

Lowland amphibians - recalculation of data on effects of diluted thyroxine

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ABSTRACT

Our previous paper described methodological problems and a generally acceptable pooling method for metamorphosis experiments and application of that method to the results of multicentre experiments performed over the course of two decades (1990 - 2010) on highland amphibians (*Rana temporaria*) treated with a homeopathically prepared high dilution of thyroxine ("30x"). Differences between treatment groups thus calculated were in line with those obtained with other pooling methods: Thyroxine 30x does slow down metamorphosis in highland amphibians. This follow up paper provides a broader background on metamorphosis physiology and describes application of the pooling method to experiments with *Rana temporaria* from lowland biotopes both with a moderate dilution of thyroxine ("8x") and with 30x. Analogously prepared water was used for control (water 8x or 30x). Development was, again as above, monitored by documenting the number of animals that had entered the 4-legged stage. Experiments were carried out between 1990 and 2000 by different researchers independently and in blind. As it is well known, metamorphosis can be speeded up by thyroxine 10⁻⁸ mol/l; interestingly, thyroxine 8x may produce a reverse, i.e. inhibiting effect ($p < 0.01$). In contrast to the inhibiting effect of thyroxine 30x on highland larvae (see above), 2-legged lowland larvae did not react to thyroxine 30x ($p > 0.05$). However, an inhibiting effect on lowland larvae was found when animals were treated from the spawn stage on ($p < 0.01$).

Keywords: homeopathy, dilution, bio-assay, thyroxine, amphibians

Introduction

Research topics in homeopathy

A number of studies regarding fundamental research on homeopathy are available in the literature [1-13]. For example, intoxication studies are an interesting tool for research in this field. In such studies, an organism is first intoxicated with an agent in a sufficiently high dose and then an attempt at detoxification or "cure" is made by application of the same agent prepared according to homeopathic procedures, i.e. in a process of stepwise dilution and agitation ("potentization") [5,10,13].

This paper review and discusses research results obtained between 1990 and 2000 by the author and his workgroup with a non-invasive experimental model in amphibians, as well as by independent colleagues that took part in multi-researcher studies employing the same model.

Sensitivity of amphibian metamorphosis to substances prepared according homeopathic procedures was first described by Koenig in the 1930s [14]. In some of the experiments reported here, animals were hyperstimulated with thyroxine before an attempt at “cure” was made by the application of thyroxine in potentiated form.

Physiology of amphibian metamorphosis

Thyroxine (tetraiodothyronine, T_4 , a thyroid hormone) plays an important role in the regulation of metamorphosis speed in amphibians. When thyroxine is added to an aquarium water to attain final concentration 1.1 or 2.2×10^{-8} mol/l, it induces or accelerates the metamorphosis of amphibians respectively [15,16]. Thyroxine concentration 1.1×10^{-7} mol/l or lower causes an acceleration of development to such extent that deformities appear in the animals [17]. In previous experiments, L-thyroxine sodium pentahydrate (Sigma) at concentration 1.1×10^{-8} mol/l in the basin water caused 10-30% metamorphosis acceleration. Lack of thyroxine due to thyroidectomy brings metamorphosis to a standstill [16,17].

It was further found that hypophysectomized *Rana pipiens* larvae immersed in different concentrations of thyroxine at an early non-feeding stage before gill appearance only reach certain developmental stages and then remain in them. This was inferred from experiments where immersion at concentration 2.2×10^{-12} mol/l DL-thyroxine sufficed only to reach an early two-legged stage, whilst immersion at concentrations about 2.2×10^{-10} mol/l and 6.7×10^{-10} mol/l were necessary in order for larvae to reach the four-legged and juvenile stage, respectively [18].

Amphibian larvae are reported to be sensitive to thyroxine from very early stages on – even before gill reduction [19-22]. Premature tail shrinkage can be induced in early stages; this effect, however, can be achieved in two-legged tadpoles after a much shorter period of thyroxine treatment. This is the outcome of a study where larvae from non-feeding stages up to the two-legged stage reacted with tail shrinkage to 5.6×10^{-8} mol/l thyroxine solution at 23°C. Tail shrinkage occurred in all tadpoles, the latent period of response was 14.2 days in animals in the larvae non-feeding stage and 4.9 days in two-legged tadpoles [22]. It is generally agreed that most larval tissues become responsive to thyroid hormones well before significant amounts of thyroid hormones are available [23]. Sensitization appears to develop successively according to type of tissue, the hind-limb buds are the last ones to respond in early tadpole development. Gill shrinkage induced by exogenous thyroxine has been reported for a urodele [21].

