Int J High Dil Res, accepted for March 2012

Lowland amphibians - Recalculation of data on the effects of diluted thyroxine

Peter Kiefer[1], Gerhard Lingg[1] and P. C. Endler[1]

[1] Interuniversity College for Health and Development Graz / Castle of Seggau
***Correspondig author**

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 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 in respect to fundamental research on homeopathy have been presented in 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 at a sufficiently high dose and then an attempt at detoxification or "cure" is made by application of the same agent prepared according to instructions derived from homeopathy, i.e. in a process of stepwise dilution and agitation ("potentisation") (5,10,13).

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

Sensitivity of amphibian metamorphosis to substances prepared according to instructions derived from homeopathy 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 potentized form.

Physiology of amphibian metamorphosis

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

It has further been found that hypophysectomized *Rana pipiens* larvae, when immersed in different concentrations of thyroxine at an early nonfeeding stage before gill appearance, reach only certain developmental stages and then remain at those stages. This has been inferred from experiments in which immersion at a concentration of 2.2 x 10^{-12} mol/l DL-thyroxine sufficed to reach an early 2-legged stage, whilst immersion at concentrations of about 2.2 x 10^{-10} mol/l and 6.7 x 10^{-10} mol/l were necessary in order for the larvae to reach the 4-legged stage 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 2-legged tadpoles after a much shorter period of thyroxine treatment. This is the outcome of a study in which larvae from the non-feeding stages on up to the 2-legged stages reacted with tail shrinkage to a thyroxine solution of 5.6 x 10^{-8} mol/l at 23°C. Tail shrinkage occurred in all tadpoles, the latent period of response being 14.2 days in animals at the larvae nonfeeding stage and 4.9 days in 2-legged tadpoles (22). It is generally agreed that most larval tissues become responsive to thyroid hormones at a stage in larval development well before significant amounts of thyroid hormones are available (23). Sensitization appears to develop successively by type of tissue, the hind-limb buds being the last tissue to respond in early tadpole development. Gill shrinkage induced by exogenous thyroxine has been reported for a urodele (21).

In general, thyroxine plays a more important role as an active hormone in premetamorphic tadpoles than it is thought to play in mammals development (24). Responses to tri-iodo-thyronine (T_3) can be provoked after only 2-4 days, i.e. earlier than those to T_4 (20). No literature has been found on the effects of thyroxine sodium pentahydrate.

The natural plasma level of iodine / thyroxine changes during and after spontaneous metamorphosis, with a slow increase during the 2-legged stages, a rapid increase during the 4-legged stage with culmination shortly before onset of tail reduction, and then a rapid decrease during tail reduction (25). This high plasma level is interpreted as being due to an increasing synthesis of thyroid hormone before tail resorption begins (26), due to its increasing release into the circulation and to increased tissue saturation with thyroxine. Tissue would become increasingly avid for thyroxine in the time before the plasma level increases (23).

In general, as is known from experiments with radio-iodine, iodine begins to be trapped and stored already at the nonfeeding 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 2-legged stage (28) and peaks upon transition to the 4-legged stage (29,30). This is assumed to be the period of most active synthesis and storage of thyroid hormone, before the stored hormone is released to mediate climax (see above). After the tadpole has entered the 4-legged stage, but before tail shrinkage, iodine absorption is reduced to a few percent (23). This results from thickening of the skin, shrinkage of the gills and cessation of feeding. Experiments have shown that reactions can still be induced when iodine is injected, but not when it is added to the water containing the animals (28,31). However there is already an accumulation of iodine in the gut in this phase (32).

When the animals are treated with moderate doses of $NaClO_4$ in order to block their thyroid gland before the 2-legged stages, metamorphosis does not proceed. In a variant of this experiment, performed at a small scale, tadpoles were treated with $NaClO_4$ when their hindlegs were already developed, whereupon their metamorphosis continued (*Bufo viridis*, (25)). Furthermore, it is known that tadpoles are most sensitive to stress during climax, which is when most of the physiological transformations occur (25).

However, thyroxine not only enhances metamorphosis; when applied at a concentration of 1.1×10^{-6} mol/l (L-thyroxine sodium pentahydrate) in the basin water at the 2-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% of that of 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 the tadpoles in the control group had already started to enter the 4-legged stage.

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

Research question

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

Between 1990 and 2010, the following types of study were performed: treatment from the 2-legged stage (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 agitated ("potentized") form.

Our initial choice of the amphibian model was motivated by the fact that during metamorphosis, a rapid increase of the thyroxine level occurs in the 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 potentized 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 from different stages on (spawn or 2-legged, respectively) with different dilutions of thyroxine prepared according to instructions derived from homeopathy ("potentisation", Table 1). Analogously potentized water was used for control. For details on the preparation process, see below.

