1   **EVIDENCE OF TEMPERATURE-DEPENDENT EFFECTS ON THE**

2   **ESTROGENIC RESPONSE OF FISH:**

3   **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.

77 Currently, procedures for assessing the risk that chemicals pose in the environment

78 incorporate a safety or uncertainty factor (US EPA, 2004) and, in general, it is

79 assumed that a ten-fold margin is sufficient to protect against combined effects

80 resulting from multiple exposures. However, growing evidence that even relatively

81 low numbers of chemicals can act together in the low concentration range to elicit

82 significant effects undermines the traditional risk assessment paradigm that there is a

83 threshold level below which a chemical is not considered to pose a threat (the NOEC;

84 no observed effect concentration). Hence, even when an uncertainty factor is applied,

85 there can still be a risk of significant mixture effects.

86 Growing realisation of this issue has fuelled concerns that risk assessment procedures

87 may further underestimate risk by failing to consider how the toxicological response

88 to chemical challenge may be influenced by the conditions of exposure. Standard

89 toxicity tests fail to consider that environmental exposures occur under variable and

90 suboptimal regimes. Hence, the confounding effects of a wide range of physico-

91 chemical factors, which vary over spatial and temporal scales, may be overlooked

92 when extrapolating from the laboratory to predict risks that exist in the real world

93 (Vignati et al., 2007). The relevance of confounding factors in the risk assessment of

94 chemicals is an issue that has, as yet, received little attention, although there is some

95 evidence that parameters such as temperature and salinity can influence toxicity

96 (Heugens et al., 2001). Hence, the interactive effects of environmental variables, as

97 well as chemical mixtures, warrant further attention in risk assessment methodology.

98 The influence of confounding factors in the risk assessment of chemicals is extremely

99 pertinent in view of climate change. This phenomenon will create multiple stress

100 exposure situations, in which organisms may respond in an unpredictable manner to

101 chemical challenge.   In particular, there is evidence to suggest that the projected rise

102 in average temperatures may increase chemical toxicity. For example, a review by

103 Cairns et al. (1975) revealed that, in general, aquatic organisms are more susceptible

104 to metal and pesticide toxicity at higher temperatures. This interaction is likely to

105 occur as a result of temperature-related effects on the physiological processes that

106 determine the rates of chemical uptake, elimination and detoxification (Heugens et al.,

107 2003). However, although there would appear to be a positive relationship between

108 temperature and acute toxicity in terms of lethal concentrations and survival times,

109 less is known about the influence of temperature on sub-lethal endpoints. This is

110 more relevant in the real world, in which organisms are more commonly exposed to

111 mixtures of chemicals at concentrations that are not associated with overt toxicity.

112 The aim of this study was to investigate the influence of temperature on the estrogenic

113 response of fish to a defined mixture of chemicals. The effects of this mixture have

114 been characterised under standard test conditions, both in terms of the induction of the

115 egg yolk precursor protein, vitellogenin (VTG), and its impact on reproduction (Brian

116 et al., 2005; 2007). The influence of temperature on the VTG response at the

117 physiological and molecular level was investigated under two different thermal

118 regimes; one above and one below the standard test temperature. Previous research

119 on salmonid fish that were injected with natural steroid estrogen indicates that an

120 increase in temperature will be associated with increased potency (Korsgaard et al.,

121 1986; Mackay and Lazier, 1992). However, waterborne exposure to mixtures of

122 chemicals that are both anthropogenic and natural in origin, might not elicit the same

123 temperature-dependent response. The results will reveal whether temperature is a

124 confounding factor in the risk assessment of estrogenic chemicals, and give an insight

125 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

149 described in Brian et al. (2005). Dosing commenced one week before the start of each

150 exposure study. This conditioning process ensured that the chemical concentrations

151 in the tanks were accurate. The exposure concentrations were verified by performing

152 analytical chemistry on water samples collected immediately prior to the addition of

153 the fish. A further set of water samples were collected on the final day of exposure.

154 The analytical methods are described in Brian et al. (2005).

155 *2.2 Protocol*

156 One week prior to exposure, whilst the experimental tanks were being conditioned,

157 male fathead minnows were selected from a stock of mixed-sex adult fish that had

158 been maintained at a constant temperature of $25\pm1°C$. These fish were transferred

159 into holding tanks where they were equilibrated to either 20 or 30°C by altering the

160 temperature of the influent water by 1°C per day until the target temperature was

161 achieved. The temperature of the holding tanks was then kept constant until the end

162 of the week, when the fish were randomly allocated to experimental tanks maintained

163 at the same temperature.

164 During the equilibration period and the experiment, the fish were fed twice daily:

165 once with frozen brine shrimp and once with flaked fish food. The photoperiod was

166 maintained on a 16hr light/8hr dark cycle with 20 minute dawn and dusk transition

167 periods. The water temperature in the fish tanks was recorded daily using an Oxi 315i

168 digital meter and Cell Ox 325 probe (WTW; Weilheim, Germany) to ensure that it

169 remained within 1°C of the target temperature. In addition, dissolved oxygen levels

170 and various water quality measurements were recorded routinely and the dosing rate

171 was monitored throughout the experiment.