As a whole, thyroxine plays a more important role as active hormone in pre-metamorphic tadpoles than it is thought to play in mammals development [24]. Responses to triiodothyronine (T_3) can be provoked after only two to four days, i.e. earlier than T_4 [20]. No literature was found on the effects of thyroxine sodium pentahydrate.

The natural plasma level of iodine / thyroxine changes during and after spontaneous metamorphosis, with slow increase during the two-legged stage, rapid increase during the four-legged stage with culmination shortly before onset of tail reduction, and then rapid decrease during tail reduction [25]. This high plasma level is interpreted as due to increased synthesis of thyroid hormone before tail reabsorption begins [26], due to higher release into the circulation and increased tissue saturation with thyroxine. Tissue would become increasingly avid for thyroxine before plasma level increases [23].

As a rule and as known from experiments with radioiodine, iodine begins to be trapped and stored already at the non-feeding stages prior to the appearance of thyroid follicles and independent of thyroid-stimulating hormone (TSH) stimulation [27,28]. Iodine uptake rises about 15-fold during the two-legged stage [28] and peaks upon transition to the four-legged stage [29,30]. This is assumed to be the period of most active synthesis and storage of thyroid hormone, before stored hormone is released to mediate climax (see above).

After tadpoles entered the four-legged stage, but before tail shrinkage, iodine absorption is reduced to a small percentage [23]. This results from thickening of the skin, shrinkage of the gills and cessation of feeding. Experiments showed that reactions can still be induced when iodine is injected, but not when it is added to the water around animals [28,31]. However there is already accumulation of iodine in the gut during this phase [32].

When animals are treated with moderate doses of NaClO_4 in order to block their thyroid gland before the two-legged stage, metamorphosis does not proceed. In a variant of this experiment performed in small scale, tadpoles were treated with NaClO_4 when their hind legs were already developed, whereupon metamorphosis continued (*Bufo viridis*, [25]). Furthermore, tadpoles are known to be most sensitive to stress during climax, which is when most physiological transformations occur [25].

However, thyroxine not only enhances metamorphosis; when applied at concentration 1.1×10^{-6} mol/l (L-thyroxine sodium pentahydrate) in the basin water at the two-legged stage, it blocks amphibian development leading to deformation and ultimately death. This was found inadvertently in early experiments (personal communication by Scherer).

Interestingly, in the treated group body length increased to about 150% compared to the control group, and tail length decreased to about 50%. Front limbs only occurred in the control group, but not in the thyroxine 10^{-6} -group. All tadpoles treated with thyroxine 1.1×10^{-6} mol/l died on the sixth day of exposure, before their front limbs had appeared. At this time tadpoles in the control group had already started to enter the four-legged stage.

No literature was found on dose-effect relationship when low concentrations of thyroxine are applied to an induced/accelerated metamorphosis state. In any case, no effects are expected at concentration 1.1×10^{-15} mol/l. This is the detection threshold of measurements performed at our laboratory during high dilution experiments to check contamination (K. Hagmueller, Institute for Zoology, University of Graz). Pilot studies on dilutions of thyroxine prepared according to homeopathic technique (1.1×10^{-6} , final concentration in the basin water 1.1×10^{-11} , “thyroxine 6x”) showed interesting but as yet inconclusive results [33, p.37].

Research question

The research question is whether thyroxine at different potencies (“8x” = 1.1×10^{-8} mol/l = final concentration in the basin water 1.1×10^{-13} mol/l, or “30x”, i.e. at concentration beyond Avogadro’s limit) has any influence on metamorphosis speed in *Rana temporaria* and if so, whether such influence can be enhanced by pretreating (hyperstimulation) animals with thyroxine.

Between 1990 and 2010, the following types of study were performed: treatment from the two-legged stage on (with three sub-studies, i.e. “type I” lowland animals and thyroxine 8x, “type II” lowland animals and thyroxine 30x, “type III” highland animals and thyroxine 30x – each with two sub-types, i.e. inert animals and hyperstimulated animals); treatment from the spawn stage (“type IV”, hyperstimulated lowland animals treated with thyroxine 30x). These experiments were inspired by the appeal of intoxication studies as an interesting tool for research in the field of homeopathy: an organism is first intoxicated with an agent at sufficiently high dose and then an attempt at detoxification or “cure” is made by applying the same agent in diluted and agitated (“potentiated”) form.

Our initial choice of the amphibian model was motivated by the fact that during metamorphosis rapid increase of the thyroxine level occurs in animals that may justify the notion of an “exceptional” (albeit not intoxicated) state (studies of sub-type “inert animals”). In studies of sub-type “hyperstimulated animals”, animals are artificially stimulated (i.e. “intoxicated”) with thyroxine, before an attempt at “cure” is made by applying thyroxine in diluted and potentiated form.