Table 1. *Effect of diluted 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	2-legged	8x, 24h	I.I	I.II
II	lowland	2-legged	30x, 24/48h	II.I	II.II
III	lowland	spawn	30x, 48h	-	III

Development was monitored by documenting the number of animals that had entered the 4-legged stage. As a rule experiments were carried out by different researchers in parallel. All experiments

were performed blind. Each of the laboratories had its own independent authority responsible for the blinding procedure. The same blinding method was used in each centre. 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 their glass vials to avoid any extraneous influences 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 the 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 all animals had reached the 4-legged stage: frequencies (2-legged test animals / 2-legged control animals / 4-legged test animals / 4-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 a moderate dilution, prepared according to instructions derived from homeopathy (thyroxine 8x) on metamorphosis in lowland *Rana temporaria* (34). For experiments of type I.I inert, i.e. unpretreated lowland animals were used and for those of type I.II lowland animals which had been hyperstimulated with thyroxine 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 experiment of type I.I was carried out by Conrad Heckmann, Tubingen 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 at which the hindlegs of the 2-legged tadpoles are straddled such that one can only just see through the triangle formed by thigh, shank, and tail (see (42), figure 1, left). This point of development occurs during Gosner's stage 31 (36). The tadpoles were observed until their forelegs, which are preformed under the skin, broke through and the animals had thus entered the 4-legged stage (see (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 the County of Styria. Some counting results were also documented photographically.

Basins contained 6 l of water each (see (42), figure 2). Water samples from type I experiments were analyzed by the Institute of Hygienics and the Institute of Endocrinology of Graz University prior to the experiment. Neither pollutants such as heavy metals or chlorine nor iodine were found.

20 animals were allotted to each of a total of 60 white plastic basins according to a random procedure. This was done in the same way in each of the centres: 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 the animals were distributed homogenously in terms of their level of activity and swimming behaviour in the main tub. The experimental design was the same at each centre, involving a total of 20 basins with five basins for each of four different treatment groups (two inert groups for the experiment of type I.I and two hyperstimulated groups for that of type I.II, see below). The basins were arranged in five rows of four, each row containing two basins from each treatment group. The spatial arrangement of groups within rows rotated from row to the next, i.e. basins with identical treatment groups were arranged in diagonals, and was left unchanged throughout the experiment to avoid the danger of cross-contamination through splashing. Indirect natural light was used, temperature was 20°C +/- 1°C. The tadpoles were fed with blanched greens (lettuce) *ad libitum*.

Preparation and administration of test solutions

A stock solution of tetra-iodo-thyronine sodium pentahydrate (T_4, Sigma) in 40% ethanol (vol/vol) was prepared (1.1×10^{-4} mol/l). For preparation of the test dilution thyroxine 8x, this stock solution (1.1×10^{-4} mol/l) was further diluted with pure double distilled water in 4 steps of 1:10 at ambient temperature, and agitated after each step of dilution according to standardized instructions derived from homeopathy (37). Using disposable pipettes, 1 ml of the precedent dilution was added to 9 ml of water in a 20 ml vial. Then the vial was banged 30 times against a rubber impediment at intervals of approximately 0.5 sec to create mechanical shocks. For preparation of control, 40% ethanol (vol/vol) was analogously further diluted with pure double distilled water in 4 steps of 1:10 and agitated after each step of dilution (water 8x). Probes prepared with the same method were checked for T_4 concentration by chemo luminescence prior to the experiment. Final thyroxine concentration of the dilution thyroxine 8x in the basin water was 1.1×10^{-13} mol/l after the first application.

Two groups of animals were exposed to the 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 one can infer from the preparation protocol, the pretreatment (control versus hyperstimulation, groups ".I" and ".II", see below) consisted in immersing the animals in a thyroxine or control solution

containing 4 ppbv of ethanol. After the first application of thyroxine or control solution in the actual treatment phase all four groups were immersed in a thyroxine or control solution containing 40 ppqv of ethanol. In this way it was ensured that any differences in the speed of metamorphosis within either the two pretreated or the two unpretreated groups would not be attributable to ethanol.

3 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 intervals of 24 hours.

Data base

Animals were blindly treated 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). In each centre 5 basins (i.e. 100 animals) were used for each of the four treatment groups. A total of 1200 animals were involved.

There was no loss of animals in the 2-legged stage. On the few occasions when an animal died in the 4-legged stage it was counted as 4-legged and removed from the basin.

Comparison and evaluation of data

Comparison and evaluation of data has in detail been described in (42). Measurement times are defined on the basis of values yielded by both the thyroxine 30x and the water 30x groups in order 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 onto 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 the test- and the control-group.

