The Berlin Journal on Research in Homeopathy, June 1991,
Vol. 1, Nr. 3:151-160

Effects of Highly Diluted Succussed Thyroxine on Metamorphosis of Highland Frogs

by

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Summary
These experiments, performed in Graz and in Utrecht, investigate the influence of extremely dilute thyroxine in a special "homeopathic" preparation on two transitions in the metamorphosis of highland amphibia: a) from the 2-legged to the 4-legged stage; b) from the tailed 4-legged stage to the untailed stage, the juvenile frog (performed in the two laboratories in Graz) or to the stage with reduced tail, respectively (performed in Utrecht).

A homeopathic dilution (SOX) of thyroxine in a small but significant range slows down the metamorphosis of highland tadpoles as compared to the reference solution H_2O.30X. This effect could be observed for both transitions. The retardation of metamorphosis was independently shown in all 3 laboratories during the course of the metamorphosis season of the highland amphibia (from August to October).

In one experiment (Graz), the number of juvenile frogs climbing out of the water in the final stages of metamorphosis was counted. Fewer "climbers" were observed under treatment with solution T_4.30X.

Keywords: Homeopathic effects, diluted hormones, thyroxine, amphibia, metamorphosis, high Dilution

1. Introduction

Important basic research has been done on the nature of homeopathic preparations hitherto: A *memory* or *information* function is documented in succussed solvents, even in dilutions beyond the Avogadro' s value. This might be based on special configurations of the solvent as well as on extremely weak (electromagnetic?) fields [1 - 10], as postulated on the basis of various physical methods (measurements of nuclear magnetic resonance [11], Raman spectroscopy [12]; measurements of specific resonance patterns of solvents [13]; for a survey, see 14-16). Apart from biochemical [17], cellular biological [18], botanical [19,20] and human medical studies [21], a number of zoological and veterinary medical studies have been performed in research on homoeo-pathy up to now.
Models where animals are intoxicated with substances in pharmacological doses have shown that homeopathic preparations of certain substances, sometimes even the toxin itself, but in high dilution, can help to detoxify the organism [22-27]. Studies of immunological aspects have been performed [28], in part by investigating the influence of a dilute hormone [29]. Furthermore, the effect of homeopathic dilutions of metal salts
on the metamorphosis and the death rate of amphibia has been described [30-32].
The model introduced in this paper is based on the fact that hormones of the thyroid glandplay an important role in the metamorphosis of amphibia. It is well known that during metamorphosis, thyroxine stimulates different processes by differential activation of genes, for example the melting down of the muscles of the tail [33-35]. Small amounts of thyroxine (tetraiodothyronine, T_4; e.g. 10^{-7} parts by weight) or triiodothyronine in water accelerate the metamorphosis of tadpoles

of anures, causing the juveniles to leave the water at body sizes below normal values. If, on the other hand, the thyroid gland is removed or blocked, no metamorphosis takes place at all.

These commonly known physiological facts on thyroxine have led to the speculation that homeopathically prepared, highly diluted thyroxine may influence metamorphosis and, as such, can be used as a possible model for research in homeopathy. Our experiments, performed in parallel in Graz and in Utrecht, investigate the influence of the special homeopathic preparation of thyroxine (solution T_4.30X) on two transitions in the metamorphosis of highland frogs: a) from the 2-legged to the 4-legged stage; b) from the tailed 4-legged stage to a stage with further reduced tail. The dilutional step 10^{-30} was chosen according to data from human medical investigations reported in homoeopathic literature [36].

2. Methods

Animals

Rana temporaria tadpoles were taken from a highland pool in the Koralpe region of Styria, Austria (1600m above sea level). For this highland frog population, metamorphosis takes place from August to November, which is very late in the year as compared to *Rana temporaria* from lower sites. Its metamorphosis proceeds rather slowly in comparison to that of lowland frogs, which furthermore starts earlier in the year. In the natural biotope, we observed that a large number of highland tadpoles died during the winter, before metamorphosis was complete (see below).