172   In the first experiment, temperature-related effects were explored by comparing the

173   VTG levels in the plasma of fish exposed to the mixture of estrogenic chemicals at 20

174   and 30°C for a period of two weeks. The response at each of these temperatures was

175   also related to that observed in a parallel exposure, conducted at 25°C, as well as that

176   reported by Brian et al. (2005) in a previous experiment. A subsequent experiment

177   was also carried out to investigate whether temperature influenced the response after

178   24 hours and seven days. In these more short-term studies, the expression of the VTG

179   gene in liver tissue was analysed alongside the induction of VTG protein. These two

180   closely related endpoints were analysed together to gain an insight into the molecular

181   basis for temperature-related effects on the VTG response.

182   *2.3 Sampling and Analysis*

183   At the end of the experiment, the fish were sacrificed by overdose with anaesthetic

184   (MS222; Sigma Aldrich). Six fish were sampled from each tank at each time point

185   (i.e. after two weeks exposure in the first and after 24 hours and seven days in the

186   second experiment, respectively). Their lengths and weights were recorded before

187   blood samples were collected from the caudal peduncle using heparinised capillary

188   tubes. Blood samples were centrifuged at 4000g for 5 minutes and the plasma drawn

189   off and stored at –20ºC for the determination of VTG protein levels. This was carried

190   out using a carp-VTG ELISA previously been validated for the measurement of VTG

191   in fathead minnow (Tyler et al. 1999).

192   Liver tissues were also collected from fish exposed to the mixture for 24 hours and

193   seven days. These were placed in RNA-free tubes, in which they were snap-frozen

194   and stored at -80 ºC. Total RNA was extracted using TriReagent (Sigma Aldrich).

195   The samples were then treated with DNase1 (Invitrogen). Total RNA concentrations

196   were then determined by UV spectrophotometry before differential gene expression

197   was performed by real-time QPCR, using an ABI Prism 7900HT sequence detection

198   system (Applied Biosystems) with one step SYBR green master mix (Qiagen). The

199   reactions were set up in triplicate in 96 well plates: each reaction was 25 μl in volume

200   and initially contained 10μg of total RNA.

201   The primers used to analyse VTG gene expression in this species were designed by

202   Miracle et al. (2006) using sequence information from Korte et al. (2000; GenBank

203   acc. no. AF130354). The sequence of the forward and reverse primers was; 5'-CAC

204   AAT CCC AGC TCT GCG TGA-3' and 5' TGG CCT CTG CAG CAA TAT CAT-

205   3', respectively. Following an initial RT step, during which samples were incubated

206   at 50°C for 30 min, amplification was measured over 40 cycles of 95°C for 20s, 60°C

207   for 20s and 72°C for 10s. The VTG gene expression level in each fish was evaluated

208   with respect to a serial dilution of a sample from a female fish, which was run in all

209   assay plates. This approach is similar to that used by Schmidt et al. (2002), although

210   these authors used an exposed male fish as a reference.   Gene expression levels are

211   therefore presented as relative values, with the female being assigned a value of 100

212   and the responses of the males being presented proportionally. The expression of β-

213   actin was also quantified, with a view to its use as a housekeeper, or internal control,

214   to account for small differences in the amount of starting material between samples.

215   However, subsequent analysis revealed an effect of estrogen treatment, as per Filby

216   and Tyler (2007). Hence, the VTG gene expression data was analysed without the use

217   of a reference gene.

218   *2.4 Statistical Analysis*

219 The chemical concentrations in the fish tanks were analysed statistically to ensure that

220 there were no differences between the exposure levels in each temperature group.

221 The mean measured concentration at the beginning and end of each experiment was

222 calculated for each chemical. This then was converted into a proportional value by

223 dividing by the nominal concentration. Comparisons were then made between tanks

224 with the same nominal exposure levels in each of the temperature groups. This was

225 achieved using paired t-tests in Minitab 13.1 (Minitab Inc. State College, PA, USA).

226 The VTG protein concentrations were log transformed prior to normalisation, which

227 allowed the data to be plotted on a percentage response scale. The normalisation

228 procedure was carried out by subtracting the mean baseline response from all other

229 values. The baseline was determined by pooling the responses of fish maintained in

230 each of the NC tanks, which did not differ significantly from one another, along with

231 any other groups that did not respond to treatment. The corrected VTG values were

232 then divided by the mean response in the PC tank, which represented the maximum

233 response. This was determined from the 30°C exposure only, as opposed to pooling

234 the data from both PC tanks, as the response was greatest at this temperature. This

235 procedure enabled the response in all other treatment groups could be plotted on a

236 graded effect scale of between zero and a hundred. The percentage VTG response

237 was then plotted against the mixture dilution, on a log scale, which produced typical

238 concentration-response curves, similar to those reported in Brian et al. (2005).

239 The effect of treatment on VTG protein induction and gene expression was explored

240 by determining the response, at each time-point, under the different thermal regimes.

241 The data were fitted to a sigmoidal dose-response model, with variable slope, using a

242 four parameter logistic equation. The top and bottom of the curve were constrained to

243   the mean of the responses observed following exposure to the highest and lowest

244   mixture dilutions, respectively. Best-fits were then determined for the median effect

245   concentration (EC_{50}), based on the nominal mixture dilution, under each thermal

246   regime. These values were then compared to assess whether there was any effect of

247   temperature. These analyses were performed using the non-linear regression function

248   of GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The ratio between

249   the levels of VTG protein:gene expression in each treatment group were also

250   calculated, as per Mackay and Lazier (1993). The efficiency with which the

251   molecular signal was translated into a proteomic response at each temperature was

252   then compared using the paired t-tests (Minitab 13.1).

