EVIDENCE OF TEMPERATURE-DEPENDENT EFFECTS ON THE ESTROGENIC RESPONSE OF FISH: IMPLICATIONS WITH REGARD TO CLIMATE CHANGE

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ABSTRACT

Chemical risk assessment is fraught with difficulty due to the problem of accounting for the effects of mixtures. In addition to the uncertainty arising from chemical-to-chemical interactions, it is possible that environmental variables, such as temperature, influence the biological response to chemical challenge, acting as confounding factors in the analysis of mixture effects. Here, we investigate the effects of temperature on the response of fish to a defined mixture of estrogenic chemicals. It was anticipated that the response to the mixture may be exacerbated at higher temperatures, due to an increase in the rate of physiological processing. This is a pertinent issue in view of global climate change. Fathead minnows (Pimephales promelas) were exposed to the mixture in parallel exposure studies, which were carried out at different temperatures (20 and 30°C). The estrogenic response was characterised using an established assay, involving the analysis of the egg yolk protein, vitellogenin (VTG). Patterns of VTG gene expression were also analysed using real time QPCR. The results revealed that there was no effect of temperature on the magnitude of the VTG response after two weeks of chemical exposure. However, the analysis of mixture effects at two additional time-points (24 hr and 7 d) revealed that the response was induced more rapidly at the higher temperature. This trend was apparent from the analysis of effects both at the molecular and biochemical level. Whilst this indicates that climatic effects on water temperature are not a significant issue with regard to the long-term risk assessment of estrogenic chemicals, the relevance of short-term effects is, as yet, unclear. Furthermore, analysis of the patterns of VTG gene expression versus protein induction give an insight into the physiological mechanisms responsible for temperature-dependent effects on the reproductive phenology of species such as roach. Hence, the data contribute to our understanding of the implications of global climate change for wild fish populations.

1. INTRODUCTION

In recent years, the legislation concerning the production and release of chemicals has tightened considerably, leading to significant improvements in environmental quality. However, in spite of these efforts, there is evidence to suggest that wildlife and human health may be adversely affected by exposure to chemicals, even at low and environmentally relevant concentrations (e.g. Jobling and Tyler, 2006; Koppe et al., 2006). This has prompted concerns that the science on which chemical regulations and policy decisions are currently based is not sound (Munns, 2006). Existing procedures for assessing environmental risk assign a major role to standard toxicity tests, in which the sensitivity of a particular species to an individual substance is determined under otherwise constant and favourable conditions in the laboratory (Heugens et al., 2001). This approach has the capacity to underestimate risks that exist in the real world, where exposures are to mixtures of chemicals under variable exposure regimes.

Increasing recognition of the risk of interactive effects has prompted considerable research into the mixtures issue. For example, the European Commission recently funded an investigation into the combined effects of estrogenic chemicals, which are ubiquitous in the environment. This revealed that these similarly acting chemicals have the capacity to act together in an additive manner to affect fish physiology and demonstrated that there is a risk of combined effects, even when each component is present at a low, individually ineffective concentration (Brian et al., 2005). Further research has demonstrated the potential for combined effects on various reproductive endpoints, highlighting how the current emphasis on single chemicals may overlook risks at the population level (Brian et al., 2007). This has significant implications for risk assessments, which consider the hazard posed by each chemical independently.

Currently, procedures for assessing the risk that chemicals pose in the environment incorporate a safety or uncertainty factor (US EPA, 2004) and, in general, it is assumed that a ten-fold margin is sufficient to protect against combined effects resulting from multiple exposures. However, growing evidence that even relatively low numbers of chemicals can act together in the low concentration range to elicit significant effects undermines the traditional risk assessment paradigm that there is a threshold level below which a chemical is not considered to pose a threat (the NOEC; no observed effect concentration). Hence, even when an uncertainty factor is applied, there can still be a risk of significant mixture effects.

Growing realisation of this issue has fuelled concerns that risk assessment procedures may further underestimate risk by failing to consider how the toxicological response to chemical challenge may be influenced by the conditions of exposure. Standard toxicity tests fail to consider that environmental exposures occur under variable and suboptimal regimes. Hence, the confounding effects of a wide range of physico-chemical factors, which vary over spatial and temporal scales, may be overlooked when extrapolating from the laboratory to predict risks that exist in the real world (Vignati et al., 2007). The relevance of confounding factors in the risk assessment of chemicals is an issue that has, as yet, received little attention, although there is some evidence that parameters such as temperature and salinity can influence toxicity (Heugens et al., 2001). Hence, the interactive effects of environmental variables, as well as chemical mixtures, warrant further attention in risk assessment methodology.

The influence of confounding factors in the risk assessment of chemicals is extremely pertinent in view of climate change. This phenomenon will create multiple stress exposure situations, in which organisms may respond in an unpredictable manner to

chemical challenge. In particular, there is evidence to suggest that the projected rise in average temperatures may increase chemical toxicity. For example, a review by Cairns et al. (1975) revealed that, in general, aquatic organisms are more susceptible to metal and pesticide toxicity at higher temperatures. This interaction is likely to occur as a result of temperature-related effects on the physiological processes that determine the rates of chemical uptake, elimination and detoxification (Heugens et al., 2003). However, although there would appear to be a positive relationship between temperature and acute toxicity in terms of lethal concentrations and survival times, less is known about the influence of temperature on sub-lethal endpoints. This is more relevant in the real world, in which organisms are more commonly exposed to mixtures of chemicals at concentrations that are not associated with overt toxicity.

The aim of this study was to investigate the influence of temperature on the estrogenic response of fish to a defined mixture of chemicals. The effects of this mixture have been characterised under standard test conditions, both in terms of the induction of the egg yolk precursor protein, vitellogenin (VTG), and its impact on reproduction (Brian et al., 2005; 2007). The influence of temperature on the VTG response at the physiological and molecular level was investigated under two different thermal regimes; one above and one below the standard test temperature. Previous research on salmonid fish that were injected with natural steroid estrogen indicates that an increase in temperature will be associated with increased potency (Korsgaard et al., 1986; Mackay and Lazier, 1992). However, waterborne exposure to mixtures of chemicals that are both anthropogenic and natural in origin, might not elicit the same temperature-dependent response. The results will reveal whether temperature is a confounding factor in the risk assessment of estrogenic chemicals, and give an insight into the potential implications of climate change with regard to ecotoxicology.