Staging

A special staging system was used to describe the development of the tadpoles. For the experiments we chose only those two-legged tadpoles

Experiment	Start (date)	50% transV (days)	90% transV (date)	N	P
Uidl	7th August	2.5	llth August	180	>0.05
Uid2	7th August	2.5	11th August	180	<0.05
Gidl	21st August	5	27th August	160	>0.05
Gid2	2 1st August	5.5	27th August	128	>0.05
Gid3	21st August	4	2nd September	160	<0.01
Gid4	4th September	4	20th September	54	<0.01
Gid5	19th October	7.5	1st November	47	>0.05
Ggnl	21st August	4	7th September	100	>0.05
Ggn2	29th August	15	24th September	90	<0.01
Ggn3	12th September	3.5	22nd September	40	<0.05

Table 1:

Dates of the experiments regarding the transition of 2-legged to 4-legged tadpoles. U, Utrecht; G, Graz; id, indoors; gn, greenhouse; start, start of the experiment; 50%transV, 50%-transition value (see text); 90%trans.V, 90%-transition value; N, number of animals; P, probability of significance of bias between the groups (see results).

which had weakly developed hind legs, comparable to stage 31 according to Gosner's staging table [37]. The tadpoles were observed until they entered the four-legged stage, and further on until the tail was reduced and they were ready to leave the water.

The development of the tadpoles is not fixed in time, but shows variation from set to set (Table 1). The speed of metamorphosis in each transition can be described by the *transition values,* which are expressed by the number of days after which the rate of transformed animals was either 50 % or 90%.

Laboratories involved

One set of experiments was performed in an empty university *greenhouse* in Graz, the other set *indoors* at a private site associated with the Ludwig Boltzmann Institute. Two independent researchers, one at each site, were involved. In Utrecht, all experiments were performed *indoors* at the State University.

Preparation of testing solutions

The tadpoles were observed under the influence of thyroxine (Firma Sigma) specially prepared in an aqueous solution of $1:10^{3}$ parts by weight. The stock solution had a concentration of $1:10^{4}$ parts by weight; it was diluted in steps of 1:10. The diluted solution was succussed according to standardized homeopathic instructions [38]. At every step the bottle was partly filled with the dilution and pushed down for 10 times (Austria) (e.g. against a rubber impediment) to establish mechanical shocks. It was succussed 100 times in 15 seconds in Utrecht. The test solution prepared in this way was called "solution T_4.30X".

For the reference solution, pure demineralized water was used instead of thyroxine and again prepared according to homeopathic standards. It was called "solution H_2O.30X".

For the experiments in Graz, two sets of solution T_4.30X and solution H_2O.30X were prepared by the Dr. Peithner Co., Vienna. The single set of dilutions for the experiments in Utrecht was prepared by VMS, Alkmaar. Both sets used in Graz were controlled and applied independently and blindly. The testing solutions in Utrecht were also applied blindly.

Solution coding

The solutions for the experiments in Graz were coded by Prof.Dr. G. Fachbach (Department for Animal Morphology and Histology of the Institute of Zoology, University of Graz). The codes of the testing solutions were announced only after the experiments were completed.

Check for contamination

The obvious contaminants of thyroxine, T_3 (triiodothyronine), as well as TSH (thyroid stimulating hormone) in the probes used in Graz were checked by radioimmunoassay (Dr. W. Passath, 1st Medical Clinic, University of Graz). The iodide content was checked by gas chromatography (Prof. G. Knapp, Meßstelle für Spurenanalyse, Graz). Contamination of the solutions mentioned above were excluded up to the accuracy of the methods used.

The tap water was investigated as to ionic contents: slight bias was proved for some ions, before and after the experiments and between the two types of treatment (solution T_4.30X and solution H_2O.30X, respectively), but this must be further investigated. Furthermore, the water in the basins treated with solution T_4.30X, showed more optical transparency and less green coloring. This effect may be due to bacteria, to chlorophyll from the greens fed to the tadpoles, as well to suspended substances (bits of vegetable, tadpole excrement). The observations (seeresults) suggest that these differences in transparency of the water are caused, at least partly, by different activities of the tadpoles under treatment. Algae developed only in the basins in the greenhouse experiments but not in those *indoors.* Since the experiments show similar results in the *greenhouse* and *indoors,* it can be assumed that the content of algae had no significant influence.