253   **3. RESULTS**

254   *3.1. Analytical Chemistry*

255   The analysis of the chemical concentrations in each fish tank revealed that there was

256   good agreement between the nominal and actual exposure levels at both temperatures

257   during each experiment (Figure 1). No significant differences were detected between

258   the actual exposure levels in each temperature group in the first experiment. In the

259   second experiment, however, slightly higher levels of NP and OP were detected at

260   30ºC than at 20 ºC. In the case of OP, there was a statistically significant difference

261   (t=-4.91, p<0.01, n=6). However, this pattern was not consistent across all chemicals:

262   the concentrations of E2, EE2 and BPA were close to nominal in both temperature

263   groups. As the mixture was delivered to the tanks as a single stock, any "real"

264   discrepancies in the exposure levels should have been apparent for all chemicals. It

265   was therefore concluded that the differences in the levels of the alkylphenols between

266   the two temperature groups probably occurred as a result of an analytical anomaly, as

267 opposed to a real difference, and that the actual exposure levels were the same across

268 all experiments.

269 *3.2. VTG Protein Induction*

270 The analysis of the levels of VTG protein after two weeks of exposure to the mixture

271 in the first experiment revealed clear and consistent concentration-response curves.

272 There was no evidence of a difference in the response of fish maintained at 20 and

273 30°C (Figure 2). The best estimates for the log EC_{50} values, derived from the non-

274 linear regression model, with 95% confidence intervals, were 0.221 (0.176-0.266) and

275 0.219 (0.176-0.264) at the lower and upper temperature, respectively. The estimates

276 did not differ from those determined in the parallel exposure, which was conducted at

277 25°C, and were consistent with previous data documenting the concentration-response

278 to the same mixture (Brian et al., 2005). Hence, there was no evidence of an effect of

279 temperature on the induction of VTG protein in fish exposed to the mixture for a

280 period of two weeks.

281 In contrast, the results of the second experiment, which compared the response of fish

282 to the mixture at 20 and 30 °C at two earlier time points, revealed a temperature-

283 dependent effect after 24 hours of exposure (Figure 3). The best fits and confidence

284 intervals for the log EC_{50} values were 0.847 (0.611-1.08) and 0.335 (0.259-0.413) in

285 the 20 and 30°C groups, respectively. This difference was highly significant

286 ($p<0.0001$). After seven days, however, these EC_{50} values had gone down to 0.369

287 (0.303-0.436) and 0.325 (0.258-0.393) at 20 and 30°C, respectively, and the difference

288 between them was no longer statistically significant. This pattern indicates that the

289 proteomic VTG response is initially more sensitive to the effects of temperature, with

290 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:

314   the proteomic response is exceptional as it can vary over several orders of magnitude.

315   Furthermore, the ratio between the levels of VTG protein:gene expression revealed a

316   significant effect of temperature at both time points ($p < 0.001$), reflecting a difference

317   in the efficiency of gene translation and/or post-translation processing under each

318   thermal regime.

319   **4. DISCUSSION**

320   The results of the first experiment were somewhat surprising in that there was no

321   evidence of a temperature-dependent effect on the estrogenic response to the mixture,

322   in terms of the induction of proteomic VTG. This was not consistent with findings

323   from earlier studies on salmonid species. Korsgaard et al. (1986) reported that the

324   VTG response of Altlantic salmon (Salmo salar) injected with E2 at regular intervals

325   over a 10-day period was strongly influenced by temperature. Male post smolts that

326   were acclimated and maintained at 3°C showed little or no VTG response, whereas

327   those maintained at 10 or 15°C during treatment showed a greater accumulation of

328   VTG, both in terms of hepatic RNA and alkali-labile phosphorous levels in plasma,

329   at higher ambient temperatures. The authors suggested that this might be due to the

330   inhibition of VTG gene expression at lower temperatures. Similarly, an investigation

331   into the estrogen responsiveness of juvenile rainbow trout (Oncorhynchus mykiss)

332   revealed that both the rate and the magnitude of the VTG response increased with

333   temperature. Mackay and Lazier (1993) reported that VTG protein could be detected

334   in the serum of fish maintained at 15°C within 24 hours of exposure to E2, compared

335   to 72 hours at 9°C. After ten days, VTG protein response was 10-fold higher in fish

336   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

361   likely to be responsible for the absence of a temperature-dependent response in our

362   study. Analysis of the VTG response of goldfish was particularly interesting in that it

363   revealed that the effects of temperature were more pronounced during the early stages

364   of exposure: after 24 hours, the levels of VTG protein were 10 000 times higher in

365   fish maintained at 30°C than at 10°C, whereas after five and ten days, the response

366   differed by a factor of 100 and 10, respectively. This response pattern, which was not

367   reported in the earlier studies, provides a potential explanation for the apparent lack

368   of temperature-dependent effects in the present study.