2. MATERIALS AND METHODS

2.1 Experimental Design

The design of this investigation was based on a previous study by Brian et al. (2005) that aimed to characterise the response of fish to a defined mixture of estrogenic chemicals in terms of the induction of plasma VTG. The mixture comprised of the endogenous steroidal estrogen, 17β-estradiol (E2) and the synthetic steroidal estrogen, 17α–ethinylestradiol (EE2), as well as three environmentally relevant chemicals that have the capacity to mimic the actions of estrogen, namely 4-tert-nonylphenol (NP), 4-tert-octylphenol (OP) and bisphenol-A (BPA). The chemicals were combined at a fixed ratio that was based on their potency with regard to the induction of VTG (Brian et al. 2005).

A master stock of the mixture, containing each component at its EC50 concentration, was prepared in a carrier solvent (dimethylformamide; DMF). This master stock of 0.9ng/l EE2, 25ng/l E2, 7µg/lNP, 45µg/l OP and 150µg/l BPA was then diluted to produce five further stocks that were 0.5, 0.3, 0.2, 0.1 and 0.05 of the original concentration. This dilution series was sufficient to cover the full extent of the concentration response curve (Brian et al., 2005). Negative and positive control (NC and PC) tanks were run alongside those containing the mixture. The NC and PC were dosed with DMF at the same rate as those dosed with the mixtures. The PC was also dosed with EE2 to produce a tank water concentration of 10ng/l, which produces a maximal response in terms of the induction of VTG (Panter et al., 2002).

Each of the stock solutions were diluted 1:15000 with de-chlorinated tap water before entering the experimental tanks. The set-up of this flow-through exposure system is

described in Brian et al. (2005). Dosing commenced one week before the start of each exposure study. This conditioning process ensured that the chemical concentrations in the tanks were accurate. The exposure concentrations were verified by performing analytical chemistry on water samples collected immediately prior to the addition of the fish. A further set of water samples were collected on the final day of exposure. The analytical methods are described in Brian et al. (2005).

2.2 Protocol

One week prior to exposure, whilst the experimental tanks were being conditioned, male fathead minnows were selected from a stock of mixed-sex adult fish that had been maintained at a constant temperature of 25±1°C. These fish were transferred into holding tanks where they were equilibrated to either 20 or 30°C by altering the temperature of the influent water by 1°C per day until the target temperature was achieved. The temperature of the holding tanks was then kept constant until the end of the week, when the fish were randomly allocated to experimental tanks maintained at the same temperature.

During the equilibration period and the experiment, the fish were fed twice daily: once with frozen brine shrimp and once with flaked fish food. The photoperiod was maintained on a 16hr light/8hr dark cycle with 20 minute dawn and dusk transition periods. The water temperature in the fish tanks was recorded daily using an Oxi 315i digital meter and Cell Ox 325 probe (WTW; Weilheim, Germany) to ensure that it remained within 1°C of the target temperature. In addition, dissolved oxygen levels and various water quality measurements were recorded routinely and the dosing rate was monitored throughout the experiment.

In the first experiment, temperature-related effects were explored by comparing the VTG levels in the plasma of fish exposed to the mixture of estrogenic chemicals at 20 and 30°C for a period of two weeks. The response at each of these temperatures was also related to that observed in a parallel exposure, conducted at 25°C, as well as that reported by Brian et al. (2005) in a previous experiment. A subsequent experiment was also carried out to investigate whether temperature influenced the response after 24 hours and seven days. In these more short-term studies, the expression of the VTG gene in liver tissue was analysed alongside the induction of VTG protein. These two closely related endpoints were analysed together to gain an insight into the molecular basis for temperature-related effects on the VTG response.

2.3 Sampling and Analysis

At the end of the experiment, the fish were sacrificed by overdose with anaesthetic (MS222; Sigma Aldrich). Six fish were sampled from each tank at each time point (i.e. after two weeks exposure in the first and after 24 hours and seven days in the second experiment, respectively). Their lengths and weights were recorded before blood samples were collected from the caudal peduncle using heparinised capillary tubes. Blood samples were centrifuged at 4000g for 5 minutes and the plasma drawn off and stored at –20ºC for the determination of VTG protein levels. This was carried out using a carp-VTG ELISA previously been validated for the measurement of VTG in fathead minnow (Tyler et al. 1999).

Liver tissues were also collected from fish exposed to the mixture for 24 hours and seven days. These were placed in RNA-free tubes, in which they were snap-frozen and stored at -80 ºC. Total RNA was extracted using TriReagent (Sigma Aldrich). The samples were then treated with DNase1 (Invitrogen). Total RNA concentrations

were then determined by UV spectrophotometry before differential gene expression was performed by real-time QPCR, using an ABI Prism 7900HT sequence detection system (Applied Biosystems) with one step SYBR green master mix (Qiagen). The reactions were set up in triplicate in 96 well plates: each reaction was 25 μl in volume and initially contained 10μg of total RNA.

The primers used to analyse VTG gene expression in this species were designed by Miracle et al. (2006) using sequence information from Korte et al. (2000; GenBank acc. no. AF130354). The sequence of the forward and reverse primers was; 5'-CAC AAT CCC AGC TCT GCG TGA-3' and 5' TGG CCT CTG CAG CAA TAT CAT-3', respectively. Following an initial RT step, during which samples were incubated at 50°C for 30 min, amplification was measured over 40 cycles of 95°C for 20s, 60°C for 20s and 72°C for 10s. The VTG gene expression level in each fish was evaluated with respect to a serial dilution of a sample from a female fish, which was run in all assay plates. This approach is similar to that used by Schmidt et al. (2002), although these authors used an exposed male fish as a reference. Gene expression levels are therefore presented as relative values, with the female being assigned a value of 100 and the responses of the males being presented proportionally. The expression of β-actin was also quantified, with a view to its use as a housekeeper, or internal control, to account for small differences in the amount of starting material between samples. However, subsequent analysis revealed an effect of estrogen treatment, as per Filby and Tyler (2007). Hence, the VTG gene expression data was analysed without the use of a reference gene.

2.4 Statistical Analysis

The chemical concentrations in the fish tanks were analysed statistically to ensure that there were no differences between the exposure levels in each temperature group. The mean measured concentration at the beginning and end of each experiment was calculated for each chemical. This then was converted into a proportional value by dividing by the nominal concentration. Comparisons were then made between tanks with the same nominal exposure levels in each of the temperature groups. This was achieved using paired t-tests in Minitab 13.1 (Minitab Inc. State College, PA, USA).

