr.	Experiment	Tadpole mass [mg]	Body length [mm]	Tail length [mm]	Total tadpole length [mm]	GOSNER-stage	Development rate in days
1	1 tadpole in stagnant polluted water	929 a	18.00 a	38.00 a	56.00 a	41.0 a	26.0 a
2	1 tadpole in flowing polluted water	1009 a	17.00 a	34.00 b	51.00 b	41.0 a	30.0 ab
3	1 tadpole in stagnant fresh water	822 b	15.00 b	32.00 bc	47.00 c	40.0 a	27.0 ab
4	1 tadpole in flowing fresh water	811 b	15.00 b	28.00 def	43.00 def	40.0 a	27.0 ab
5	5 tadpole in stagnant polluted water	566 ± 154 cd	14.40 ± 0.89 bc	28.10 ± 2.84 def	42.50 ± 3.54 def	39.0 ± 2.4 ab	32.7 ± 5.8 abc
6	5 tadpoles in flowing polluted water	340 ± 136 e	11.50 ± 1.70 fg	22.80 ± 3.96 gh	34.30 ± 5.44 hi	34.4 ± 4.2 def	28.5 ± 2.1 ab
7	5 tadpoles in stagnant fresh water	712 ± 105 c	14.50 ± 1.00 bc	30.50 ± 1.29 cd	45.00 ± 2.16 cd	41.3 ± 1.0 a	27.0 ± 0.0 ab
8	5 tadpoles in flowing fresh water	459 ± 264 d	12.10 ± 2.07 def	25.00 ± 5.70 gh	37.10 ± 7.54 gh	36.4 ± 5.3 cd	32.3 ± 6.8 abc
9	10 tadpoles in stagnant polluted water	464 ± 120 d	13.17 ± 1.22 cd	27.50 ± 3.18 efg	40.67 ± 3.85 efg	37.1 ± 4.1 bc	39.0 ± 13.4 bcd
10	10 tadpoles in flowing polluted water	266 ± 196 e	10.60 ± 2.39 g	21.20 ± 5.15 h	31.80 ± 7.48 i	33.0 ± 3.9 ef	46.6 ± 13.5 de
11	10 tadpoles in stagnant fresh water	470 ± 69 d	13.30 ± 1.42 de	26.50 ± 2.24 fg	39.80 ± 3.09 fg	40.9 ± 2.2 a	28.5 ± 3.0 ab
12	10 tadpoles in flowing fresh water	286 ± 135 e	11.20 ± 1.99 fg	22.15 ± 4.26 gh	33.35 ± 5.96 hi	33.1 ± 3.7 ef	42.9 ± 14.3 cd
13	25 tadpoles in stagnant polluted water	292 ± 123 e	10.76 ± 1.84 fg	22.61 ± 3.48 gh	33.37 ± 5.14 hi	33.7 ± 3.4 ef	57.5 ± 15.3 e
14	25 tadpoles in flowing polluted water	223 ± 139 e	10.23 ± 2.29 g	20.25 ± 4.49 h	30.48 ± 6.73 i	31.7 ± 3.3 f	56.8 ± 12.2 e
15	25 tadpoles in stagnant fresh water	315 ± 92 e	11.25 ± 1.27 fg	22.80 ± 2.50 gh	34.05 ± 3.63 hi	34.7 ± 2.1 cde	39.4 ± 8.6 bcd
16	25 tadpoles in flowing fresh water	223 ± 89 e	10.54 ± 1.57 g	20.89 ± 2.73 h	31.43 ± 4.22 i	32.2 ± 3.6 ef	43.2 ± 10.7 cd

Discussion

All results suggest that tadpoles of *Leptodactylus melanonotus* grow best when they are housed solitary, whereas crowding delays their growth. My results confirm the results of previous studies on other species like *Rana sylvatica* (Wilbur 1977) or *Pseudacris triseriata* (Smith 1983), but provide additional information that suggests the strongest differentiation to occur in groups with extremely low numbers of individuals. Even in groups of only two or three individuals, the growth rate decreased and development periods tended to be longer than in individually housed tadpoles. A similar result was recently published by Reynolds et al. (2011) on *Bufo americanus*.