The main evaluation was performed by 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). Furthermore, aggregate values obtained for treatment with thyroxine 8x versus water 8x both for the inert and the hyperstimulated groups were analyzed by logistic regression analysis. In addition, Cox's proportional hazards model was applied. This method considers the time required to reach the 4-legged stage and takes account of each data set with all reference points (days 1-10) (34). In both tests the pooled data were assessed by determining the P values over the accumulated raw data at the level of basins as well as the p-values at the level of individual experiments. 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 experiments of type II.I inert, i.e. unpretreated lowland animals were used and for those of type II.II lowland animals which had been hyperstimulated with thyroxine (1.1×10^{-8} mol/l). Temperature was

21°C +/– 1°C. Experiments were performed by 1 researcher (Scherer). For further details on methods, see explanations on study type I above.

Preparation and administration of test solutions

For preparation of the test dilution thyroxine 30x, the stock solution (1.1×10^{-4} mol/l) (see studies of type I above) was further diluted with pure double distilled water in 26 steps of 1:10 and agitated after each step of dilution according to a standardized protocol. Analogously, 40% ethanol (vol/vol) was further diluted with pure double distilled water in 26 steps of 1:10 (water 30x). Probe dilutions were added at intervals of 24h or 48h, 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, as 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 if an 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. Their development was monitored by documenting the number of animals that had entered the 4-legged stage. Temperature was 21+/–1°C. The experiment was performed by 1 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 the experiments of type I.I (with *not* hyperstimulated animals) performed by Scherer and Zausner, the number of animals that reached the 4-legged stage at defined measuring points was slightly smaller in the group treated with thyroxine 8x than in the group treated with water 8x. In the laboratory of Lassnig, no difference was found between the groups. Heckmann found slightly higher values in the thyroxine 8x-group than in the control group (35). The overall difference at the 70% measuring point was not statistically significant ($p > 0.05$). Use of other statistical methods used led to similar results (for details, see (34). 1 SD was +/– 6% both in the test and the control group and the effect size was 0.3 (small).

In the experiments of type I.II (with hyperstimulated animals), the number of animals that reached the 4-legged stage was smaller in the hyperstimulated thyroxine 8x-group than in the hyperstimulated water 8x-group. The inhibiting effect at the 90% measuring point was statistically significant in the laboratory of Scherer ($p < 0.05$) and of Zausner ($p < 0.01$), but not of Lassnig ($p > 0.05$). When the data were pooled, the effect was significant ($p < 0.01$). 1 SD was about +/– 14% both in the test and the control group and the effect size was 0.82 (large) (Figure 1).

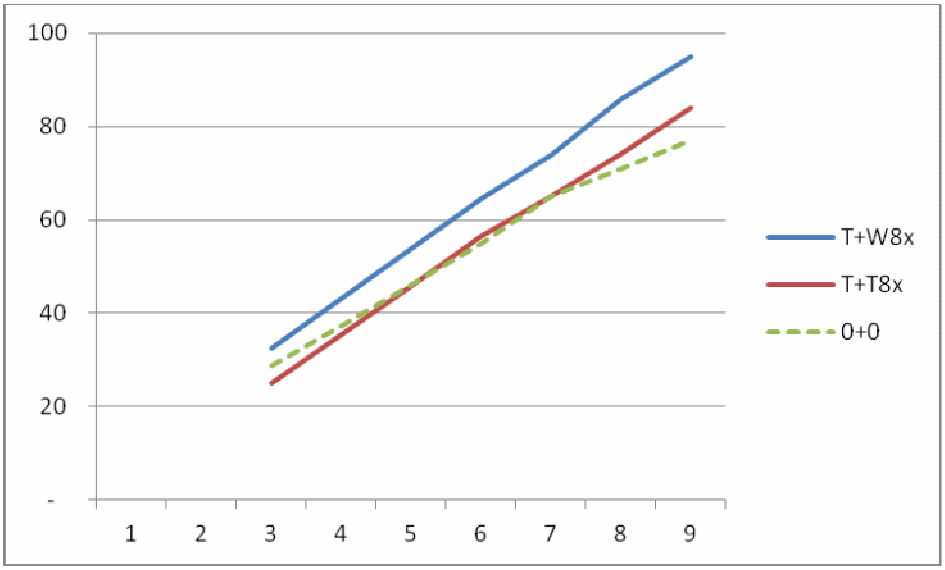

Figure 1. The influence of moderately diluted agitated thyroxine, tested versus analogously prepared water, on hyperstimulated lowland amphibians. Pooled results from three researchers. Ordinate = cumulative frequency of 4-legged tadpoles in % (= N). Abscissa = points in time. Blue dotted line: frequency of animals treated with water 8x; red dotted line: with thyroxine 8x; Blue line: frequency of hyperstimulated animals treated with water 8x; red line: of hyperstimulated animals treated with thyroxine 8x, green dotted line: of not hyperstimulated not treated animals. For explanation, see text. Data combined and recalculated from (39).