The VTG protein concentrations were log transformed prior to normalisation, which allowed the data to be plotted on a percentage response scale. The normalisation procedure was carried out by subtracting the mean baseline response from all other values. The baseline was determined by pooling the responses of fish maintained in each of the NC tanks, which did not differ significantly from one another, along with any other groups that did not respond to treatment. The corrected VTG values were then divided by the mean response in the PC tank, which represented the maximum response. This was determined from the 30°C exposure only, as opposed to pooling the data from both PC tanks, as the response was greatest at this temperature. This procedure enabled the response in all other treatment groups could be plotted on a graded effect scale of between zero and a hundred. The percentage VTG response was then plotted against the mixture dilution, on a log scale, which produced typical concentration-response curves, similar to those reported in Brian et al. (2005).

The effect of treatment on VTG protein induction and gene expression was explored by determining the response, at each time-point, under the different thermal regimes. The data were fitted to a sigmoidal dose-response model, with variable slope, using a four parameter logistic equation. The top and bottom of the curve were constrained to

the mean of the responses observed following exposure to the highest and lowest mixture dilutions, respectively. Best-fits were then determined for the median effect concentration (EC_{50}), based on the nominal mixture dilution, under each thermal regime. These values were then compared to assess whether there was any effect of temperature. These analyses were performed using the non-linear regression function of GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The ratio between the levels of VTG protein:gene expression in each treatment group were also calculated, as per Mackay and Lazier (1993). The efficiency with which the molecular signal was translated into a proteomic response at each temperature was then compared using the paired t-tests (Minitab 13.1).

3. RESULTS

3.1. Analytical Chemistry

The analysis of the chemical concentrations in each fish tank revealed that there was good agreement between the nominal and actual exposure levels at both temperatures during each experiment (Figure 1). No significant differences were detected between the actual exposure levels in each temperature group in the first experiment. In the second experiment, however, slightly higher levels of NP and OP were detected at 30ºC than at 20 ºC. In the case of OP, there was a statistically significant difference ($t=-4.91$, $p<0.01$, $n=6$). However, this pattern was not consistent across all chemicals: the concentrations of E2, EE2 and BPA were close to nominal in both temperature groups. As the mixture was delivered to the tanks as a single stock, any "real" discrepancies in the exposure levels should have been apparent for all chemicals. It was therefore concluded that the differences in the levels of the alkylphenols between the two temperature groups probably occurred as a result of an analytical anomaly, as

opposed to a real difference, and that the actual exposure levels were the same across all experiments.

3.2. VTG Protein Induction

The analysis of the levels of VTG protein after two weeks of exposure to the mixture in the first experiment revealed clear and consistent concentration-response curves. There was no evidence of a difference in the response of fish maintained at 20 and 30ºC (Figure 2). The best estimates for the log EC_{50} values, derived from the non-linear regression model, with 95% confidence intervals, were 0.221 (0.176-0.266) and 0.219 (0.176-0.264) at the lower and upper temperature, respectively. The estimates did not differ from those determined in the parallel exposure, which was conducted at 25ºC, and were consistent with previous data documenting the concentration-response to the same mixture (Brian et al., 2005). Hence, there was no evidence of an effect of temperature on the induction of VTG protein in fish exposed to the mixture for a period of two weeks.

In contrast, the results of the second experiment, which compared the response of fish to the mixture at 20 and 30 ºC at two earlier time points, revealed a temperature-dependent effect after 24 hours of exposure (Figure 3). The best fits and confidence intervals for the log EC_{50} values were 0.847 (0.611-1.08) and 0.335 (0.259-0.413) in the 20 and 30ºC groups, respectively. This difference was highly significant ($p<0.0001$). After seven days, however, these EC_{50} values had gone down to 0.369 (0.303-0.436) and 0.325 (0.258-0.393) at 20 and 30ºC, respectively, and the difference between them was no longer statistically significant. This pattern indicates that the proteomic VTG response is initially more sensitive to the effects of temperature, with a 2.6-fold difference in the potency of the mixture being detected after 24 hours.

291 However, temperature-related effects were transient and were detected only during the
292 early stages of exposure. After 7 days, there was no evidence of a difference in the
293 VTG protein levels in fish maintained under each thermal regime.

294 *3.3. VTG Gene Expression*

295 A similar pattern was evident from the analysis of the VTG gene expression data after
296 24 hours (Figure 4). This revealed a clear difference between the response exhibited
297 by the fish at each temperature ($p<0.0001$), with a log EC_{50} value of 1.15 (0.904-1.39)
298 and 0.444 (0.369-0.519) at 20 and 30ºC, respectively. The increase in the potency of
299 the mixture at the higher temperature was of a similar magnitude to that reported for
300 VTG protein. In contrast with the proteomic response, however, there was a reversal
301 in this pattern after 7 days of exposure, by which time the gene expression levels had
302 risen in fish maintained at 20ºC to a greater extend than in those maintained at 30ºC.
303 This meant that there was a small, but statistically significant difference between the
304 best estimates for the log EC_{50} values at each temperature ($p<0.01$). These values
305 were 0.397 (0.323-0.464) and 0.540 (0.449-0.630) at the lower and upper temperature,
306 respectively.

307 *3.2. Gene Expression vs. Protein Induction*

308 Analysis of the ratios between each of the VTG responses (Table 1) revealed that the
309 quantity of VTG protein per unit of gene expression increased from day 1-7. This is
310 consistent with there being a time lag between the molecular response, in terms of an
311 increase in VTG gene transcription, and its translation into VTG protein at a higher
312 organisational level. In general, the ratios also appeared to increase with the exposure
313 concentration, which may reflect differences in the response range for each endpoint:

the proteomic response is exceptional as it can vary over several orders of magnitude. Furthermore, the ratio between the levels of VTG protein:gene expression revealed a significant effect of temperature at both time points ($p<0.001$), reflecting a difference in the efficiency of gene translation and/or post-translation processing under each thermal regime.