Methods

Rana temporaria larvae from different biotopes (lowland, i.e. 200 – 400 m above sea level, or highland, i.e. 1,400 – 1,600 m above sea level) were treated at different stages (spawn and two-legged) with different dilutions of thyroxine prepared according homeopathic technique (“potentiation”, Table 1). Analogously potentiated water was used as control. For details on the preparation process, see below.

Table 1. Effect of diluted and agitated thyroxine on amphibian metamorphosis. Overview on studies performed. For further information, see text.

Type of study	Source of animals	Onset of study	Dilution used	A - inert	B - hyperstimulated
I	lowland	two-legged	8x, 24h	I.I	I.II
II	lowland	two-legged	30x, 24/48h	II.I	II.II
III	lowland	spawn	30x, 48h	-	III

Development was monitored by documenting the number of animals that entered the four-legged stage. As a rule experiments were carried out by different researchers in parallel. All experiments were performed blind. Each laboratory had its own independent authority responsible for the blinding procedure. The same blinding method was used at each center. Substances were prepared in pairs. All substances were prepared in glass vials identifiable by the plaintext designation on the pull-off label. Blinding was performed within pairs. All solutions were left in the glass vials to avoid any extraneous influence through decanting. The plaintext labels were then removed by the blinding authority and replaced with labels bearing encoded designations. The code was not made known until after presentation of the results. For reasons of laboratory convenience (danger of cross-contamination due to intricate handling) we abstained from using more than one vial per substance.

For this survey, chi-square tests were performed for the measuring point when in experiments of type I.I and II.I (inert animals) about 70% and in experiments of type I.II, II.II or III (hyperstimulated animals; hyperstimulation in itself speeds up development by about 20%) about 90% of animals had reached the four-legged stage: frequencies (two-legged test animals / two-legged control animals / four-legged test animals / four-legged control animals) were entered in two-by-two-tables.

At that measuring point, the effect size (Cohen’s d, standardized difference of means = absolute difference between means of verum and control group, divided by standard deviation (SD)) was calculated. An effect size is considered small when higher than 0.2, medium when higher than 0.5 and large when higher than 0.8. Details on further evaluation are described below.

Study type I, lowland animals and thyroxine 8x

Study type I concerns the influence of thyroxine in moderate dilution prepared according to homeopathic technique (thyroxine 8x) on metamorphosis in lowland *Rana temporaria* (34). For type I.I experiments inert, i.e. non-pretreated lowland animals were used, and for type I.II experiments lowland thyroxine-hyperstimulated animals were used.

Laboratories and researchers

The experiments of types I.I and I.II were carried out in parallel by 3 researchers: Waltraud Scherer-Pongratz, Boltzmann Institute Graz, Christa Zausner-Lukitsch, Institute of Zoology, Vienna University, and Heimo Lassnig, Federal Institute of Veterinary Investigation, Graz [34]. An additional type I.I experiment was carried out by Conrad Heckmann, Tübingen University [35].

Animals, staging, water and further laboratory conditions

Rana temporaria larvae were taken from lowland pools ca. 300 m above sea level in Styria, Austria. The starting stage was defined as the point when the hind legs of two-legged tadpoles are straddled so that one can merely see through the triangle formed by thigh, shank, and tail (see ref 42, figure 1, left). This point of development occurs during Gosner's stage 31 [36]. Tadpoles were observed until their forelegs, which are preformed under the skin, broke through and animals had thus entered the four-legged stage (see ref 42, figure 1, right).

At a certain point of development, the forelegs break through almost instantaneously. Thus, this parameter seems well suited for defining the final stage. Inter-rater reliability of counting was assessed in collaboration with different authorities from the Institute of Zoology of Graz University as well as from the Environmental Agency of Styria County. Some counting results were also documented photographically.

Basins contained 6 l of water each (see ref 42, figure 2). Water samples from type I experiments were analyzed by Institute of Hygienics and Institute of Endocrinology of Graz University prior to the experiment. Pollutants such as heavy metals, chlorine or iodine were not found.

Twenty animals were allotted to each of a total of 60 white plastic basins according to a random procedure. This was performed in the same way in all centers, 20 basins were used in each laboratory. One by one, 20 animals were fished out of the main tub and distributed over the basins so that there was one in each. This was repeated 19 times. The purpose of this procedure was to ensure that animals were distributed homogeneously as a function of their level of activity and swimming behavior in the main tub. The experimental design was the same at each center, involving a total of 20 basins distributed five basins for each of four different treatment groups (two inert groups for type I.I experiment and two hyperstimulated groups for type I.II, see below). Basins were arranged in five rows of four, each row contained two basins from each treatment group. The spatial arrangement of groups within rows alternated from one row to the next, i.e. basins with identical treatment groups were arranged in diagonals, and this arrangement was left unchanged all throughout the experiment to avoid the danger of cross-contamination through splashing. Indirect natural light was used, temperature was 20°C +/- 1°C. Tadpoles were fed blanched greens (lettuce) *ad libitum*.