Exposure to test solutions

The white alimentary plastic basins (34 x 22 x 14.5 cm; Miraplast, Austria) were filled with 5 1 of tap water. Temperature was kept between 18°-23°C in the experiments *indoors,* and between 21°-27°C in the experiments in the greenhouse. All experiments were run under natural light cycles. Two drops of solution T_4.30X or solution H_2O.30X (control) were added blindly to the corresponding basins, followed by gentle stirring, every other day.

The transition from the two-legged to the four-legged tadpoles was examined in *Observation A.* For *Observation B,* the four-legged tadpoles were

transferred into basins filled with only 0.5 l of tap water to prevent the tailless stages, the juvenile frogs, from drowning (Graz experiments). Because the experiments in Utrecht were stopped earlier than those in Graz (at a stage with reduced tail) the further observations in Utrecht were also done in basins containing 5 1 of tap water. The tadpoles were fed cooked greens (lettuce) ad libitum in both Observations A and B.

Further experiments *{Observation* C) dealt with tadpoles which had been removed directly from the lake at the four-legged stage without any treatment. They were not fed during the transition to juvenile frogs in the two days of observation.

In *Observation D,* animals treated with substances during step A and B which spontaneously climbed out of the water towards the end of metamorphosis (from the 10th day of treatment onward) were counted. Before this time, all animals that climbed up the wall to a certain height were put back in the water. The number of climbers was determined after 1,2, 3 and 4 minutes, respectively. This experiment was repeated 5 times daily. The basins were covered during the breaks to prevent the juveniles from climbing out.

Avoidance of contamination

Each basin was used for only one experiment. Metal tools (nets and spoons) for handling the tadpoles were treated with dry heat for 1 hour, water splashes from the basins were removed with soapy water [16].

After completion of the experiments, the animals were set out in biotopes where they would not be taken for further animal experiments.

Data base

Two sets of basins (n = 1 to 5 each) for treatment with solution T_4.30X and H_2O.30X, respectively, were used for the experiments on the transition of the tadpoles in all observation sessions (A, B, C, D). Each basin contained 4 to 25 animals. This bias was due to natural support from the lake. In each experiment, however, there were the same numbers of animals per basin. For *Observation D* one pair of basins was taken from *Observation B.* Here, 25 animals were treated with solution T_4.30X and 22 with solution H_2O.30X. The positions of the basins were changed regularly to avoid any influence of spatial factors (e.g. gradient changes of light or temperature).

Evaluation of the data

In *Observation A,* the number of tadpoles that had reached the four-legged stage was added up for each set and compared to the sum of tadpoles with only two or three legs. These *cumulative frequencies* were proved as a 4-field table in a chi-square test for each measuring a point in time (every other day) in each single experiment. In *Observations B* and *C,* the numbers of juvenile frogs (Graz experiment) and the number of tadpoles with partly reduced tail (Utrecht experiment), respectively, were compared to the number of still fully-tailed, 2-, 3- or 4- legged tadpoles, again proving the bias similarly by chi-square test.

In a further step of evaluation, to get a more general view, the successive experiments of the types *A,B,C* were treated as one experiment A,B and C and evaluated as mentioned above. This was possible by normalization of the duration of the experiments in the following way: tg is defined as the start of visible development and is not equal to the start of exposition of the animals in the water tanks; t_{max} is equal to the *90% transition value* for the control animals, by the time when 90% had reached the expected stage (see Table 1). The time between t_0 and t_{max} is divided into four quartiles. Therefore, t_0 is in the beginning of the first quartile, and t_{max} is the time at the end of the 4th quartile. The rate measurements refer to the end of each quartile.