4. DISCUSSION

The results of the first experiment were somewhat surprising in that there was no evidence of a temperature-dependent effect on the estrogenic response to the mixture, in terms of the induction of proteomic VTG. This was not consistent with findings from earlier studies on salmonid species. Korsgaard et al. (1986) reported that the VTG response of Altlantic salmon (Salmo salar) injected with E2 at regular intervals over a 10-day period was strongly influenced by temperature. Male post smolts that were acclimated and maintained at 3°C showed little or no VTG response, whereas those maintained at 10 or 15°C during treatment showed a greater accumulation of VTG, both in terms of hepatic RNA and alkali-labile phosphorous levels in plasma, at higher ambient temperatures. The authors suggested that this might be due to the inhibition of VTG gene expression at lower temperatures. Similarly, an investigation into the estrogen responsiveness of juvenile rainbow trout (Oncorhynchus mykiss) revealed that both the rate and the magnitude of the VTG response increased with temperature. Mackay and Lazier (1993) reported that VTG protein could be detected in the serum of fish maintained at 15°C within 24 hours of exposure to E2, compared to 72 hours at 9°C. After ten days, VTG protein response was 10-fold higher in fish exposed at 15°C. A similar pattern was evident from the analysis of gene expression.

337 In view of the published evidence, there are several possible explanations for the
338 absence of temperature-dependent effects in this experiment. Firstly, it is possible
339 that the influence of temperature is chemical specific: both of the previous studies
340 investigated the effects of temperature on the estrogenic response to E2 on its own,
341 whereas our study assessed the effects of a mixture. This was believed to be more
342 representative of a real world exposure situation, as well as increasing the likelihood
343 of detecting an effect of temperature in the event that this was specific to a particular
344 type of chemical. However, this possibility was considered unlikely: although a wide
345 range of structurally diverse chemicals have estrogenic properties, which is reflected
346 in the composition of the mixture, they share a common mechanism (i.e. estrogen
347 receptor binding). Hence, we concluded that any temperature-dependent effects on
348 the VTG response would have been evident from the analysis of fish exposed to the
349 mixture, as well as those exposed to E2 alone.

350 We then considered whether the effects of temperature could be related to the route
351 of chemical exposure (i.e. injection vs. waterborne exposure) or whether the response
352 was likely to be species specific (i.e. salmonid vs. cyprinid fish). Salmon and trout
353 live in coldwater habitats and spawn once during their annual reproductive cycle,
354 whereas fathead minnows have adapted to live at much higher temperatures and have
355 a prolonged breeding season, spawning on a continuous cycle, every few days, for
356 several months of the year. It is therefore possible that they differ in their sensitivity
357 to the effects of temperature due to differences in their reproductive biology.

358 More recently, however, it has been demonstrated that the VTG response of goldfish
359 (Carrasius aurarus) exposed to waterborne E2 is strongly influenced by temperature
360 (Ishibashi et al., 2001), which suggests that neither of the factors outlined above are

likely to be responsible for the absence of a temperature-dependent response in our study. Analysis of the VTG response of goldfish was particularly interesting in that it revealed that the effects of temperature were more pronounced during the early stages of exposure: after 24 hours, the levels of VTG protein were 10 000 times higher in fish maintained at 30°C than at 10°C, whereas after five and ten days, the response differed by a factor of 100 and 10, respectively. This response pattern, which was not reported in the earlier studies, provides a potential explanation for the apparent lack of temperature-dependent effects in the present study.

Here, the effects of temperature on the VTG response of fathead minnows were assessed after a two-week exposure period, in order that the data could be compared to an existing dataset (Brian et al., 2005). However, patterns of VTG induction in goldfish maintained at different temperatures indicate that the effects of temperature become increasingly difficult to detect with increasing duration of exposure and, whilst there was a difference in the VTG response at each time point, it was not possible to determine whether there was any effect on the maximal response because the VTG levels in fish maintained at the lower temperature did not plateau over the course of the ten day exposure. It is therefore possible that, after a more prolonged period, the effects of temperature become less apparent and, ultimately, cannot be detected. This would explain why the VTG response in the first experiment in the present study appeared to be unaffected by thermal regime.

As a result, a second experiment was carried out to determine whether temperature-dependent effects on the VTG response could be detected at an earlier stage of exposure. Suitable time points for assessing the response were identified using data from a preliminary study, in which we characterised the VTG response of fish in the

PC groups at several time-points throughout the course of the two-week exposure. The results confirmed our suspicions: there was a significant effect of temperature on the first and second day of exposure, which became less pronounced between days four and seven, and disappeared after an exposure period of two weeks (data not shown). As a result, it was decided to sample fish exposed to the mixture at two time points: after 24 hours and seven days. In this experiment, we investigated the effects of temperature on an additional endpoint: levels of VTG gene expression were analysed alongside the induction of VTG protein.

The determination of VTG protein revealed a significant effect of temperature after 24 hours of exposure. The difference was most pronounced when comparing the responses of fish exposed to the 0.5 mixture dilution; these were approximately 20% and 80% at 20 and 30°C, respectively. After seven days, however, this effect could no longer be detected. This indicates that the rate of VTG induction was affected, such that the response reached its maximum level more rapidly in fish maintained at the higher temperature. Conversely, at the lower temperature, fish accumulated VTG at a slower rate, but ultimately, after seven days, there was no difference between the responses achieved under either thermal regime. It was somewhat surprising that the effects were so transient, given that temperature-dependent effects on VTG induction in goldfish were apparent after ten days of exposure. The magnitude of the effect was also greater in goldfish. This may reflect the wider temperature differential assessed by Ishibashi et al. (2001), compared to the present study (10 vs. 20°C).

Analysis of temperature-related effects on VTG gene expression revealed a similar pattern after 24 hours, with the fish maintained at 30°C exhibiting a greater response. In contrast, after seven days, there was a reversal in this trend. Published data on the

kinetics of the VTG response demonstrate that this molecular response is induced rapidly and reaches a plateau within three days of exposure (Schmid et al., 2002), indicating that, after seven days, the levels are likely to have stabilised. Differences in VTG gene expression levels could be explained by a compensatory mechanism if, for example, the efficiency with which this genetic information is translated at the biochemical level increases with temperature. The likelihood of temperature-related effects on gene translation can be investigated by comparing the ratio of VTG protein per unit gene expression, which revealed that translation efficiency was higher in the 30°C treatment group at each time point. This pattern is consistent with the findings of Mackay and Lazier (1993) and supports their assertion that temperature-dependent effects on the induction of VTG protein occur as a result of both differences in gene transcription and translation efficiency.