Table 2 is attached (xls)

Table 2: Details on the sub-experiments on the influence of moderately diluted 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.

Study type II, lowland animals and thyroxine 30x

In experiments of types II.I and II.II, there were no statistically significant differences between the test and the control group ($p > 0.05$).

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

It was found that animals treated with the test solution metamorphosed more slowly than the control animals, i.e. the effect of thyroxine 30x was (again, as in the previous studies) opposed to the usual effect of thyroxine. The number of test animals that reached the 4-legged stage at defined points in

time was smaller in the group treated with thyroxine 30x at some, but not at all points in time, compared to water 30x (Figure 2). At the 90%-measuring point, this difference was significant ($p < 0.01$).

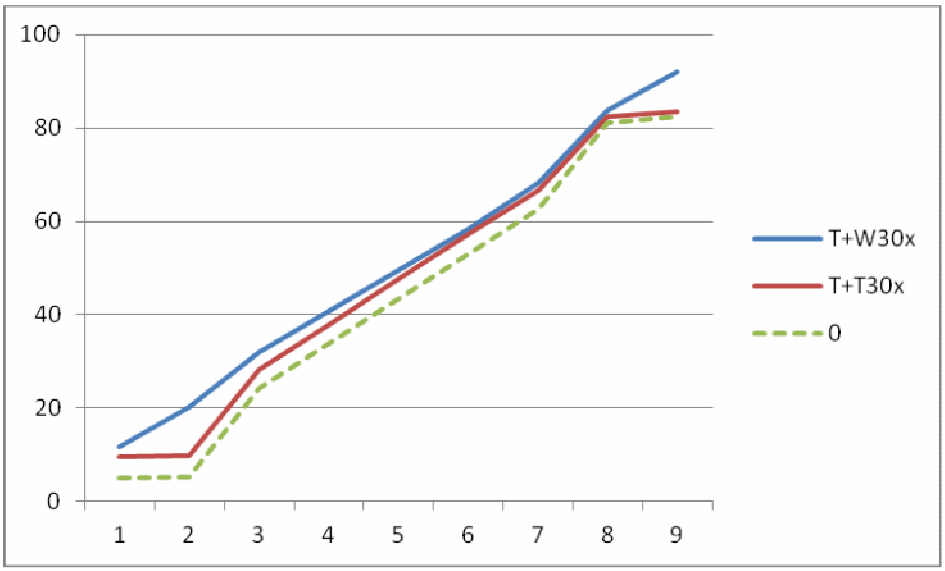

Figure 2. The influence of extremely diluted agitated thyroxine, added from the spawn stage on, on hyperstimulated lowland amphibians. N = 500 per group. Green dotted line: non-hyperstimulated animals treated with inert water. For further explanation, see legend to Figure 1 and text. Data recalculated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

Table 3 is attached (xls)

Table 3. Details on the experiment on the influence of extremely diluted agitated thyroxine, added from the spawn stage on, on hyperstimulated lowland amphibians. For explanation, see table 2.

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 agitated ("potentized") form. The initial choice of the amphibian model was motivated by the fact that during metamorphosis a rapid increase in thyroxine blood level occurs in the animals. We believe this to justify the notion of an "exceptional" (albeit not intoxicated) state (studies of type ".I"). In studies of type ".II", animals are artificially hyperstimulated with thyroxine (i.e. "intoxicated") before an attempt at "cure" is made by applying thyroxine in potentized form.

It was found that inert lowland amphibians do not visibly react to thyroxine 8x, but that thyroxine 8x can slow down metamorphosis in lowland amphibians when these have been pretreated (hyperstimulated) with thyroxine. In other words, pretreatment with thyroxine can enhance a reverse or "curative" effect of thyroxine 8x. Furthermore, it was found that amphibians from lowland biotopes do not visibly react to the high dilution thyroxine 30x. In contrast, thyroxine 30x does slow down metamorphosis in inert highland amphibians (42). This was observed by 5 researchers in most of altogether 20 experiments, and it seems to be the most reliable bio-assay found in research in amphibians and diluted agitated thyroxine so far. However, pretreatment (hyperstimulation) of such highland animals with thyroxine does not lead to a more marked effect of thyroxine 30x; rather the effect was smaller than when no pretreatment was applied (42).