Furthermore, the so-called "survival analysis" (Number Cruncher Statistical System -40) was used to determine the statistical significance of the bias in the number of remaining 2-legged animals *(Observation* A) and of remaining 4-legged animals *(Observation B)* between the two groups according to the treatment. This test sums up the data from all quartiles and is more stringent than the chi-square tests applied to the data from the single quartiles.

Important sections of the statistical analysis of the results from Graz and Utrecht were mutually supervised.

3. Results

Observation A: The influence of the solutions T_4.30X and H_2O.30X on the speed of metamorphosis from the 2-legged to the 4-legged stage.

Ten experiments were performed in Graz and in Utrecht between August and October 1990. In the following, they are treated as three groups of experiments, according to the time and to the place of performance.

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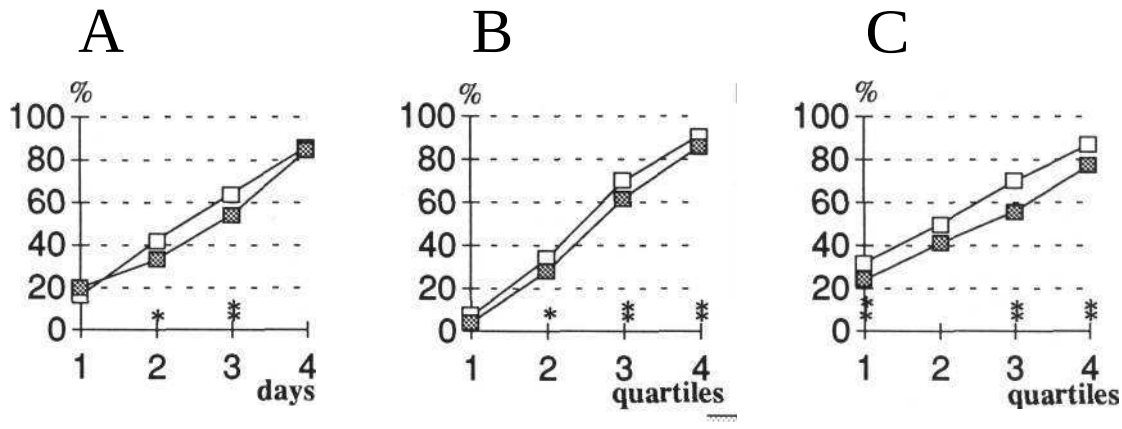

Fig. 1:

The transition of 2-legged to 4-legged tadpoles. Ordinate: cumulative frequency of four-legged animals treated with dilution T_4.30X (black squares) and dilution H_2O.30X (white squares); Abscissa: the number of quartiles of normalized duration of experiments (see methods). Three groups of experiments (see Tab. 1): A, *indoors* in Utrecht; *B, indoors* in Graz; C, in the *greenhouse in Graz; *, P < 0.05; **, P* < 0.01, evaluation with chi-square test.

a) Two successive experiments, each with 180 animals (total = 360), were performed *indoors* in Utrecht. Both were completed in August. Figure 1 A: As a parameter for metamorphosis speed, the cumulative frequency of 4-legged animals F_4 is shown for all quartiles (see Methods). Two curves document the increase of F_4 for animals treated with solution T_4.30X (black squares) and solution H_2O.30X (white squares). The F_4 values for T_4.30X animals are below the F_4 values for reference (H_2O.30X) at about 10%; this is true for the second quartile ($P < 0.05$) and the third quartile (chi-square test: $P < 0.01$).

b) Five experiments were performed *indoors* in Graz from August to October (Table 1). These successive experiments included n = 160,128, 160, 54 and 47 animals for a total of n = 549. The decreasing number of test animals is due to poor support from the lake. In Figure IB, the two curves again show generally lower F_4 values for T_4.30X animals. Significant differences occur in the second ($P < 0.05$), third and fourth quartile (chi-square test: $P < 0.01$).

c) Three experiments were performed in a greenhouse in Graz; all were completed in September (Table 1). These successive experiments included n = 100,90 and 40 animals, in all n = 230 animals. In Figure 1C, the two curves show significant differences in the first, third and fourth quartiles (chi-square test: $P < 0.01$).