The results of this investigation provide convincing evidence that temperature has a confounding effect on the estrogenic response of fish and that this is manifested both at the molecular and physiological level. Initially, the fish exhibited a more pronounced response to the mixture at the higher temperature, which made the mixture appear more potent in this treatment group. Presumably, this occurred as a result of temperature-dependent effects on the rate of physiological processing (Heugens et al., 2003). The effects on VTG protein levels were transient, however, and the positive relationship between temperature and gene expression after 24 hours was subsequently reversed. In contrast, the difference between the ratio of the proteomic and molecular responses increased with the duration of exposure, suggesting that the equilibrium between the transcriptional and/or translational factors varies, depending on the thermal regime. As such, it would be interesting to

433 determine whether this has implications at higher levels of biological organisation,
434 affecting parameters such as fitness and fecundity.

435 Whilst there was evidence of temperature dependent effects on the VTG response
436 during the fist seven days of exposure, after two weeks, the potency of the mixture
437 did not differ between each treatment group and the effects were consistent with
438 those reported in an earlier study (Brian et al., 2005). From this, we can conclude
439 that these estrogenic chemicals continue to act in an additive, predictable manner
440 within the temperature range studied here. Hence, temperature-dependent effects are
441 unlikely to be a significant confounding factor in the risk assessment of chemicals in
442 a continuous exposure situation, such as this, as the effects of this factor are restricted
443 to the early stages of exposure. However, the influence of temperature may become
444 more relevant in the environment, where exposures may be pulsed or intermittent.
445 An increase in the rate of response under these conditions may have developmental
446 or behavioural implications for fish, as well as being associated with physiological
447 effects as a result of increased energy expenditure. Further research is required to
448 establish the ecotoxicological significance of these effects in the short-term.

449 The data also provide an insight into the molecular and physiological mechanisms
450 responsible for temperature-dependent effects on the timing of reproduction in wild
451 fish. This is relevant in view of recent research into patterns of ovarian development
452 and the date of the onset of spawning in roach (Rutilus rutilus) in Lake Geneva,
453 which has revealed that the time of breeding in this species has advanced by two
454 weeks in less than twenty years. This has been associated with an increase in annual
455 mean water temperature of only one degree (Gillet and Quetin, 2006). Temperature-
456 dependent effects on fish reproduction are unlikely to be restricted to Lake Geneva:

457 there is growing evidence of an upward trend in the temperature of surface waters
458 across Europe. For example, the Environment Agency of England and Wales has
459 reported a warming rate of as much as 0.65°C per decade in some areas (Hammond
460 and Pryce, 2007). The phenological changes that are likely to be associated with this
461 rapid rate of warming have significant ecological implications in terms of adaptation
462 and survival of offspring due to factors such as food availability.

463 In species such as the roach, the effects of temperature on the timing of reproduction
464 can be explained in terms of the seasonal cycle of gonad development. This process
465 begins in the autumn, when VTG synthesis is induced by endogenous E2. It is then
466 transported from the liver, in the plasma, into the gonads, where is taken up by the
467 oocytes, via a receptor mediated process. The rate of gonad development is closely
468 associated with temperature: VTG is taken up by the oocytes more rapidly in autumn
469 and spring than during the colder winter months, when VTG synthesis is inhibited
470 (Rinchard et al., 1997).

471 The results of the present study indicate that the effects of temperature on VTG
472 synthesis are mediated both at the molecular and physiological level. Whilst an
473 increase in temperature from 20 to 30°C was associated with only transient effects on
474 the VTG response of fathead minnows, it is possible that greater effects would have
475 been observed across a lower temperature differential (e.g. 10 to 20°C), due to the
476 presence of a thermal threshold, below which VTG gene expression is inhibited
477 (Korsgaard et al., 1986). This would explain why mild spring conditions and
478 shortened winters, when water temperatures do not exceed this critical threshold, are
479 associated with a significant advancement in the date of spawning: an increase in
480 VTG synthesis accelerates the rate of gonad development, thereby reducing the time

taken for oocytes to reach the size required for ovulation (1.4mm diameter in roach; Mann, 1973). This means that the fish are ready to spawn as soon as they are given the appropriate environmental cues.

5. CONCLUSIONS

The results of this investigation indicate the temperature is not a major confounding factor determining the way in which fish respond to estrogenic chemicals in the long term. Whilst the rate of response increased with temperature, there was no effect on the magnitude of the response at the end of the exposure period. However, a review of the literature suggests that the induction of VTG may be inhibited below a critical thermal threshold. This means that more pronounced effects might have occurred if we had compared the effects of temperature on either side of this threshold, although this design was not consistent with the aims of this study (i.e. to assess the ecotoxicological significance of elevated water temperature). The data therefore indicate that an increase in the temperature of surface waters is not particularly important from a long-term risk assessment perspective. The implications of short-term changes in the rate of response are difficult to anticipate, yet could be of relevance. Furthermore, the patterns observed provide a useful insight into the physiological mechanisms responsible for temperature-dependent effects on the date of spawning, which may have profound implications at the population level. Data that enable us to elucidate the way in which temperature exerts its effects at the molecular and physiological level are likely to be of value in helping to improve our understanding of the risks associated with the climate change.

6. ACKNOWLEDGEMENTS

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569 Table 1: Mean of the VTG responses of fish in each treatment group after i. 24 hours
570 and ii. seven days of exposure to the mixture. Gene expression is presented in relative
571 units, based on the levels measured in a reference sample (see text for details). The
572 ratio of protein to gene expression was calculated for treatment groups in which there
573 was a clear VTG response (i.e. significant induction above the baseline). The effect
574 of temperature on the amount of protein per unit RNA was statistically significant
575 after 24 hours and 7 days.
576

i. 24 hours

VTG Response	Gene expression (relative units)		Protein induction (μg/ml plasma)		Ratio (protein:RNA)	
	20ºC	30ºC	20ºC	30ºC	20ºC	30ºC
Treatment						
N. Control	0.00	0.00	0.03	0.12	-	-
0.05 dilution	0.01	0.00	0.64	0.10	-	-
0.1 dilution	0.05	0.00	0.76	0.03	-	-
0.2 dilution	0.05	0.56	0.03	1.89	-	3.35
0.3 dilution	0.75	1.25	1.11	3.96	1.49	3.17
0.5 dilution	0.48	5.79	1.13	25.4	2.35	4.38
1:0 dilution	2.06	11.48	3.34	35.5	1.62	3.09
P. Control	4.08	11.31	8.14	53.7	2.00	4.75

ii. 7 days

VTG Response	Gene expression (relative units)		Protein induction (μg/ml plasma)		Ratio (protein:RNA)	
	20ºC	30ºC	20ºC	30ºC	20ºC	30ºC
Treatment						
N. Control	0.00	0.00	0.04	0.05	-	-
0.05 dilution	0.01	0.00	0.35	0.03	-	-
0.1 dilution	0.04	0.03	0.36	0.53	8.96	15.5
0.2 dilution	0.07	0.19	0.68	5.98	9.70	31.5
0.3 dilution	5.19	1.20	47.5	86.8	9.16	72.3
0.5 dilution	8.26	4.09	89.8	240	10.9	58.6
1:0 dilution	15.7	16.9	365	1012	23.3	59.9
P. Control	18.7	13.7	830	1546	44.3	113