369   Here, the effects of temperature on the VTG response of fathead minnows were

370   assessed after a two-week exposure period, in order that the data could be compared

371   to an existing dataset (Brian et al., 2005). However, patterns of VTG induction in

372   goldfish maintained at different temperatures indicate that the effects of temperature

373   become increasingly difficult to detect with increasing duration of exposure and,

374   whilst there was a difference in the VTG response at each time point, it was not

375   possible to determine whether there was any effect on the maximal response because

376   the VTG levels in fish maintained at the lower temperature did not plateau over the

377   course of the ten day exposure. It is therefore possible that, after a more prolonged

378   period, the effects of temperature become less apparent and, ultimately, cannot be

379   detected. This would explain why the VTG response in the first experiment in the

380   present study appeared to be unaffected by thermal regime.

381   As a result, a second experiment was carried out to determine whether temperature-

382   dependent effects on the VTG response could be detected at an earlier stage of

383   exposure. Suitable time points for assessing the response were identified using data

384   from a preliminary study, in which we characterised the VTG response of fish in the

385   PC groups at several time-points throughout the course of the two-week exposure.

386   The results confirmed our suspicions: there was a significant effect of temperature on

387   the first and second day of exposure, which became less pronounced between days

388   four and seven, and disappeared after an exposure period of two weeks (data not

389   shown). As a result, it was decided to sample fish exposed to the mixture at two time

390   points: after 24 hours and seven days. In this experiment, we investigated the effects

391   of temperature on an additional endpoint: levels of VTG gene expression were

392   analysed alongside the induction of VTG protein.

393   The determination of VTG protein revealed a significant effect of temperature after

394   24 hours of exposure. The difference was most pronounced when comparing the

395   responses of fish exposed to the 0.5 mixture dilution; these were approximately 20%

396   and 80% at 20 and 30°C, respectively. After seven days, however, this effect could

397   no longer be detected. This indicates that the rate of VTG induction was affected,

398   such that the response reached its maximum level more rapidly in fish maintained at

399   the higher temperature. Conversely, at the lower temperature, fish accumulated VTG

400   at a slower rate, but ultimately, after seven days, there was no difference between the

401   responses achieved under either thermal regime. It was somewhat surprising that the

402   effects were so transient, given that temperature-dependent effects on VTG induction

403   in goldfish were apparent after ten days of exposure. The magnitude of the effect

404   was also greater in goldfish. This may reflect the wider temperature differential

405   assessed by Ishibashi et al. (2001), compared to the present study (10 vs. 20°C).

406   Analysis of temperature-related effects on VTG gene expression revealed a similar

407   pattern after 24 hours, with the fish maintained at 30°C exhibiting a greater response.

408   In contrast, after seven days, there was a reversal in this trend. Published data on the

409   kinetics of the VTG response demonstrate that this molecular response is induced

410   rapidly and reaches a plateau within three days of exposure (Schmid et al., 2002),

411   indicating that, after seven days, the levels are likely to have stabilised. Differences

412   in VTG gene expression levels could be explained by a compensatory mechanism if,

413   for example, the efficiency with which this genetic information is translated at the

414   biochemical level increases with temperature. The likelihood of temperature-related

415   effects on gene translation can be investigated by comparing the ratio of VTG protein

416   per unit gene expression, which revealed that translation efficiency was higher in the

417   30°C treatment group at each time point. This pattern is consistent with the findings

418   of Mackay and Lazier (1993) and supports their assertion that temperature-dependent

419   effects on the induction of VTG protein occur as a result of both differences in gene

420   transcription and translation efficiency.

421   The results of this investigation provide convincing evidence that temperature has a

422   confounding effect on the estrogenic response of fish and that this is manifested both

423   at the molecular and physiological level. Initially, the fish exhibited a more

424   pronounced response to the mixture at the higher temperature, which made the

425   mixture appear more potent in this treatment group. Presumably, this occurred as a

426   result of temperature-dependent effects on the rate of physiological processing

427   (Heugens et al., 2003). The effects on VTG protein levels were transient, however,

428   and the positive relationship between temperature and gene expression after 24 hours

429   was subsequently reversed. In contrast, the difference between the ratio of the

430   proteomic and molecular responses increased with the duration of exposure,

431   suggesting that the equilibrium between the transcriptional and/or translational

432   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

481   taken for oocytes to reach the size required for ovulation (1.4mm diameter in roach;

482   Mann, 1973). This means that the fish are ready to spawn as soon as they are given

483   the appropriate environmental cues.

5. CONCLUSIONS

484

485   The results of this investigation indicate the temperature is not a major confounding

486   factor determining the way in which fish respond to estrogenic chemicals in the long

487   term. Whilst the rate of response increased with temperature, there was no effect on

488   the magnitude of the response at the end of the exposure period. However, a review

489   of the literature suggests that the induction of VTG may be inhibited below a critical

490   thermal threshold. This means that more pronounced effects might have occurred if

491   we had compared the effects of temperature on either side of this threshold, although

492   this design was not consistent with the aims of this study (i.e. to assess the ecotoxico-

493   logical significance of elevated water temperature). The data therefore indicate that

494   an increase in the temperature of surface waters is not particularly important from a

495   long-term risk assessment perspective. The implications of short-term changes in the

496   rate of response are difficult to anticipate, yet could be of relevance. Furthermore,

497   the patterns observed provide a useful insight into the physiological mechanisms

498   responsible for temperature-dependent effects on the date of spawning, which may

499   have profound implications at the population level. Data that enable us to elucidate

500   the way in which temperature exerts its effects at the molecular and physiological

501   level are likely to be of value in helping to improve our understanding of the risks

502   associated with the climate change.