In other words, both the experiments in Graz and in Utrecht, when evaluated by chi-square tests, show similarly that the probability of completing metamorphosis was generally lower for the group treated with solution T_4.30X than for the reference group.

In order to determine the statistical significance of the remaining fraction of 2-legged animals, data of all experiments were pooled and evaluated by survival analysis. A significantly larger fraction

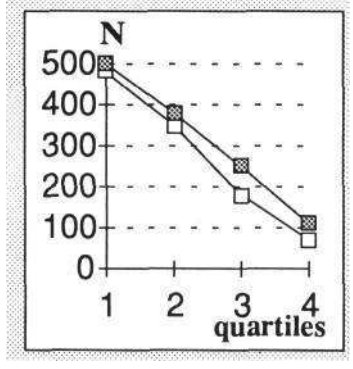

Fig. 2:

Remaining tadpoles before entering the 4-legged stage in the Utrecht and Graz experiments if treated as one experiment. N, number of animals. The bias, evaluated with survival analysis, is statistically highly significant for the sum of all quartiles. For more details, see Fig. 1.

($p < 0.01$) of remaining 2-legged tadpoles was observed in the group treated with solution T_4.30X as compared to the controls (Figure 2). This also demonstrates a slowing down of metamorphosis in the presence of solution T_4.30X. When the data were analyzed separately, only the data from Graz showed significant differences (Graz 1: $p < 0,05$; Graz 2: $p < 0,05$; Utrecht: $p > 0,05$).

It should be mentioned here that only a limited comparison of the experiments in Utrecht, in Graz *indoors* and in Graz in the *greenhouse* can be made. The Utrecht experiments were performed earlier than the Graz experiments, and they showed a higher metamorphosis speed than the Graz experiments (see Table 1).

Observations: Influence of solutions T_4.30X and H_2O.30X on the speed of metamorphosis from the tailed 4-legged stage to the untailed stage (Graz) or to the stage with reduced tail (Utrecht).

Seven experiments were performed in Graz and in Utrecht between August and November.

(a) Two experiments were performed in Utrecht in August, again with a total of 360 animals. In Figure 3A, the two curves show a significant difference only in the third quartile. Here again, the cumulative frequency of animals with reduced tail (F_{ut}) was lower at about 8% for the T_4.30X animals (chi-square test: $P < 0.05$).

(b) Three experiments were performed in the *greenhouse* in Graz; all were completed in September. The experiments included n = 100,90 and 40 animals, for a total of 230. The two curves show significant differences in the third and fourth quartiles (chi-square test: $P < 0.01$).

(c) Two experiments were performed *indoors* in Graz; they were completed in September and November, respectively. These successive experiments included n = 160 and 47 animals; total n = 207 animals. During the fourth quartile, only 47 animals were observed. In Figure 3C, the bias was significant in the second ($P < 0.05$), third and fourth quartile (chi-square test: $P < 0.01$).

In other words, the probability of completing metamorphosis was generally lower for the group treated with solution T_4.30X (chi-square test).

To determine the statistical significance of the remaining fraction of 4-legged animals, normalized data from all the experiments were again pooled and evaluated according to survival analysis. A significantly larger fraction ($P < 0.01$) of remaining 4-legged tadpoles was observed in the group treated with solution T_4.30X as compared to controls. Similar to *Observation A*, if the data were analyzed separately with this test, the differences were significant only for the data from Graz (Graz 1: $p<0,05$; Graz 2: $p< 0,01$), but not for the data from Utrecht.