7. FIGURES

Figure 1: Nominal versus measured concentrations of each chemical in experiment one and two. The blue diamonds and red squares represent the average of the concentration measured at the start and end of the exposure in tanks maintained at 20 and 30ºC, respectively. The abbreviations are as follows; EE2= 17α–ethinylestradiol; E2= 17β-estradiol; NP= 4-tert-nonylphenol; OP= 4-tert-octylphenol and BPA= bisphenol-A.

Figure 2: (i) shows the normalised VTG protein concentrations in fish exposed to various dilutions of the mixture for a period of two weeks. Each dot represents the VTG response of an individual fish. The blue and red circles represent the responses of fish maintained at 20 and 30ºC, respectively. (ii) shows the estrogenic responses of fish maintained at a standard test temperature of 25ºC (black circles). The best fits of the responses observed at 20 and 30ºC are represented by the blue and red line, respectively. The broken lines represent the 95% confidence intervals.

Figure 3: The blue and red lines represent the best fits of the responses at 20 and 30ºC, respectively. The broken lines represent the 95% confidence limits. (i) shows normalised VTG protein concentrations in fish exposed to various dilutions of the mixture for 24 hours. There was a statistically significant difference between the response observed under each thermal regime, such that the potency of the mixture increased by a factor of 2.5 with a temperature rise of 10ºC. (ii) shows the same response after seven days of exposure, by which time the difference between the best fits determined at each temperature had largely disappeared.

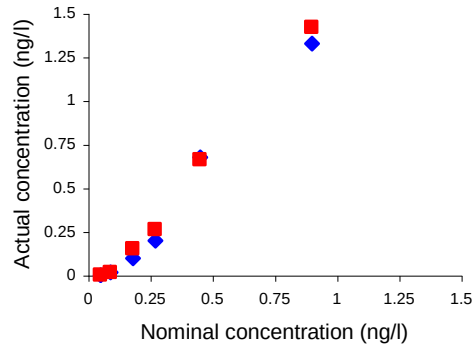
Figure 4: The blue and red lines represent the best fits of the responses at 20 and 30ºC, respectively. The broken lines represent 95% confidence limits. (i) shows

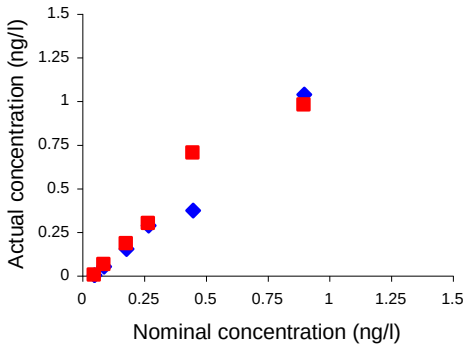
normalised patterns of VTG gene expression in fish exposed to the mixture for 24 hours. There was a statistically significant difference between the gene expression levels of fish maintained in each temperature group, such that the potency of the mixture was almost doubled at 30ºC, relative to the response observed at 20ºC. (ii) shows the molecular response after seven days of exposure, by which time there was no statistically significant difference between the effects observed under each thermal regime.

Figure 1

Experiment 1 Experiment 2

i. EE2





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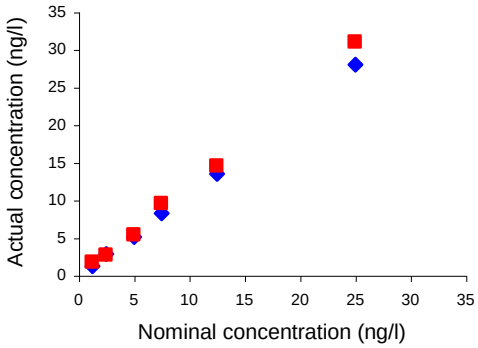

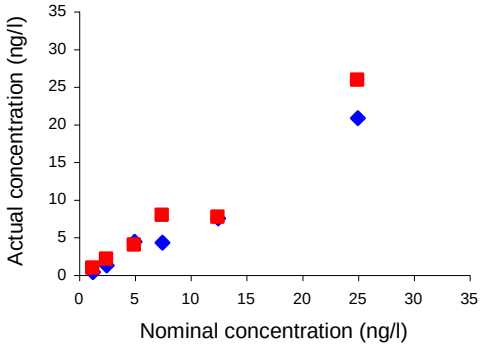

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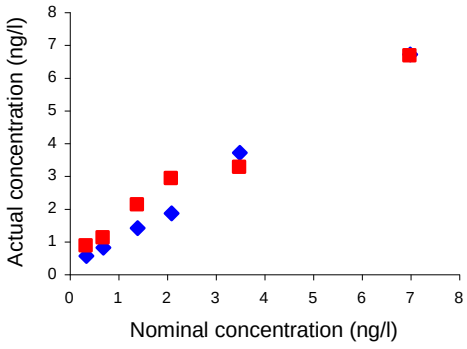
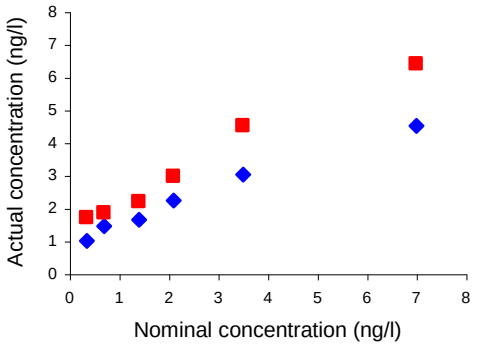
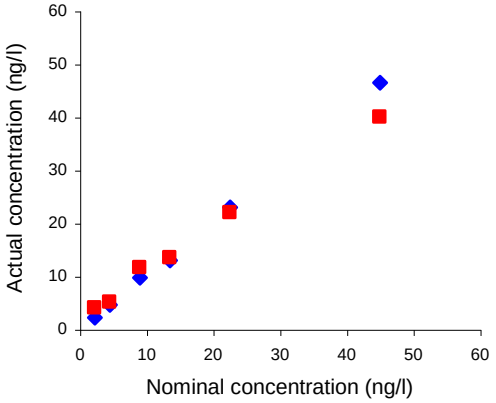
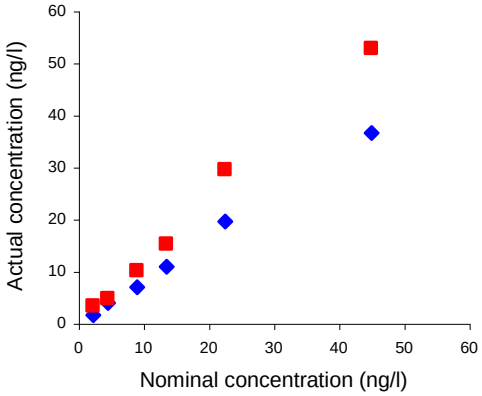