6. ACKNOWLEDGEMENTS

503

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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

577   i. 24 hours

578

579 VTG Response	Gene expression (relative units)		Protein induction (μg/ml plasma)		Ratio (protein:RNA)	
581	20ºC	30ºC	20ºC	30ºC	20ºC	30ºC
582 Treatment						
583						
584 N. Control	0.00	0.00	0.03	0.12	-	-
585 0.05 dilution	0.01	0.00	0.64	0.10	-	-
586 0.1 dilution	0.05	0.00	0.76	0.03	-	-
587 0.2 dilution	0.05	0.56	0.03	1.89	-	3.35
588 0.3 dilution	0.75	1.25	1.11	3.96	1.49	3.17
589 0.5 dilution	0.48	5.79	1.13	25.4	2.35	4.38
590 1:0 dilution	2.06	11.48	3.34	35.5	1.62	3.09
591 P. Control	4.08	11.31	8.14	53.7	2.00	4.75

592

593   ii. 7 days

594

595 VTG Response	Gene expression (relative units)		Protein induction (μg/ml plasma)		Ratio (protein:RNA)	
597	20ºC	30ºC	20ºC	30ºC	20ºC	30ºC
598 Treatment						
599						
600 N. Control	0.00	0.00	0.04	0.05	-	-
601 0.05 dilution	0.01	0.00	0.35	0.03	-	-
602 0.1 dilution	0.04	0.03	0.36	0.53	8.96	15.5
603 0.2 dilution	0.07	0.19	0.68	5.98	9.70	31.5
604 0.3 dilution	5.19	1.20	47.5	86.8	9.16	72.3
605 0.5 dilution	8.26	4.09	89.8	240	10.9	58.6
606 1:0 dilution	15.7	16.9	365	1012	23.3	59.9
607 P. Control	18.7	13.7	830	1546	44.3	113

608

609 **7. FIGURES**

610 Figure 1: Nominal versus measured concentrations of each chemical in experiment

611 one and two. The blue diamonds and red squares represent the average of the

612 concentration measured at the start and end of the exposure in tanks maintained at 20

613 and 30ºC, respectively. The abbreviations are as follows; EE2= 17α–ethinylestradiol;

614 E2= 17β-estradiol; NP= 4-tert-nonylphenol; OP= 4-tert-octylphenol and BPA=

615 bisphenol-A.

616 Figure 2: (i) shows the normalised VTG protein concentrations in fish exposed to

617 various dilutions of the mixture for a period of two weeks. Each dot represents the

618 VTG response of an individual fish. The blue and red circles represent the responses

619 of fish maintained at 20 and 30ºC, respectively. (ii) shows the estrogenic responses of

620 fish maintained at a standard test temperature of 25ºC (black circles). The best fits of

621 the responses observed at 20 and 30ºC are represented by the blue and red line,

622 respectively. The broken lines represent the 95% confidence intervals.

623 Figure 3: The blue and red lines represent the best fits of the responses at 20 and

624 30ºC, respectively. The broken lines represent the 95% confidence limits. (i) shows

625 normalised VTG protein concentrations in fish exposed to various dilutions of the

626 mixture for 24 hours. There was a statistically significant difference between the

627 response observed under each thermal regime, such that the potency of the mixture

628 increased by a factor of 2.5 with a temperature rise of 10ºC. (ii) shows the same

629 response after seven days of exposure, by which time the difference between the best

630 fits determined at each temperature had largely disappeared.

631 Figure 4: The blue and red lines represent the best fits of the responses at 20 and

632 30ºC, respectively. The broken lines represent 95% confidence limits. (i) shows

633   normalised patterns of VTG gene expression in fish exposed to the mixture for 24

634   hours. There was a statistically significant difference between the gene expression

635   levels of fish maintained in each temperature group, such that the potency of the

636   mixture was almost doubled at 30ºC, relative to the response observed at 20ºC. (ii)

637   shows the molecular response after seven days of exposure, by which time there was

638   no statistically significant difference between the effects observed under each thermal

639   regime.

640

641 Figure 1

642   Experiment 1   Experiment 2

643 i. EE2
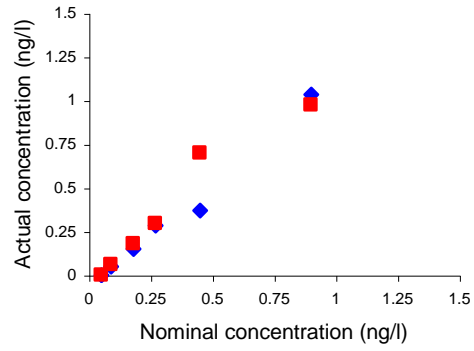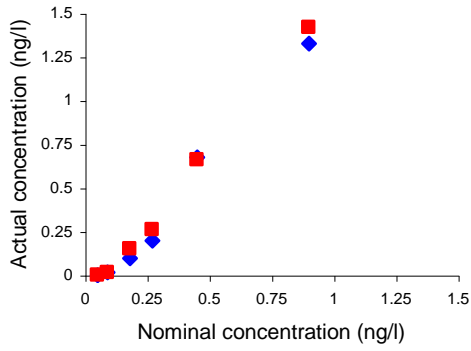
644