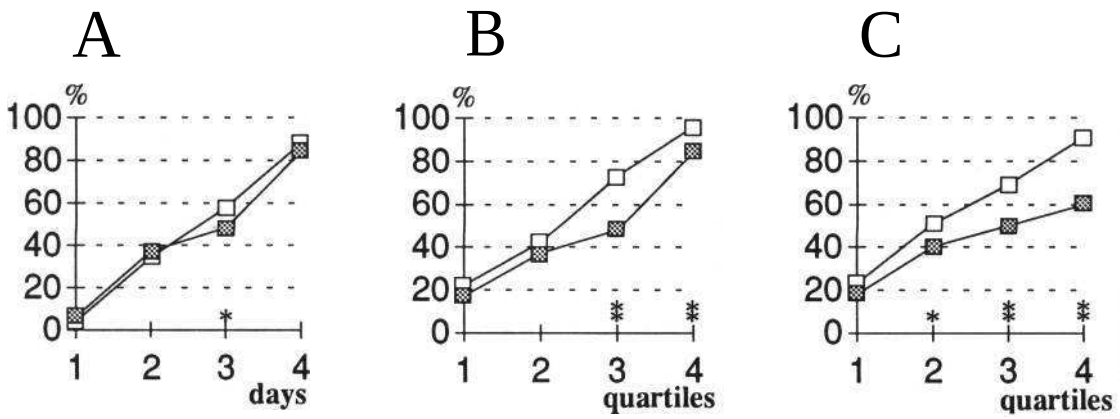

Fig. 3:

The transition of 4-legged tadpoles to tadpoles with reduced tail (A) or to juvenile frogs (B, C), respectively. Three groups of experiments (see methods): A, *indoors* in Utrecht; B, in the *greenhouse* in Graz; C, *indoors* in Graz. For more details, see Fig. 1.

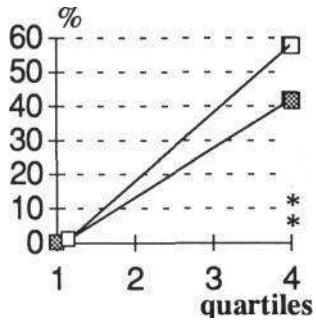

Fig.4:

The transition of 4-legged tadpoles to juvenile frogs. For more details, see Fig. 1.

Observation C: The influence of the solutions T_4.30X and H_2O.30X on the speed of metamorphosis from the freshly caught 4-legged tadpole to the juvenile frog.

The four-legged tadpoles which had been taken from the lake were observed for two days. Seventeen experiments with n = 8-26 (total 368) individuals each were performed in September and October (Graz *indoors).* Figure 4 shows the cumulative frequencies of untailed animals F_{ut} after the first and the second day of exposition, corresponding to t_0 and t_{max} according to the definition given in the methods section. After the second day, the F_{ut} value for T_4.30X animals is below the F_{ut} value for the reference at about 16% (chi-square test: $P < 0.01$).

Observation D: Number of juvenile frogs spontaneously climbing out of the water at the end of their metamorphosis under the influence of homeopathic pretreatment (solutions T_4.30X and H_2O.30X).

One experiment with n = 47 animals was performed *indoors* in Graz from October to November. The number of the animals spontaneously climbing out of the water was counted in 5 repetitions of the experiment (total: 235 cases each day) beginning on the 10th day of treatment (Fig. 5A), and after 12 or 14 days (Fig. 5B and C, resp.). The differences between the groups were significant in the first days (chi-square test: $P < 0.01$). The animals treated with solution T_4.30X showed less tendency to leave the water (Fig. 5). Differences diminished in the course of the further metamorphosis.

4. Discussion

The results presented in this study on highland frogs show that there are small but significant differences in speed of metamorphosis between the groups of animals treated with solution T_4.30X as compared to groups treated with solution H_2O.30X: In general, the differences tended to indicate that solution T_4.30X slows down development. This was proven by chi-square tests for the data from Graz and Utrecht when combined as one experiment as well as for the individual experiments from each of the three laboratories