The "Experiment with differently sized siblings" shows that the members of a cohort of *L. melanonotus* tadpoles react in a differentiated manner to different degrees of crowding, at least during the early larval stages. These results suggest that a delay in larval growth will not necessarily be permanent when it was caused by crowding. Once the faster developing tadpoles were taken away (in this experiment, the medium-sized tadpoles from the smaller ones), other, formerly smaller, tadpoles assumed the dominant role and grew faster. That would be the situation in natural habitats when the strongest tadpoles are taken out by predation. Therefore, I assume that any healthy tadpole of a *L. melanonotus* population is potentially able to assume a more dominant position and can grow faster if the competition of stronger tadpoles is eliminated. In this experiment, nineteen days after the selection of the tadpoles in groups sorted after size, the initially medium-sized tadpoles only doubled their corporal mass, whereas the body mass of initially smaller-sized siblings increased more than six times. Nevertheless, during this period, the initially smaller ones still maintained their delay in development, as is shown by their Gosner-stages (Tab. 2). Presumably,

the tadpoles first needed to recover their corporal mass and would later convert their gains into advancing in developmental stage. This result may explain why a school of tadpoles at an advanced stage dissolves into tadpoles that live individually.

Although my first experiments confirmed that growth and developmental rates are indeed density-dependent, they do not reveal the mechanisms that affects the growth and development of tadpoles. Water quality could be one of the causes that influence the development of tadpoles, because it is widely supposed that an increase in pollutants in the water chemistry over time could have detrimental effects. Unchanged water accumulates waste substances that decompose to nitrogen compounds, especially ammonium, nitrite, and nitrate. Therefore, my subsequent experiments were conducted with the aim of studying the water quality as one of the possible factors that influence the growth rates of tadpoles. These experiments with stagnant water revealed equal patterns of growth rates in polluted and fresh water and so pointed out that, irrespective of population density, polluted water did not have a delaying effect on growth during the first 23 days of life. However, compared to the fresh water treatments, stagnant polluted water adversely affected the development rate of the tadpoles of *L. melanonotus* (Fig. 4). Such a prolongation of the development period would presumably expose them more to the risk of predation under natural conditions.

These results could be confirmed through experiments with circulating water. Applying the same population density once more, the resulting growth rates were similar in circulating fresh and polluted water up to the point of time when the first tadpoles metamorphosed; however, the development period of the whole tadpole group was generally extended in polluted water (Fig. 5). In any case, the differences in development rates were comparatively greater between the density groups than between the water qualities.

In summary, polluted water did not affect the growth rate of fast-growing tadpoles but delayed the development rate of the slowly growing tadpoles of a group, presumably due to the long-term exposure in this polluted medium and presence of strong competition. It was only once the fast-growing tadpoles arrived at the climax of metamorphosis would the delayed tadpoles have a chance to accelerate their advancement in stage. If oxygen is available to a sufficient extent, the ammonium content in the water is generally quickly transformed into nitrite and nitrate with the help of aerobic nitrifying bacteria. Because the absorption of oxygen from the air was higher in flowing than in stagnant water treatments, the ammonium content remained low in flowing water, but high in stagnant polluted water. In view to the total N-content in the water it is remarkable that the waste content in the water correlates strongly with the total tadpole biomass (Tab. 5, Fig. 6). It needs to be taken into consideration that the weakly expressed response of the growth rate to water pollution could be partially biased by the assessment period, i.e., when the more advanced tadpoles had already reached their climax of metamorphosis while pollution had not yet reached its highest concentration. Later, when the weaker and therefore retarded tadpoles finally entered their metamorphosis stage, the prolonged development period might be influenced additionally by the prolonged exposure to this increasingly polluted water. Presumably, a loss of nitrogen occurred in gaseous form in the treatments with continuous water circulation, as the total N-content was then comparatively low, even in polluted water (Tab. 5).