In a pilot study it was found that thyroxine 30x can slow down metamorphosis in lowland amphibians that have been hyperstimulated with thyroxine from the spawn stage on. In other words, in such a study, 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 agitated thyroxine. This in turn seems to depend on whether the animals are 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 instructions derived from homeopathy and a naturally or artificially elevated thyroxine level in the animals during metamorphosis. It is reasonable to suppose that highland larvae of *Rana temporaria* have become adapted to an environment which necessitates a comparatively high thyroxine level or high sensitivity to thyroxine. This would be a plausible explanation for their consistent response in experiments with extremely diluted thyroxine.

The present results give rise to the idea that administering diluted agitated thyroxine to amphibian larvae during their thyroxine-controlled metamorphosis is in a certain sense analogous to the intoxication-detoxification concept used in other models of homeopathy research, although in our experimental model the intoxication dose and its effect on responsiveness do not seem to be correlated in a linear way.

Results of independent researchers that back some of our findings (45,46,47) were described in (42, see discussion section). Guedes *et al.* investigated the histological changes during tail absorption and found more apoptosis (programmed cell death) in the test group (46). Challenging work has already been performed in respect to modulation of signal proteins by dilutions prepared according to instructions derived from homeopathy (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 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 the liquid water. Biophysical theories have evolved which support the possibility of such findings (49). Physics research has revealed that water dipoles may develop phase coherent oscillations through radiation coupling (50). It has been proposed that these could be modulated as a time-ordered pattern of signals and form "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 in future be inspired by de Broglie's concept of the wave nature of particles and the particle nature of waves (52,53).

Research along the line of biophysics may be stimulated by the finding that diluted agitated substances, sealed in glass vials, may influence physiological processes (54). Using 2-legged *Rana temporaria*, in some but not all cases researchers found 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).

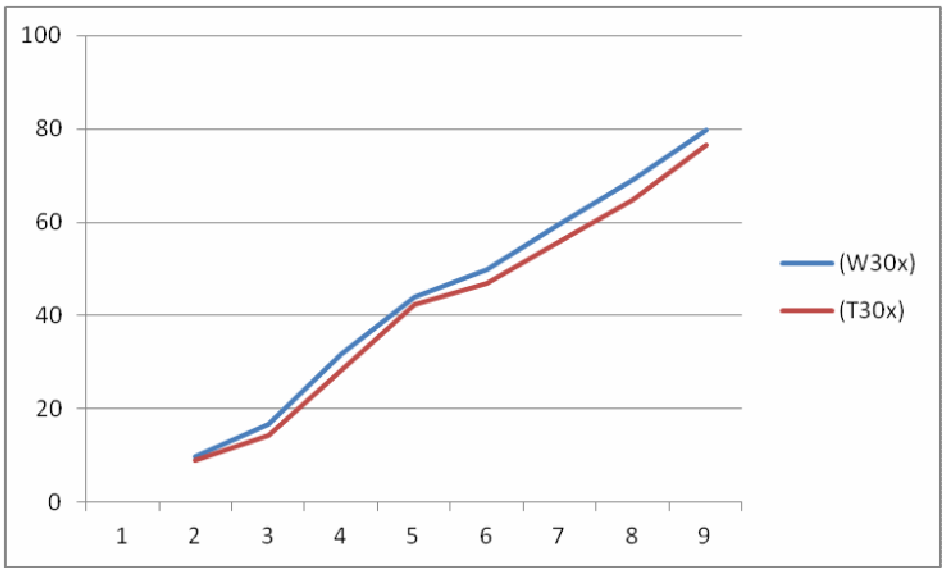

Figure 3. The influence of extremely diluted agitated thyroxine sealed in glass vials. N = 1710 per group. For further explanation, see legend to Figure 1 and text. Data recalculated from (55).

A total of 7 sets of experiments were performed. The number of animals that reached the 4-legged stage was smaller in the test group than in the control group. An inhibiting effect at the 70% measuring point was statistically significant when all the data were pooled ($p < 0.01$); for the experiments treated separately, it was significant only in the laboratory of Scherer-Pongratz ($p < 0.01$), while it was visible as a trend ($p > 0.05$) in the laboratories of Endler, Vinattieri (Turin), Hilgers (Vienna), and there was no difference between groups ($p > 0.05$) at Dieterle's main experiment (Graz). However, when in a small experiment Dieterle used quarz glass vials instead of soft soda glass, there was less metamorphosis speed in the thyroxine 30x- than in the control group (figure 4).