Preparation and administration of test solutions

A stock solution of tetraiodothyronine sodium pentahydrate (T₄, Sigma) in 40% ethanol (vol/vol) was prepared (1.1 x 10⁻⁴ mol/l). To prepare test dilution thyroxine 8x, the stock solution (1.1 x 10⁻⁴ mol/l) was further diluted with pure double distilled water in 4 1:10 steps at ambient temperature, and agitated after each dilution step according to standardized homeopathic pharmacotechnics [37]. Using disposable pipettes, 1 ml of the precedent dilution was added to 9 ml of water in a 20 ml vial. The vial was banged 30 times against a rubber impediment at approximately 0.5 sec intervals to create mechanical shocks. For preparation of control, 40% ethanol (vol/vol) was analogously further diluted with pure double distilled water in 4 1:10 steps and agitated after each dilution step (water 8x). Probes prepared by the same method were checked for T₄ concentration by means of chemiluminescence prior to the experiment. Final thyroxine concentration of dilution thyroxine 8x in the basin water was 1.1 x 10⁻¹³ mol/l after the first application.

Two groups of animals were exposed to stock solution diluted in the basin water (immersion in thyroxine 1.1×10^{-8} mol/l, hyperstimulated groups). Two other groups were kept in tap water with an analogous concentration of ethanol. One of the hyperstimulated groups and one of the inert groups were then treated with thyroxine 8x, and the others were treated with water 8x.

As inferred from the preparation protocol, pretreatment (control versus hyperstimulation, groups “I” and “II”, see below) consisted in immersing the animals in thyroxine or control solution containing 4 ppbv (part per billion per volume) ethanol. After the first application of thyroxine or control solution in the actual treatment phase, all four groups were immersed in thyroxine or control solution containing 40 ppqv ethanol. It was thus ensured that any differences in metamorphosis speed within either the two pretreated or two non-pretreated groups could not be attributable to ethanol.

Three microliters of probe dilutions (thyroxine 8x or water 8x) were added per animal and 300 ml of basin water (i.e. 60 microliters per 6 l-basin) at 24-hour intervals.

Database

Animals were treated blindly with: a. normal water + water 8x (inert control group I.I), b. normal water + thyroxine 8x (inert test group I.I), c. thyroxine 10^{-8} + water 8x (hyperstimulated control group I.II), d. thyroxine 10^{-8} + thyroxine 8x (hyperstimulated test group I.II). At each center, five basins (i.e. 100 animals) were used for each of the four treatment groups. A total of 1,200 animals were involved.

No animals in the two-legged stage were lost. The few animals in the 4-legged stage that died were counted as four-legged and removed from the basin.

Comparison and evaluation of data

Comparison and evaluation of data has been described in detail in (42). Measures time-points are defined on the basis of values yielded by both thyroxine 30x and water 30x groups to avoid artificial differences in variability. The range from 0% to 100% over which the fraction of four-legged animals progresses in the course of an experiment is divided into 10%-intervals and mapped on a corresponding relative time scale from 1 to 9. Each measurement is then assigned to the point (reference point) on the time scale to which it is closest (e.g. values between 46% and 54% are all assigned to reference point 5). These values are aggregated over all experiments within test- and control-group.

Main evaluation was performed by means of chi-square test at the 70%-measuring point for the inert groups (I.I) and at the 90%-measuring point for the hyperstimulated groups (I.II). Aggregate values obtained for treatment thyroxine 8x versus water 8x both in the inert and hyperstimulated groups were analyzed by way of logistic regression analysis. Cox's proportional hazards model was also applied. This method considers the time required to reach the four-legged stage and takes each data set with all reference points (days 1-10) into account [34]. In both tests the pooled data were assessed by determining p-values over the accumulated raw data at the basins level as well as the p-values at individual experiments level. Mean values and standard deviations were calculated.

Study type II, lowland animals and thyroxine 30x

Study type II concerns the influence of a high dilution of thyroxine “thyroxine 30x” on metamorphosis in lowland *Rana temporaria* [33,38, and personal communication by Scherer]. For type II.I experiments inert, i.e. non-pretreated lowland animals were used, and for type II.II lowland animals hyperstimulated with thyroxine (1.1×10^{-8} mol/l). Temperature was $21^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Experiments were performed by one researcher (Scherer). For further details on methods, see explanations on study type I above.