645 ii. E2
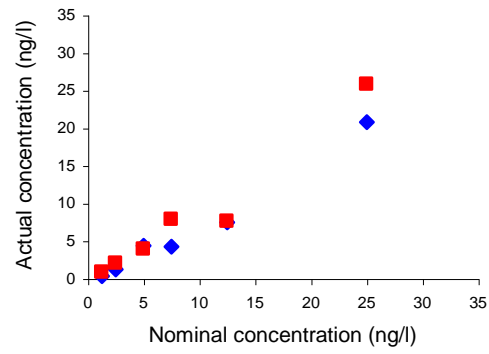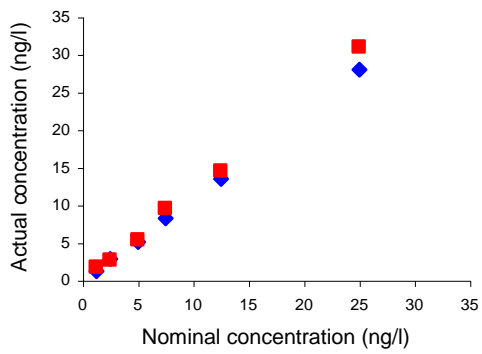
646

647 iii. NP
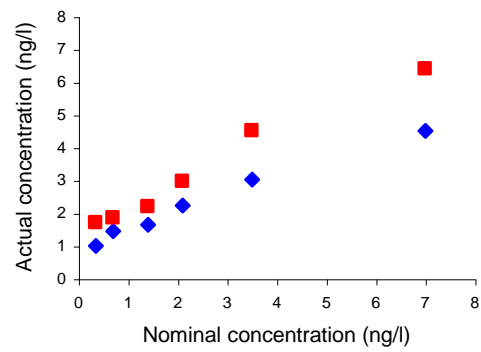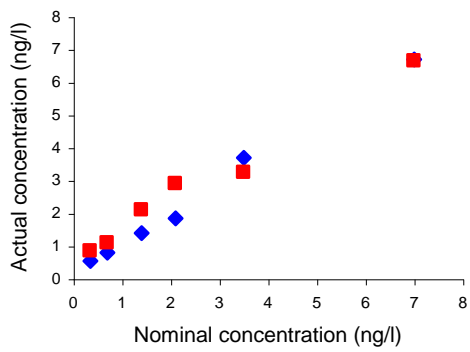
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650   iv. OP
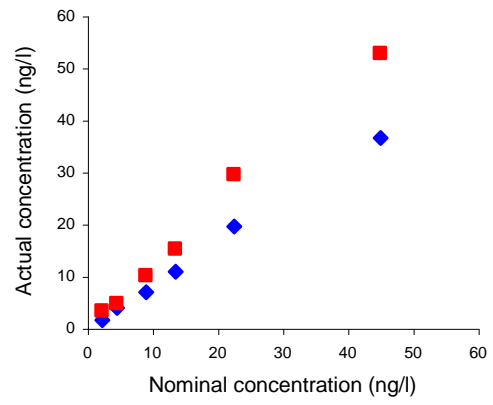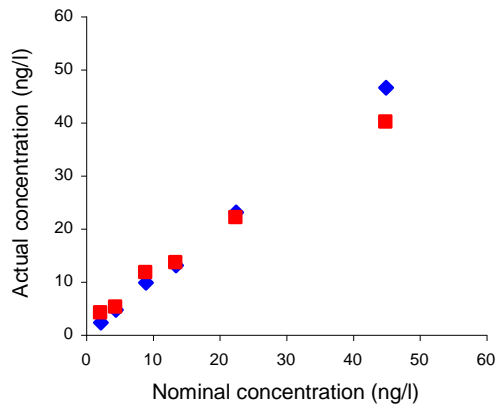
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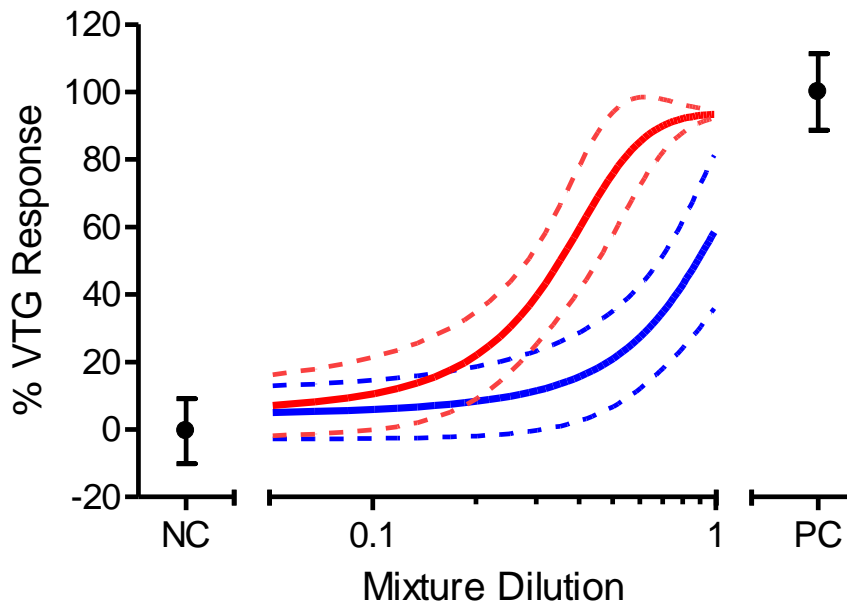652   v. BPA
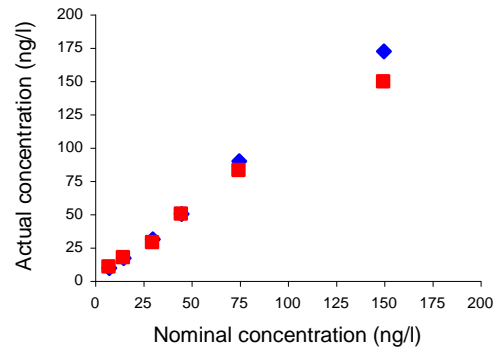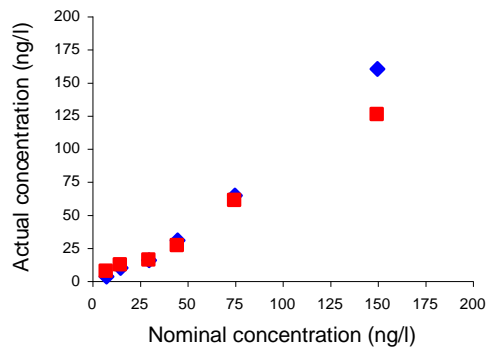
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654   Figure 2