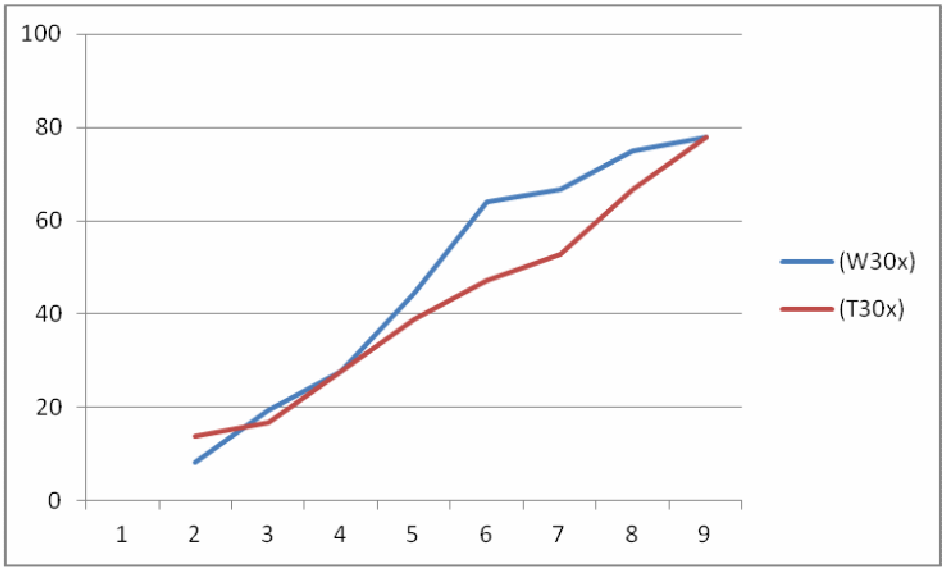

Figure 4. Result of a pilot experiment using thyroxine 30x sealed in quarz glass vials. N = 36 per group. For further explanation, see legend to figure 1 and text. Data recalculated from (55).

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

ACKNOWLEDGEMENTS

Thanks are due to all who performed experiments between 1990 and 2010. The paper presented here is part of a doctoral thesis.

REFERENCES

1. S Baumgartner: Reproductions and reproducibility in homeopathy: Dogma or Tool? *J Altern Complement Med* 11, 771-772 (2005)

2. S Baumgartner: The state of basic research on homeopathy. In: New directions in homeopathy research - Advice from an interdisciplinary conference. Eds: H Albrecht, C Witt, *KVC-Verlag*, Essen, Germany, 107–130 (2009)

3. H Albrecht, R van Wijk, S Dittloff: A new database on basic research in homeopathy. *Homeopathy* 91, 162-165 (2002)

4. P C Endler: Fundamental research into high dilution effects. A classification of non-clinical research topics. In: Fundamental Research in Ultra High Dilution and Homoeopathy. Eds: J Schulte, P C Endler, *Kluwer Academic Publishers*, Dordrecht, Netherlands (1998)

5. C Goeldner: Review und Evaluierung von neuen, mit homoeopathischen Zubereitungen durchgefuehrten toxikologischen Experimenten. Thesis, University of Graz (2006).

6. K Linde, W B Jonas, D Melchart, F Worku, H Wagner and F Eitel: Critical review and meta-analysis of serial agitated dilutions in experimental toxicology. *Hum Exp Toxicol* 13, 481-482 (1994)

7. A J Vickers: Independent replication of pre-clinical research in homoeopathy: a systematic review. *Res Compl Med / Forsch Komplementaermed* 6, 311-320 (1999)

8. H Walach, W B Jones, J Ives, R V Wijk, O Weingartner: Research on homeopathy: state of the art. *J Altern Complement Med* 11, 813-29 (2005)

9. C M Witt, M Bluth, H Albrecht, T E Weisshuhn, S Baumgartner, SN Willich: The *in vitro* evidence for an effect of high homeopathic potencies – a system review of the literature. *Complement Ther Med* 15, 128-38 (2007)

10. P C Endler, K Thieves, R Reich, P F Matthiessen, L Bonamin, C Scherr, S Baumgartner: Repetitions of fundamental research models for homeopathically prepared dilutions beyond 10e-23: a bibliometric study. *Homeopathy* 99, 25-36 (2010)

11. T Jäger T, C Scherr, M Simon, P Heusser, S Baumgartner. Effects of Homeopathic Arsenicum Album, Nosode and Gibberellic Acid Preparations on the Growth Rate of Arsenic-Impaired Duckweed (Lemna gibba L.). The Scientific World Journal 10, 2112 – 2129 (2010)

12. PC Endler, W Matzer, C Reich, T Reischl, AM Hartmann, K Thieves, A Pfleger, J Hofäcker, H Lothaller, W Scherer-Pongratz. Seasonal variation of the effect of extremely diluted agitated gibberellic acid (10e-30) on wheat stalk growth – a multi researcher study. The Scientific World Journal 11, 1667-1678 (2011)

13. C Roth: Literature review and critical analysis on the topic of “In- and detoxication experiments in homeopathy”. *Berlin Res J Hom* 1, 111-117 (1991)