Preparation and administration of test solutions

To prepare test dilution thyroxine 30x, the stock solution (1.1×10^{-4} mol/l) (see type I studies above) was further diluted with pure double distilled water in 26 1:10 steps and agitated after each dilution step according to a standardized protocol. Analogously, 40% ethanol (vol/vol) was further diluted with pure double distilled water in 26 steps 1:10 (water 30x). Probe dilutions were added at 24-h or 48-h intervals, i.e. application intervals were not uniform across experiments. For further details on methods, see explanations on study type I above.

Study type III, hyperstimulated lowland animals treated with thyroxine 30x from the spawn stage on

Study type III concerns the influence of thyroxine 30x compared to water 30x on hyperstimulated lowland *Rana temporaria* treated from the spawn stage on [44]. The objective of study type IV was to investigate whether earlier onset of pretreatment with thyroxine (1.1×10^{-8} mol/l, prepared in pure water) sensitizes lowland animals to thyroxine 30x.

The influence of thyroxine 30x on metamorphosis was studied in lowland *Rana temporaria* from the spawn stage on. Hyperstimulated animals (spawn, later larvae) were treated either with thyroxine 30x or water 30x. Development was monitored by documenting the number of animals that entered the four-legged stage. Temperature was $21 \pm 1^\circ\text{C}$. The experiment was performed by one researcher (Helmut Graunke, Interuniversity College). For further details on methods, see study types I and II above.

Results

Study type I, lowland animals and thyroxine 8x

In type I.I experiments (*non*-hyperstimulated animals) performed by Scherer and Zausner, the number of animals that reached the four-legged stage at defined measuring points was slightly smaller in the group treated with thyroxine 8x compared to water 8x. In Lassnig no difference was found between groups. Heckmann found slightly higher values in thyroxine 8x-group compared to control group [35]. Overall difference at the 70% measuring point was not statistically significant ($p > 0.05$). Use of other statistical methods led to similar results (for details, see [34]; 1 SD was $\pm 6\%$ in both test and control group, and effect size was 0.3 (small).

In type I.II experiments (hyperstimulated animals), the number of animals that reached the four-legged stage was smaller in hyperstimulated thyroxine 8x-group compared to hyperstimulated water 8x-group. The inhibiting effect at the 90% measuring point was statistically significant in Scherer ($p < 0.05$) and Zausner ($p < 0.01$) but not of Lassnig ($p > 0.05$) laboratory. When data were pooled effect was significant ($p < 0.01$); 1 SD was about $\pm 14\%$ in both test and control group, and effect size was 0.82 (large) (Figure 1).

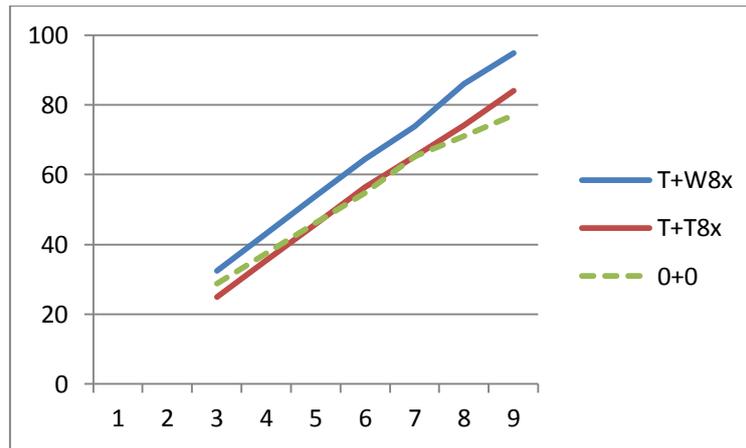


Figure 1. Influence of moderately diluted and agitated thyroxine tested versus analogously prepared water on hyperstimulated lowland amphibians. Pooled results from three researchers. Ordinate = cumulative frequency of four-legged tadpoles in % (= N). Abscissa = points in time. Blue dotted line: frequency of animals treated with water 8x; red dotted line: with thyroxine 8x; Blue line: frequency of hyperstimulated animals treated with water 8x; red line: hyperstimulated animals treated with thyroxine 8x, green dotted line: non-hyperstimulated not treated animals. For explanation, see text. Data combined and recalculated from (39).

Table 2: Details of sub-experiments on the influence of moderately diluted and agitated thyroxine on hyperstimulated lowland amphibians. ST: “steps of ten”: see Methods; black figures: raw data; blue: sums of raw data from T30x + W30x groups for calculation of “ST”; red: application of “ST” to T30x + W30x groups separately. ([Table 2 is available as a supplementary xls file](#) [58])

Study type II, lowland animals and thyroxine 30x

In types II.I and II.II experiments there was no statistically significant difference between test and control groups ($p > 0.05$).