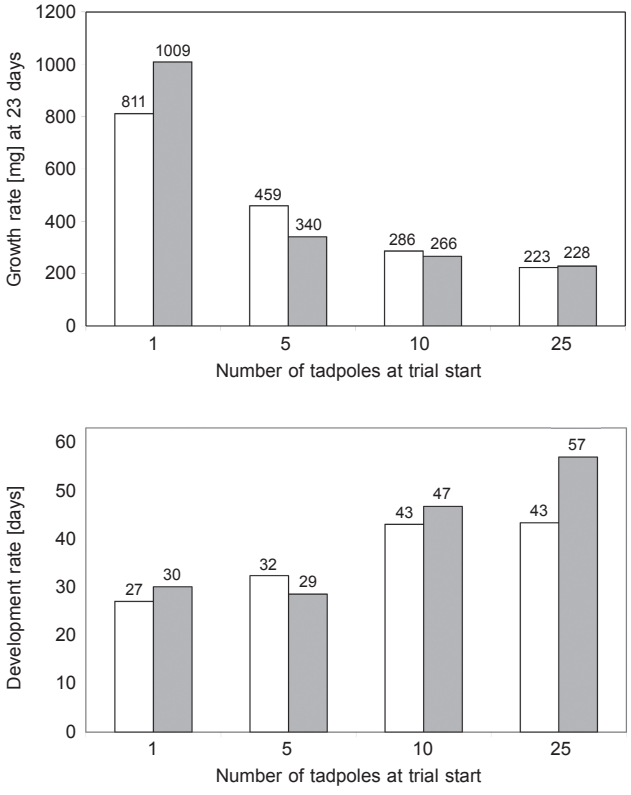

Figure 5. Growth rate at 23 days following to the start of the experiment (above) and development rate (below) of *Leptodactylus melanonotus* tadpoles reared in flowing fresh and polluted water. ☐ Flowing fresh water; ▩ Flowing polluted water.

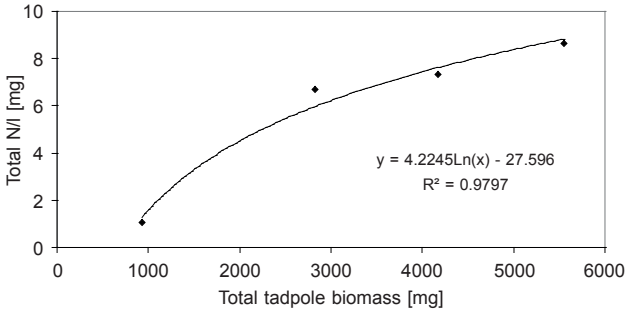

Figure 6. Relation between total tadpole biomass and total nitrogen content (as ammonium, nitrate, and nitrite in mg/l) in the corresponding polluted water sample.

Tab. 5. Water analysis in experiments with sand-coated containers in Experiment #1 (the water in the 'fresh water' setup was exchanged three days before analysis; in flowing water systems, the water quality is the same for all tadpole groups).

Nr.	Treatment	pH	DO ppm	DO%	NH_4-N	NO_3-N	NO_2-N	N-total	Total tadpole mass in mg
1	Polluted water with 1 tadpole	7.21	2.72	32.4	1.24	0.34	0.048	1.06	929
2	Polluted water with 5 tadpoles	7.45	0.73	8.6	8.6	0	0	6.69	2829
3	Polluted water with 10 tadpoles	7.36	0.63	7.3	9.4	0	0	7.31	4173
4	Polluted water with 25 tadpoles	7.61	0.93	9.6	11	0.38	0	8.64	5552
5	Flowing fresh water	7.23	4.78	49.9	0.22	1.88	0.086	0.62	11107
6	Flowing polluted water	7.55	4.00	42.2	0.4	3.25	0.170	1.10	9825

Table 6. Development rates of *Leptodactylus melanonotus* tadpoles as averages and standard deviations (from hatching to metamorphosis) and percentage of agitated tadpoles during observation periods (values in the same column with the same letter are not statistically different; Duncan-Test, $\alpha = 0.05$).