14. Koenig K: Ueber die Wirkung extrem verduennter (“homoeopathischer”) Metallsalzloesungen auf Entwicklung und Wachstum von Kaulquappen. *Zschft Ges exp Med* 56, 881-893 (1927)

15. H Giersberg: Hormone. Eds: *Springer*, Berlin, Germany (1972)

16. R Wehner, G Gehring: Zoologie. Eds: *Thieme*, Stuttgart, Germany (1990)

17. R Pitt-Rivers, W R Trotter: The Thyroid Gland. Eds: *Butterworth*, London, UK (1964)

18. J J Kollros: Mechanisms of amphibian metamorphosis: Hormones. *Amer Zool* 8, 107-114 (1961)

19. H Moser: Ein Beitrag zur Analyse der Thyroxinwirkung im Kaulquappenversuch und zur Frage des Zustandekommens der Fruehbereitschaft des Metamorphose-Reaktionssystems. *Rev Suisse Zool* 57, 1-144 (1950)

20. J R Tata: Early metamorphic competence of *Xenopus* larvae. *Develop Biol* 18, 415-441 (1968)

21. K V Prahlad, L E DeLanney: A study of induced metamorphosis in the Axolotl. *J Exp Zool* 160, 137-146 (1965)

22. H I Chou, J J Kollros: Stage modified responses to thyroid hormones in Anurans. *Gen Comp Ednocrinol* 22, 255-260 (1974)

23. M H J Dodd, J M Dodd: The Biology of Metamorphosis. In: Physiology of Amphibia, vol. 3. Eds: *Academic Press*, London, UK, 467-599 (1976)

24. V A Galton, J K S Cohen: Action of thyroid hormones in premetamorphic tadpoles: an important role for thyroxine? *Endocrinology* 107,1820-1826 (1980)

25. M R Weil: Changes in plasma thyroxine levels during and after spontaneous metamorphosis in a natural population of the green frog, *Rana clamitans*. *Gen Comp Endocrinol* 62, 8-12 (1986)

26. G Zelzer: *In vitro* Untersuchungen zur intestinalen Phenylalaninresorpion vor und waehrend der Metamorphose bei Kaulquappen von *Bufo viridis*. Thesis, Graz University (1990)

27. L Saxen: The onset of thyroid activity in relation to the cytodifferentiation of the anterior pituitary. *Acta Anat* 32, 87-100 (1958)

28. N W Kaye: Interrelationships of the thyroid and the pituitary in embryonic and premetamorphic stages of the frog *Rana pipiens*. *Gen Comp Endocrinol* 1, 1-19 (1961)

29. J M Dodd: Studies on amphibian metamorphosis using I^{131}. *J Physiolog (London)* 130, 11 (1955)

30. Y Hanaoka: Uptake of I^{131} by the thyroid gland during metamorphosis in *Xenopus laevis*. *J Fac Sci Hokkaido Univ* 16, 106-112 (1966)

31. P Neuenschwander: Ultrastruktur und Jodaufnahme der Schilddruese bei Larven des Krallenfrosches (*Xenopus laevis* daud.) *Z Zellforsch Mikrosk Anat* 130, 553-574 (1972)

32. H Lipner, S Hazen: Extra thyroidal iodine pump in tadpoles (*Rana grylio*). *Science* 138, 898-899 (1962)

33. P C Endler: Homeopathy research – an expedition report. An old healing system gains plausibility. Eds: edition@inter-uni.net, Graz, Austria (2003)

34. P C Endler, R Luedtke, C Heckmann, C Zausner, H Lassnig, W Scherer-Pongratz, M Haidvogl, M Frass: Pretreatment with thyroxine (10e-8 parts by weight) enhances a "curative" effect of homeopathically prepared thyroxine (10e-13) on lowland frogs. *Res Compl Med / Forsch Komplementaermed* 10, 137-142 (2003)

35. C Heckmann: Einfluss homoeopathisch und nicht homoeopathisch hergestellter Thyroxinloesungen auf die Metamorphosegeschwindigkeit beim Grasfrosch (*Rana temporaria* L.). Thesis, Tubingen University (1997)

36. K L Gosner: A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16, 183-190 (1960)

37. Homoeopathisches Arzneibuch, 1. Ausgabe. Deutscher Apothekerverlag, Eds: *Stuttgart and Govi Verlag*, Frankfurt, Germany (1978)

38. P C Endler, W Pongratz, R Van Wijk, G Kastberger, M Haidvogl: Effects of highly diluted succussed thyroxine on metamorphosis of highland frogs. *Berlin J Research Hom* 1, 151-160 (1991)