Study type III, hyperstimulated lowland animals treated with thyroxine 30x from the spawn stage on

Animals treated with test solution were found to metamorphose slower than control animals, i.e. effect of thyroxine 30x was (as in the previous studies) opposed to thyroxine usual effect. The number of test animals that reached the four-legged stage at defined time-points was smaller in the group treated with thyroxine 30x at some but not at all time-points compared to water 30x (Figure 2). At the 90%-measuring point, this difference was significant ($p < 0.01$).

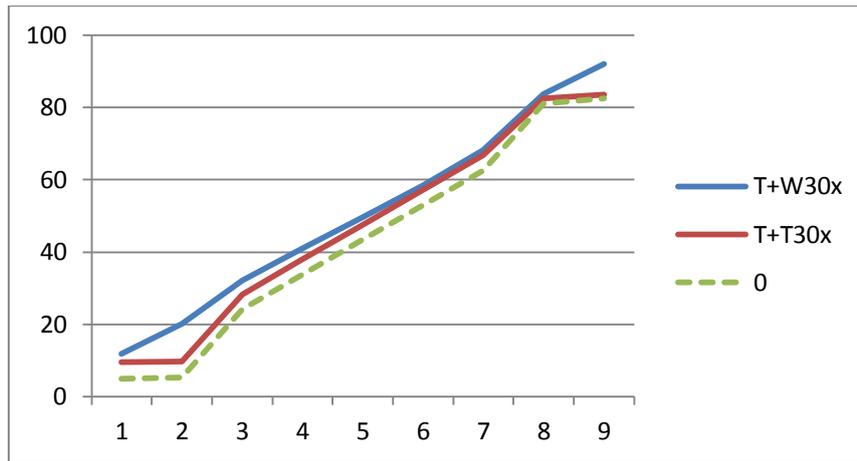


Figure 2. Influence of extremely diluted and agitated thyroxine added from the spawn stage onwards on hyperstimulated lowland amphibians. N = 500 per group. Green dotted line: non-hyperstimulated animals treated with inert water. For further explanation, see legend to Figure 1 and text. Data recalculated from (44).

Table 3. Details on the experiment on the influence of extremely diluted and agitated thyroxine added from the spawn stage onward on hyperstimulated lowland amphibians. For explanation, see table 2. ([Table 3 is available as a supplementary xls file](#) [59])

Discussion

These experiments were inspired by the appeal of intoxication studies as an interesting tool for research in the field of homeopathy: an organism is first intoxicated with a molecular agent and then an attempt at detoxification or “cure” is made by applying the same agent in diluted and agitated (“potentiated”) form. Initial choice of the amphibian model was motivated by the fact that rapid increase in thyroxine blood levels occurs during metamorphosis. We believe this justifies the notion of an “exceptional” (albeit non-intoxicated) state (type “I” studies). In type “II” studies, animals are artificially hyperstimulated by thyroxine (i.e. “intoxicated”) before an attempt at “cure” is made by applying thyroxine in potentiated form.

Inert lowland amphibians were found not to visibly react to thyroxine 8x, but thyroxine 8x can slow down metamorphosis in lowland amphibians when pretreated (hyperstimulated) with thyroxine. In other words, pretreatment with thyroxine can enhance thyroxine 8x reverse or “curative” effect. Furthermore, amphibians from lowland biotopes were found not to visibly react to high dilution thyroxine 30x.

In contrast, thyroxine 30x does slow down metamorphosis in inert highland amphibians [42]. This was observed by five researchers in most of altogether 20 experiments, and it seems to be the most reliable bioassay found in research in amphibians and diluted agitated thyroxine so far. However, pretreatment (hyperstimulation) of highland animals with thyroxine does not lead to a more marked effect of thyroxine 30x; rather the effect was smaller compared to non.pretreatment [42].

In a pilot study thyroxine 30x was found to slow down metamorphosis in lowland amphibians hyperstimulated with thyroxine from the spawn stage on. In other words, pretreatment with thyroxine can enhance a reverse or “curative” effect of thyroxine 30x. However, the special design tested has to be further

investigated before general conclusions on the possibility to influence lowland *Rana temporaria* by extremely diluted thyroxine can be drawn.

Different degrees of the experimental effect seem to be due to different degrees of amphibian sensitivity towards diluted and agitated thyroxine. This in turn seems to depend on whether animals come from lowland or highland biotopes.

From these studies we conclude that there appears to be a relationship between the effect of thyroxine prepared according to homeopathic technique and naturally or artificially elevated thyroxine levels in animals during metamorphosis. It is reasonable to suppose that highland larvae of *Rana temporaria* become adapted to an environment requiring comparatively higher thyroxine levels or high sensitivity to thyroxine. This would be a plausible explanation for the consistent response found in experiments performed with extremely diluted thyroxine.