655   i.
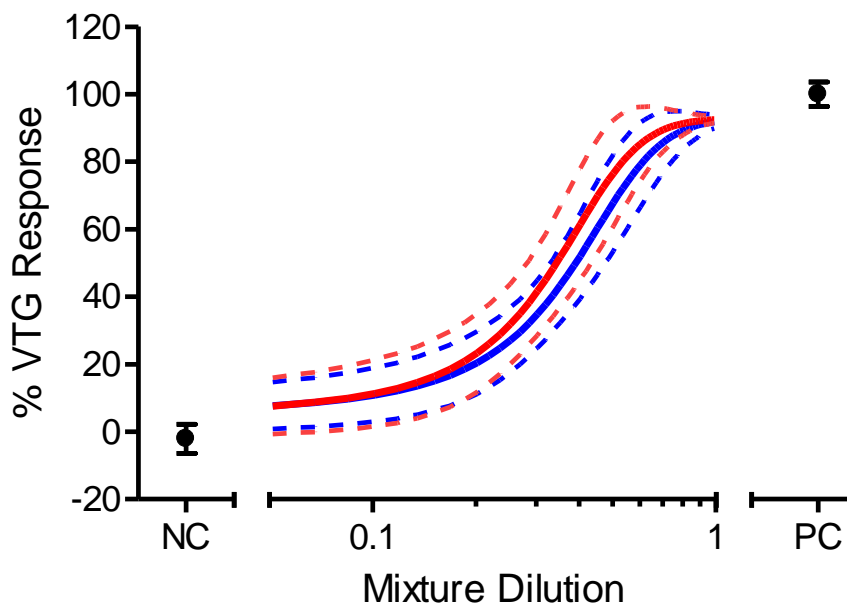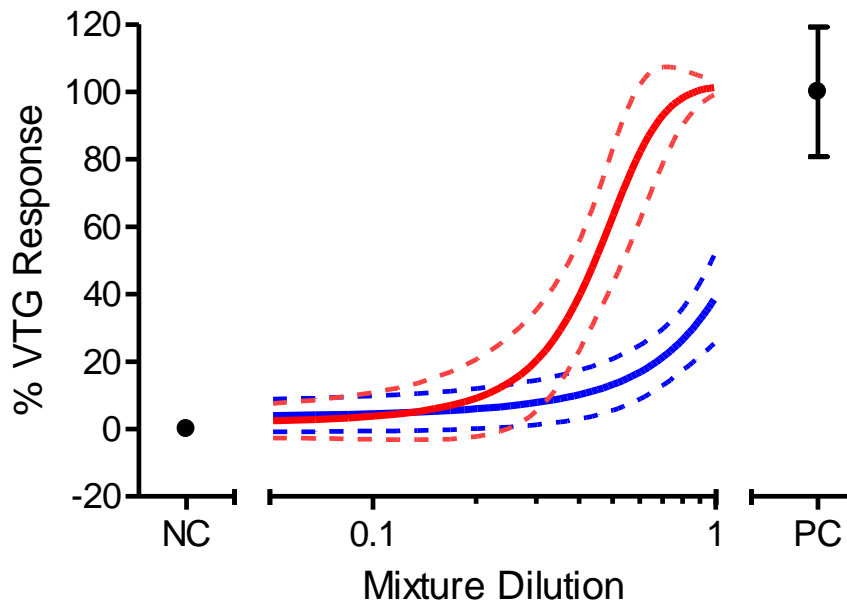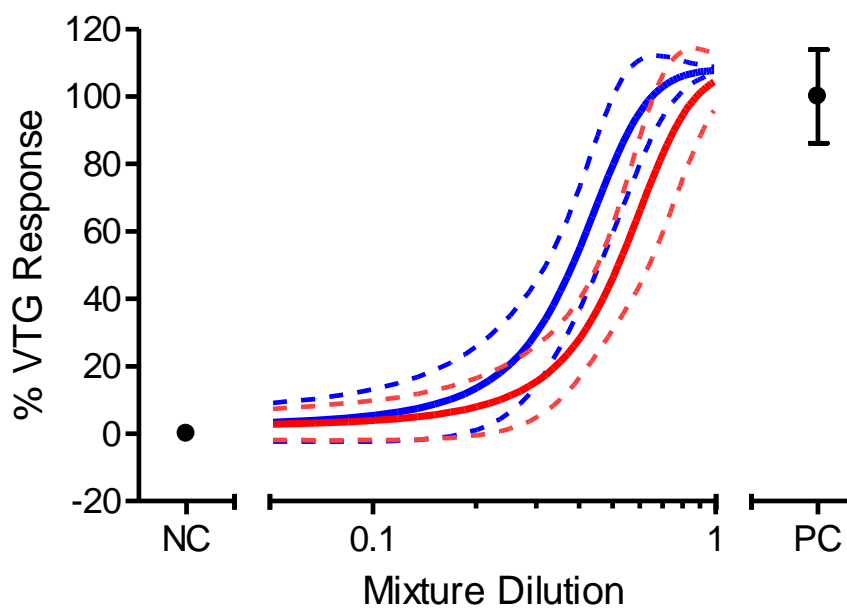