650 iv. OP





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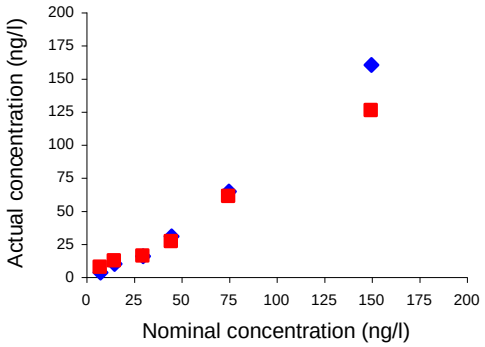
652 v. BPA



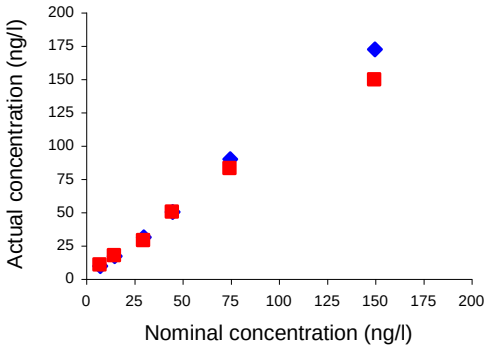


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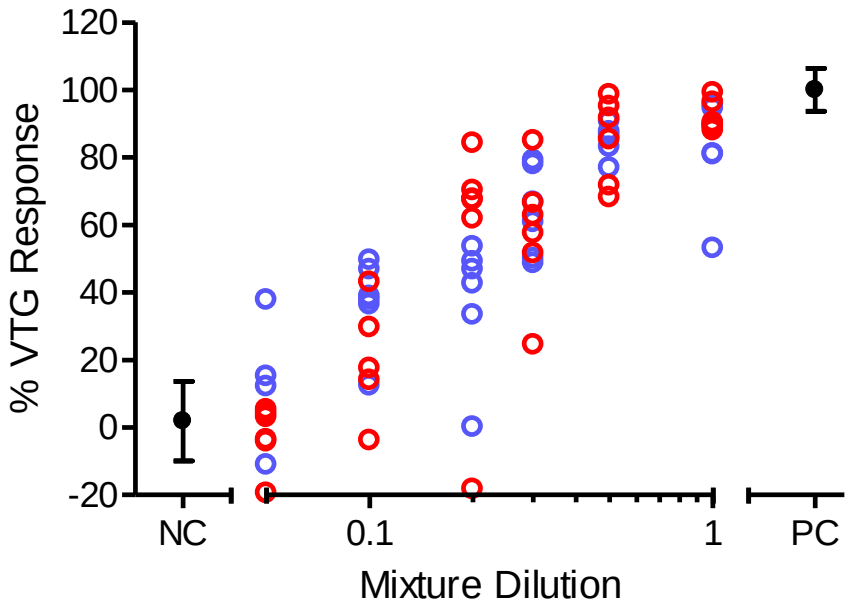
Figure 2

i.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% VTG Response
NC
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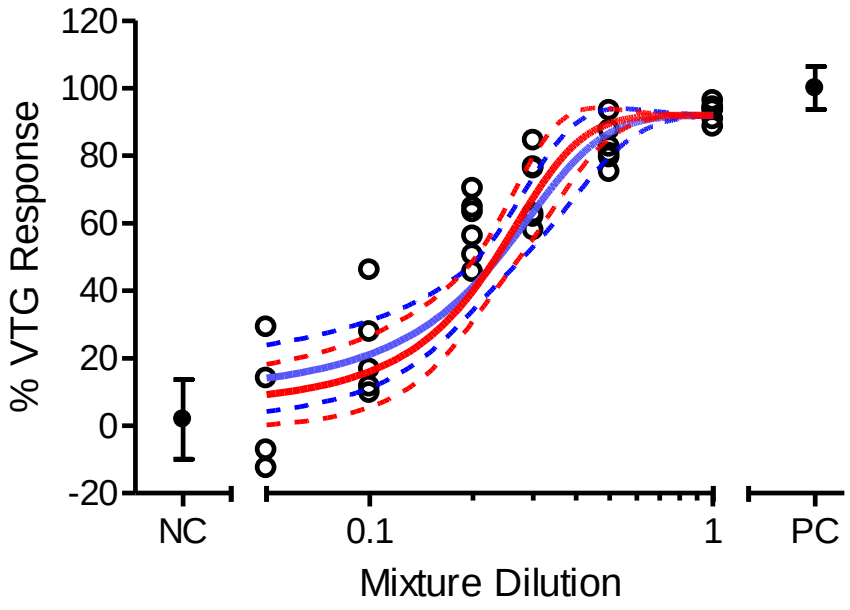
1
PC
Mixture Dilution