These results suggest that administering diluted and agitated thyroxine to amphibian larvae during thyroxine-controlled metamorphosis is in a certain sense analogous to the intoxication-detoxification concept used in other homeopathy research models, although in our experimental model the intoxication dose and its effect on responsiveness do not seem to correlate in a linear manner.

Results of independent researchers backing some of our findings [45,46,47] were described in (42, see discussion section). Guedes *et al.* investigated histological changes during tail absorption and found higher apoptosis rate (programmed cell death) in the test group [46]. Interesting work has already been performed regarding signal proteins modulation by dilutions prepared according to homeopathic technique [12,48]. However, in keeping with our principle of avoiding invasive methods we chose not to pursue this question any further.

It is interesting to note that a thyroxine-sensitive state may be influenced by diluted and agitated thyroxine, and that even after extreme dilution beyond Avogadro's limit, information from the original thyroxine molecule appears to be stored in or linked to liquid water. Some biophysical theories support the possibility of such findings [49]. Physics research revealed through radiation coupling that water dipoles might develop phase coherent oscillations [50]. It was suggested that these can be modulated as a time-ordered pattern of signals forming "liquid crystals". UV spectroscopy may be an adequate tool for research in this field [51]. We are inclined to believe that the theoretical explanation of homeopathy - just as the explanation of a wide range of other phenomena in physiology, psychology and epistemology - will be inspired in the future by de Broglie's concept of the wave nature of particles and the particle nature of waves [52,53].

Research along biophysics line may be stimulated by the finding that diluted and agitated substances sealed in glass vials may influence physiological processes [54]. Using two-legged *Rana temporaria*, researchers found in some but not all cases that animals treated with *thyroxine 30x sealed in glass vials* metamorphosed slower than control animals treated with *water 30x sealed in glass vials* (figure 3).

A total of seven sets of experiments were performed. The number of animals that reached the four-legged stage was smaller in the test compared to the control group. An inhibiting effect at the 70% measuring point was statistically significant when all data were pooled ($p < 0.01$); for the experiments treated separately, it was significant only in Scherer-Pongratz laboratory ($p < 0.01$), while it was visible as a trend ($p > 0.05$) in Endler, Vinattieri (Turin) and Hilgers (Vienna) laboratories, and there was no difference between groups ($p > 0.05$) in Dieterle main experiment (Graz). However, when in a small experiment Dieterle used quartz glass vials instead of soft soda glass, there was less metamorphosis speed in thyroxine 30x compared to control group (figure 4).

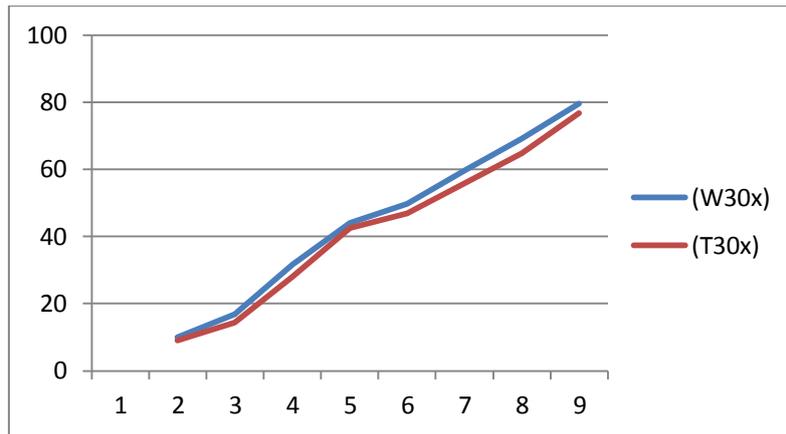


Figure 3. Influence of extremely diluted and agitated thyroxine sealed in glass vials. N = 1710 per group. For further explanation, see legend to Figure 1 and text. Data recalculated from (55).

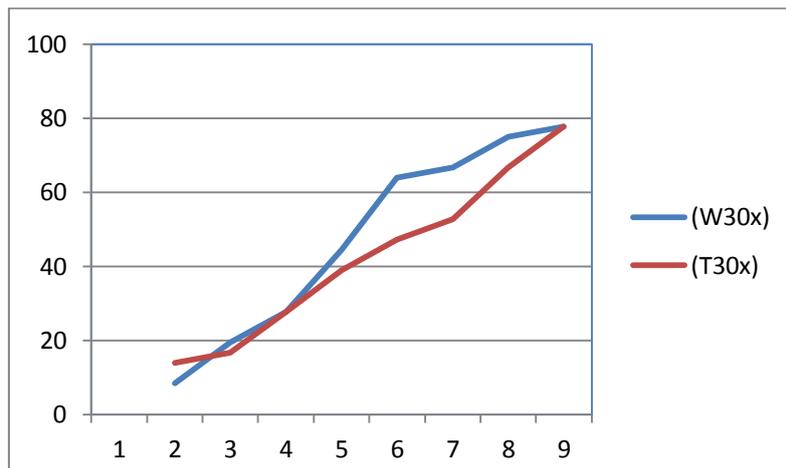


Figure 4. Result of a pilot experiment using thyroxine 30x sealed in quartz glass vials. N = 36 per group. For further explanation, see legend to figure 1 and text. Data recalculated from (55).