656

657   ii.

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Figure 3

i.

ii.
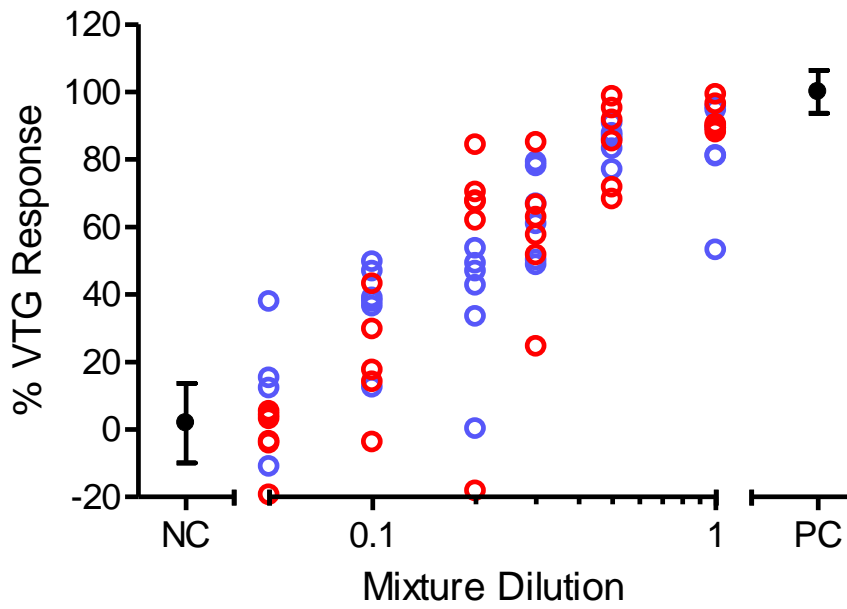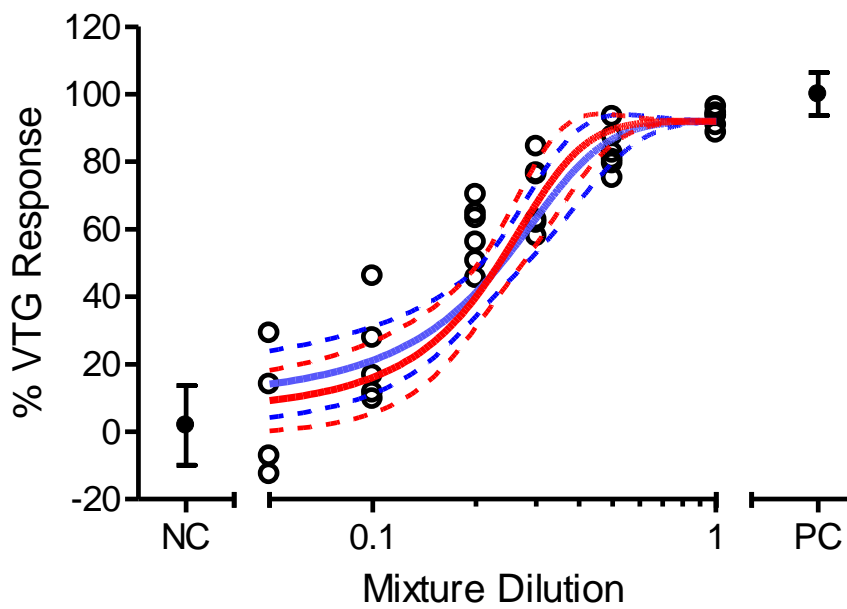
665   Figure 4

666   i.

667

668   ii.

669

670