ii.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% VTG Response
NC
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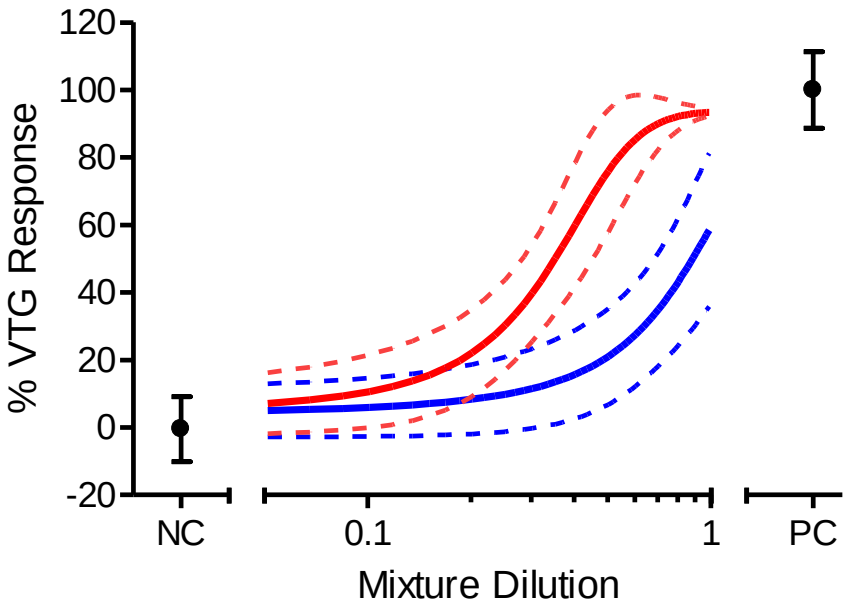
Mixture Dilution

Figure 3

i.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Mixture Dilution

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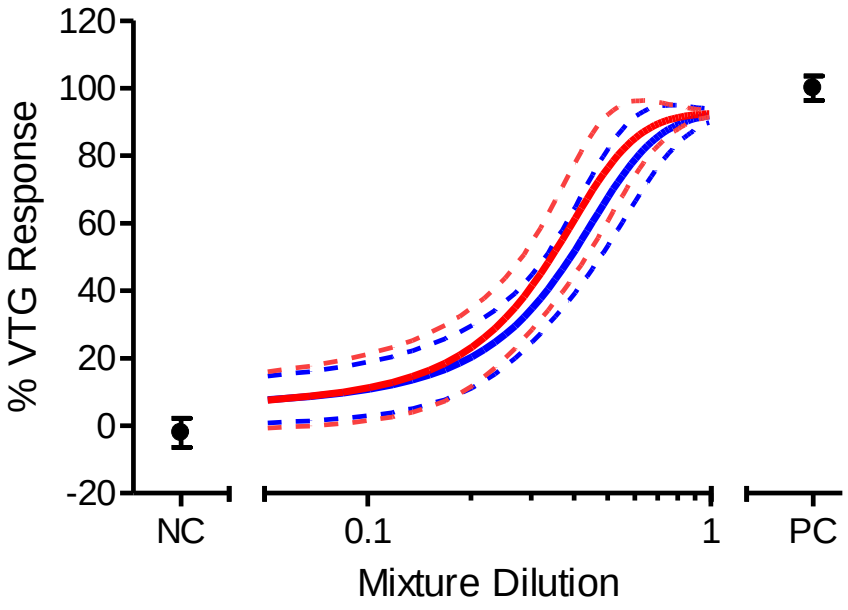
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% VTG Response
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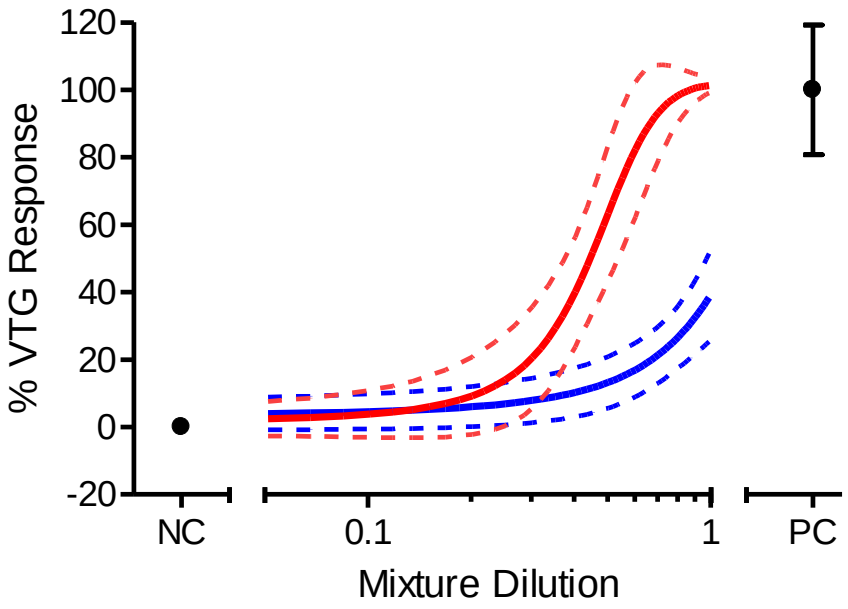
PC
Mixture Dilution

Figure 4

i.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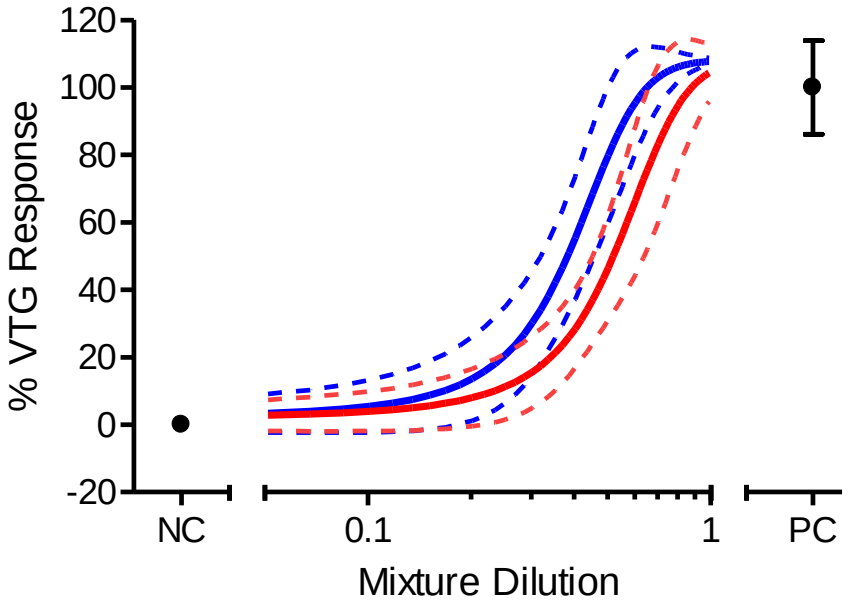
1
PC
Mixture Dilution

ii.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