A comprehensive overview on the state of repetitions of fundamental research models for dilutions beyond 10^{23} was given in ref 10. Research into homeopathy was described in the divulgation-book “Homeopathy – An Expedition Report” [33]. This book also discusses further types of studies with amphibians [56,57].

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Anfíbios de terras baixas – recálculo dos dados sobre os efeitos de tiroxina diluída

RESUMO

Nosso artigo anterior descreve problemas metodológicos e um método geralmente aceitável de agrupamento em experimentos sobre metamorfose e a aplicação deste método aos resultados de experimentos multicêntricos realizados no curso de duas décadas (1990 – 2010) em anfíbios de terras altas (*Rana temporaria*) tratados com uma alta diluição de tiroxina preparada homeopaticamente (“30x”). As diferenças calculadas entre os grupos de tratamento concordam com as obtidas através de outros métodos de agrupamento: Tiroxina 30x de fato faz mais lenta a metamorfose nos anfíbios de terras altas. Este artigo posterior oferece um panorama mais amplo sobre a fisiologia da metamorfose e descreve a aplicação do método de agrupamento a experimentos com *Rana temporaria* de biótopos de terras baixas com diluição moderada (“8x”) e 30x de tiroxina. Foi utilizado como controle água preparada analogamente (água 8x e 30x). Novamente, o desenvolvimento foi monitorado documentando o número de animais que entraram no estágio de 4 patas. Os experimentos foram realizados entre 1990 e 2000 por diversos pesquisadores independentemente e em cego. Como é sabido, a metamorfose pode ser acelerada por tiroxina 10^{-8} mol/l; de maneira interessante, tiroxina 8x pode produzir o efeito inverso, ou seja, inibidor ($p < 0.01$). Contrariamente ao efeito inibidor de tiroxina 30x nas larvas de terras altas (vide acima), as larvas de 2 patas de terras baixas não responderam a tiroxina 30x ($p > 0.05$). No entanto, foi achado um efeito inibidor sobre as larvas de terras baixas quando os animais foram tratados a partir da desova ($p < 0.01$).

Palavras-chave: homeopatia, diluição, bioensaio, tiroxina, anfíbios

Anfíbios de tierras bajas – re-cálculo de datos sobre los efectos de tiroxina diluida

RESUMEN

Nuestro artículo anterior describe problemas metodológicos y un método aceptable en general de agrupamiento en experimentos sobre metamorfosis y la aplicación de este método a los resultados de experimentos multicéntricos realizados durante dos décadas (1990 – 2010) en anfíbios de tierras altas (*Rana temporaria*) tratados con una alta dilución de tiroxina preparada homeopáticamente (“30x”). Las diferencias calculadas entre los grupos de tratamiento están de acuerdo con las obtenidas mediante otros métodos de agrupamiento: Tiroxina 30x fehacientemente enlentece la metamorfosis en anfíbios de tierras altas. Este artículo posterior ofrece un panorama más amplio de la fisiología de la metamorfosis y describe la aplicación del método de agrupamiento en experimentos con *Rana temporaria* de biótopos de tierras bajas con dilución moderada (“8x”) y 30x de tiroxina. Se utilizó como control agua preparada análogamente (agua 8x y 30x). Nuevamente, el desarrollo fue monitoreado documentando el número de animales que entraron en el estadio de 4 patas. Os experimentos fueron realizados entre 1990 e 2000 por diversos investigadores independentemente y en ciego. Como se sabe, la metamorfosis puede ser acelerada por tiroxina 10^{-8} mol/l; de modo interesante, tiroxina 8x puede producir el efecto inverso, o sea, inibidor ($p < 0.01$). Contrariamente al efecto inibidor de tiroxina 30x en larvas de tierras altas (ver antes), las larvas de 2 patas de tierras bajas no respondieron a tiroxina 30x ($p > 0.05$). Sin embargo, se encontró efecto inibidor en larvas de tierras bajas cuando los animales fueron tratados desde la freza ($p < 0.01$).

Palabras-clave: homeopatía, dilución, bioensayo, tiroxina, anfíbios



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