	Development rate in days		Percentage agitated tadpoles	
	Experiment #1	Experiment #2 (confined space)	Experiment #1	Experiment #2 (confined space)
Solitary tadpoles in sand-coated containers	28.0 ± 0.0 a	36.8 ± 4.4 a	22.3 ± 4.1 a	13.6 ± 11.8 a
Solitary tadpoles in mirror-lined containers	33.3 ± 4.2 b	40.3 ± 4.7 a	21.4 ± 4.8 a	31.0 ± 5.5 b
5 tadpoles/group	39.9 ± 3.0 c	50.1 ± 8.1 b	25.9 ± 3.5 a	31.3 ± 2.6 b

The most important result of the experiments is shown in Fig. 5, where in the flowing-water setup, all tadpole groups were exposed to the same water quality. While the groups of five tadpoles, and still more those of 10 and 25, exhibited low growth rates, the individually housed tadpoles grew at three or four times higher rates. If the N-content had been the determining cue for tadpole growth, the individually housed tadpoles could not have had such an advantage. Therefore, when the waste content in the water is neither the sole nor primary cause for the differences between the groups, other factors must account for the different growth patterns of tadpoles in groups with different numbers of individuals (Tab. 4, Fig. 5).

Previous studies on population density showed that the differences in tadpole growth and development could neither be explained by the competition for food, because feeding was ad libitum, nor with water quality alone, so that other cues must have an important controlling effect on tadpole growth if the larvae live at different population densities. Here it is important to consider how tadpoles perceive the presence and density of conspecifics, especially with regard to optical and tactile stimulants. Bisazza et al. (2002) showed that tadpoles of five anuran species were attracted to their mirror images. Based on this observation,

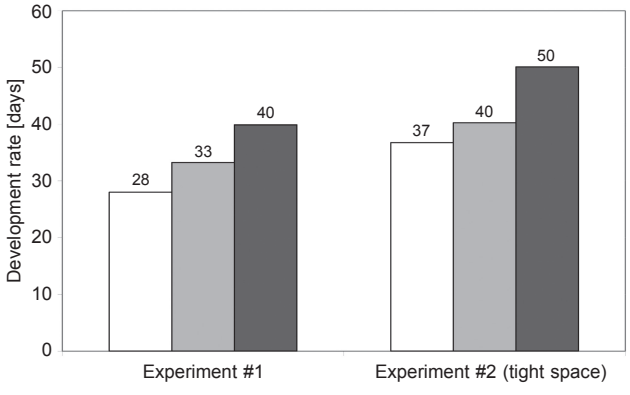

Figure 7. Development rate of *Leptodactylus melanonotus* tadpoles in two experiments with mirror-lined and sand-coated aquarium walls (the tadpoles were reared in different water volumes: Experiment #1 in 1.25 litres vs. 0.25 litres in Experiment #2). ☐ Individually housed tadpoles; ▒ Individually housed tadpoles in mirrored cells; ■ 5 tadpoles per group.

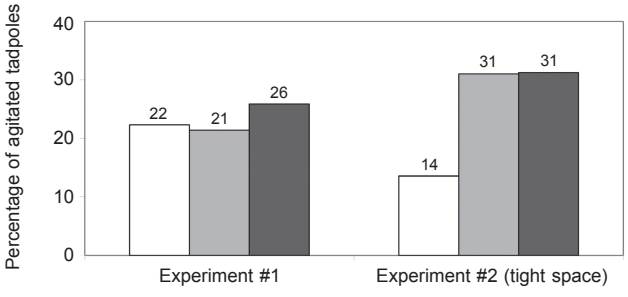

Figure 8. Percentage of agitated tadpoles during observation times in two experiments with mirror-lined and sand-coated aquarium walls (Experiment #1 in 1.25 litres vs. 0.25 litres in Experiment #2). ☐ Individually housed tadpoles; ▒ Individually housed tadpoles in mirrored cells; ■ 5 tadpoles per group.