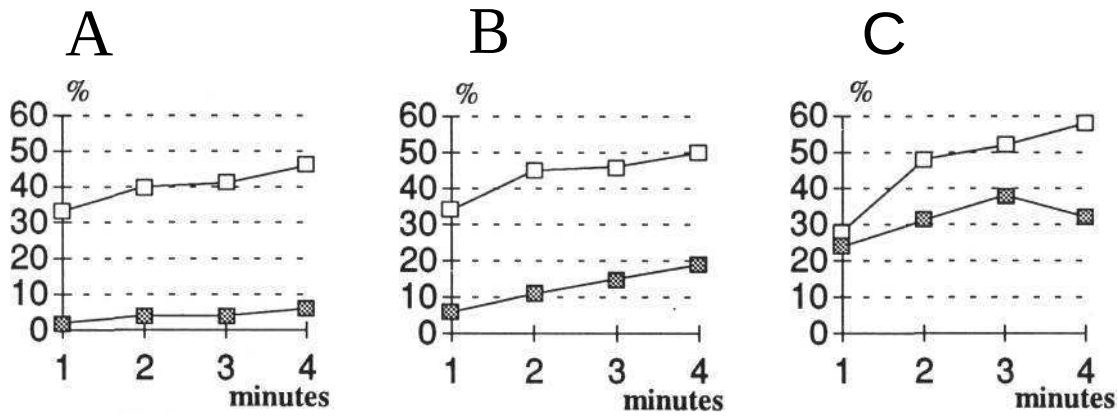

Fig. 5:

The transition from water-borne to terrestrial stage at the end of metamorphosis in frogs. Ordinate: cumulative frequency of juvenile frogs spontaneously climbing out of the water. Abscissa: number of minutes after start of the experiment; further details see Fig. 1 and text; A, observation after 10 days of exposure to test solutions; B, after 12 days; C, after 14 days.

involved (Graz *indoors*, Graz *greenhouse*, Utrecht). Survival analysis of the pooled data of all experiments (sum of all quartiles) showed significant bias. This is also true for the Graz experiments, but not for the Utrecht experiments if treated separately.

However, it is obviously sufficient to add a few drops of solution T_4.30X every other day to the tap water in the test basins to induce a reduction in the speed of metamorphosis in highland amphibia. Evidently, this inhibitory effect on metamorphosis is opposite to that known for thyroxine in pharmacological doses down to 10^{-7} parts by weight [35-37]. Homeopathic preparations are often reported to have effects opposite to pharmacologically dosed stock Solutions [8,9, 11-16,18-34]. Nothing is yet known about effects of thyroxine diluted beyond 10^{-8} parts by weight, neither in pharmacological dilutions nor in homeopathic preparations. Our findings refer to a distinct number of Steps of dilution (according to the samples of solution T_4.30X in Graz and Utrecht). Therefore, the results can be discussed only äs one aspect out of the whole homeopathic spectrum of thyroxine.

In further investigations, it will be necessary to check the homeopathic influence of T_4 samples at various numbers of dilutional Steps. Furthermore, in future experiments, the findings must be compared to a larger set of references. In addition to the reference used in this study (solution H_2O.30X), the zero control (using a probe of water which is not homeopathically prepared) and the positive control (pharmacological dose of thyroxine) must be considered as references. Independent repetitions at different locations of the basic experiment, combining *Observation A*, *B* and *D* would also be desirable.

Acknowledgments

The authors wish to express their special thanks for scientific help and advice to the Dean, Prof. Dr. Th. Kenner, Prof. Dr. G. Fachbach, Prof. Dr. K. Hagmüller, Prof. Dr. F. Moser, Dr. G. Passath, Dr. H. Guttenberger, Dr. M. Moser, Dr. F. Muhry, Dr. E. Lehner, Dr. E. and Y. dar, U. Stelzer, B. Giegler and E. Lamont, all from Graz, and to Prof. Dr. W. Timischl and Dr. F. Müller, Vienna, to Dr. P. Kokoschinegg, Salzburg, to Prof. Dr. J. Benveniste, Paris, Prof. Dr. Z. Bentwich, Rehovet, Israel, to G. Karmapa, India and to Dr. F. A. C. Wiegant, Utrecht.

Financial funding for the experimental work was provided by the Deutsche Homöopathie-Union (memberoftheHomeopathy International Group) and the Ludwig Boltzmann Gesellschaft zur Förderung der wissenschaftlichen Forschung.

A personal word:

The authors from Graz have submitted discussion of the amphibian model to thepublic with the hope that it will not be used for any investigations harmful to animals.

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