39. C Zausner, H Lassnig, P C Endler, W Scherer, M Haidvogl, M Frass, G Kastberger, R Luedtke: Die Wirkung von "homoeopathisch" zubereitetem Thyroxin auf die Metamorphose von Hochlandamphibien. Ergebnisse einer multizentrischen Kontrollstudie. *Perfusion* 17, 268-276 (2002)

40. P C Endler, J Schulte: Ultra High Dilution: Physiology and Physics. Eds: *Kluwer Academic Publishers*, Dordrecht, Netherlands (1994)

41. J Schulte, P C Endler: Fundamental Research in Ultra High Dilution and Homoeopathy. Eds: *Kluwer Academic Publishers*, Dordrecht, Netherlands (1998)

42. G Lingg, P C Endler: Recalculation of data from 1990 to 2010 on the effects of highly diluted thyroxine on the metamorphosis of highland amphibians. Accepted by *Int J High Dilution Res* (2011)

43. S U Welles, P C Endler, W Scherer-Pongratz, E Suanjak-Traidl, S Weber, M Frass, H Spranger, H Lothaller: Pretreatment with thyroxine (10e-8) and the effect of homeopathically prepared thyroxine ($10e^{-30}$) on highland frogs - a multi-researcher study. *Res Compl Med / Forsch Komplementaermed* 14, 353-357 (2007)

44. H Graunke, P C Endler, W Scherer-Pongratz, M Frass, H Lothaller: Treatment of lowland frogs from the spawn stage on with homeopathically prepared thyroxine (10e-30). *The Scientific World Journa*l 7, 1697-1702 (2007)

45. J R P Guedes, C M Ferreira, H M B Guimaraes, P H N Saldiva, V L Capelozzi: Homeopathically prepared dilution of *Rana catesbeiana* thyroid glands modifies its rate of metamorphosis. *Homeopathy* 93, 132-137 (2004)

46. J R P Guedes: Ultradiluicao homeopatica de triiodotironina altera a apoptose cellular da cauda de girinos de *Rana catesbeiana*: *in vitro*. Doctoral thesis, Sao Paulo University (2009)

48. A R Khuda-Bukhsh, S S Bhattacharyya, S Paul, S Dutta, N Boujedaini, P Belon: Modulation of signal proteins: a plausible mechanism to explain how a potentized drug Secale Cor 30c diluted beyond Avogadro's Limit combats skin papilloma in mice. *Evid Based Complement Alternat Med*, 2011 (2009)

47. Interuniversity College: Pilot trial aimed at an independent replication of a study on the possibility of influencing the speed of metamorphosis of amphibian larvae by means of homeopathically diluted thyroxin (10^{-30}), by G. Bach, KIKOM Bern University. Report to the Government of the State of Styria. Pilotversuch zur unabhaengigen Wiederholung einer Studie zur Beeinflussung der Metamorphosegeschwindigkeit von Amphibienlarven durch homoeopathisch verduenntes Thyroxin (10^{-30}) durch G. Bach, KIKOM Universitaet Bern. Bericht an das Amt der Steiermaerkischen Landesregierung, Graz, Austria (2011)

49. M Enserink. Newsmaker Interview: Luc Montagnier Science **330**, 1732 (2010)

50. E Del Giudice, G Preparata: Coherent electrodynamics in water. Eds: J Schulte, P C Endler, 89-104 (1998)

51. B Marschollek, M Nelle, M Wolf, S Baumgartner, P Heusser, U Wolf. Effects of Exposure to Physical Factors on Homeopathic Preparations as Determined by Ultraviolet Light Spectroscopy. The Scientific World Journal 10, 49 – 61 (2010).

52. A Kavarainen: Mesoscopic theory of matter and interaction with light. Principles of self organisation in ice, water and biosystems. Eds: F N Turku, *Turun Yliopiston Offsetpaino*, Turku, Finland (1992).

53. J Schulte: Bio-Information between quantum and continuum physics. Eds: J Schulte, P C Endler, 45-68 (1998)

54. R van Wijk, F A C Wiegant. Homeopathic remedies and pressure-induced changes in the galvanic resistance of the skin. Alkmaar: VSM (1988)

55. P C Endler, C Heckmann, E Lauppert, W Pongratz, J Alex, D Dieterle, C Lukitsch, C Vinattieri, C W Smith, F Senekowitsch, H Moeller, J Schulte: The metamorphosis of amphibians and information of thyroxine. In (41)

56. P C Endler, W Pongratz, G Kastberger , F A C Wiegant, J Schulte: The effect of highly diluted agitated thyroxine on the climbing activity of frogs. *J Vet Hum Tox* 36, 56-59 (1994)

57. P C Endler, W Pongratz, C W Smith, J Schulte: Non-molecular information transfer from thyroxine to frogs in respect to 'homoeopathic' toxicology. *Vet Hum Tox* 37, 259-260 (1995)