Rot-Nikcevic et al. (2006) and Gouchie et al. (2008) used mirrors to investigate if tadpoles used visual information to assess their density and claimed that tadpoles would respond similarly to their own images as they would to the presence of real conspecifics. Studying the effect of mirrors on tadpoles of *Rana sylvatica* and *Bufo americanus*, the first authors quantified the behaviour of the tadpole by assessing the percentage of tadpole movement. They measured the tadpoles' growth and development rates and concluded that the mirrors had an effect as optical stimulants and together with tactile stimulation prolonged the period of development, but at the cost of the size at metamorphosis. They associated higher density with higher tadpole activity. This phenomenon was more strongly expressed in *R. sylvatica* than in *B. americanus*. Building on these observations, I conducted mirror experiments with tadpoles of *L. melanonotus*, which is species with strong schooling tendencies. In my mirror experiment (#2) under tightly confined conditions, the tadpoles were forced much nearer to the mirrored walls as compared to the situation in the more spacious containers of Experiment #1. They were therefore more intensely stimulated to seeing themselves nearer and clearer. This confirms indirectly the observations by Lannoo (1999) about the nearsightedness of tadpoles, just as Rot-Nikcevic et al. (2006) commented that only tadpoles near to a mirror would see images of themselves in focus. These ocular peculiarities might explain the tadpoles' stronger response to mirrors in a tight space. Presumably, these experimental habitat conditions could be compared with the physical closeness in which strongly schooling tadpoles live together in nature. Therefore, the experiments with visual stimuli summarized here could explain why solitary tadpoles grow extraordinarily well and crowding alters tadpole growth and development rates.

Nevertheless, the sight of other tadpoles might not be in itself the ultimate cause for differences in tadpole development, but could trigger other, final mechanisms. Because the environmental factors described above can only partially explain how density induces an effect on tadpole growth and development, the possible role of tactile stimuli comes to the fore. Rot-Nikcevic et al. (2005) detected in the specific case of *Rana sylvatica* (but not *Bufo americanus*) that these tadpoles assessed their environment with the help of mechanical stimuli. Tadpoles of this species were exposed to visual and additional tactile stimuli. As mentioned above, these authors found elevated stress-induced whole-body corticosterone levels and suggested in the case of *R. sylvatica* a causal connection between stress and tadpole growth and development. In the mirror experiment reported on here, tadpoles of *L. melanonotus* kept in groups of five individuals responded with higher mobility than solitary tadpoles in tight mirror-walled containers. This result suggests that the disturbance amongst five tadpoles has a stronger effect due to the physical presence of, and contact with, agitated tadpoles than virtual tadpoles in mirrors. Nevertheless, the individually reared tadpoles in cells with non-reflecting walls spent yet more time resting and consequently responded with faster growth and shorter development periods than other tadpoles in equally sized containers with mirror-walls or in groups of five individuals (see the values for Experiment #2 in Table 6, and Fig. 7 and 8). Therefore, it seems that calmness due to the absence of disturbances caused by other tadpoles definitely enhances the growth and development of *L. melanonotus* tadpoles. Tadpoles are obviously stressed by experiencing other real or virtual tadpoles nearby or by having direct contact with others. Stress reduces the time available for rest and quiet digestion and increase energy expenditure by additional movement.

In conclusion, these last results point finally to additional movement and consequently to mutual disturbances as the major predictors for differences in tadpole development in closed artificial systems or small natural habitats. The tadpoles' relatively weak response to visual stimuli could be interpreted as tolerance of a species with schooling tadpoles that would nevertheless prefer to keep a proper distance from one another.

Results of further experiments will show the corresponding response of tadpoles of other anuran families, like Ranidae and Centrolenidae, to different population densities.

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