HYPOXIA DOES NOT INFLUENCE THE RESPONSE OF FISH TO A MIXTURE OF ESTROGENIC CHEMICALS

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ABSTRACT

Chemical risk assessment procedures assign a major role to standardised toxicity tests, in which the response of a particular organism to a single test substance is determined under otherwise constant and favourable conditions in the laboratory. This approach fails to consider the potential for chemical interactions, as well as failing to consider how the toxicological response varies, depending on the conditions of exposure. As yet, the issue of confounding factors on chemically-mediated effects in wildlife has received little attention, despite the fact that a range of physicochemical parameters, including temperature, water quality and pH, are known to modify chemical toxicity. Here, we consider how the estrogenic response of fish varies with regard to hypoxia. Fathead minnows (*Pimephales promelas*) were exposed to a mixture of estrogenic chemicals under hypoxic or normoxic conditions. Their estrogenic response was characterised using an *in vivo* assay, involving the analysis of the egg yolk protein, vitellogenin (VTG). The results revealed that there was no effect of hypoxia on the VTG response in either treatment group at the end of the exposure period. This suggests that this endpoint is robust and relatively insensitive to the effects of any physiological changes that arise as a result of hypoxia. The implications of these negative findings are discussed in terms of their relevance with regard to the development of risk assessment policy.

KEY WORDS:

Fathead minnow, multiple stress, hypoxia, endocrine disruption, estrogen, mixture, vitellogenin.

1. INTRODUCTION

Hypoxia is a phenomenon that occurs in both marine and freshwater environments, affecting many thousands of km^2, worldwide. In this context, it is defined as dissolved oxygen (DO) levels of less than 2.8 mg/l (*1*). These conditions are generally of detriment to the survival of aquatic organisms, having been associated with mass mortalities, benthic defaunation and declining fisheries production (*2*). Although hypoxia can occur as a result of natural stratification in some systems, through the formation of haloclines and thermoclines, the incidence and extent of this phenomenon has increased in recent decades as a result of excessive inputs of nutrients and organic matter into water bodies with poor circulation. An example is provided by the situation in the northern Gulf of Mexico, where the hypoxic region has averaged over 15,600 km^2 in size since 1993, as a result of the increased use of nitrate fertilisers (*3*). Situations such as this are likely to be exacerbated in the future due to the increase in the intensity of agricultural practices and the rate of human population growth in coastal areas, combined with the impacts of global climate change (*4*).

Fish have developed two main strategies for coping with hypoxia. The first is to invoke various behavioural and physiological responses that increase oxygen delivery. For example, ventilation rates are increased and glycolysis, with lactic acid as an end product, is induced to resist the effects of hypoxia in the short term (*5*). A second strategy, which may be invoked following prolonged exposure, is to conserve energy by metabolic suppression (*2*). This is apparent from the analysis of gene expression patterns in the mudsucker, *Gillichthys mirabili*, which revealed that cellular growth is suppressed under hypoxic conditions in order to allow energy to be channelled into

essential metabolic processes (*6*). However, in contrast with their capacity to protect against hypoxia, it would appear that these responses are associated with a reduction in the tolerance of fish to simultaneous chemical challenge. Increased toxicity under low oxygen conditions has been demonstrated for a range of micro-pollutants (e.g. cyanide, ammonia) and for some metals (e.g. copper, cadmium), leading to reduced survival of fish in multiple stress exposure situations (*7-11*). This phenomenon may be linked to the enhanced uptake of toxicants under hypoxic conditions; there is an apparent link between DO, ventilation rate and toxicity (*9*). However, the evidence surrounding this issue remains equivocal (*8*).

Currently, little is known about the potential influence of hypoxia on the response of fish to endocrine disrupting chemicals (EDCs), such as the environmental estrogens. However, this issue is pertinent for two reasons. Firstly, the input of nutrients into the environment from anthropogenic sources often coincides with the presence of EDCs (e.g. in sewage treatment works effluent), creating multiple stress exposure situations. Secondly, there is growing evidence that hypoxia can, on its own, cause endocrine-mediated disturbances in fish. Whilst the mechanism(s) responsible are still under investigation, it would appear that changes in the hormonal balance of the common carp, *Cyprinus carpio*, that occur in response to hypoxia are associated with retarded gonadal development, reduced spawning success, sperm motility, fertilisation success, hatching rate and larval survival (*12*). Subsequent research has revealed effects on sex differentiation and development in the zebrafish, *Danio rerio*, leading to male-dominated populations (*13*). In view of this evidence, it seems likely that hypoxia may act as a confounding factor in determining the way in which fish respond to chemical challenges mediated via the endocrine system.

The environmental literature rarely considers the influence of physicochemical factors on endocrine mediated effects, which is probably due, at least in part, to the difficulty in designing appropriate experiments for detecting these highly complex interactions. However, some insight into influence of hypoxia on the estrogenic response can be garnered from the biomedical field. For example, cancer research, using microarray technology, has revealed that hypoxia and estrogen interact to modulate gene expression in an *in vitro* study, involving human breast cancer cells (*14*).

In the present study, we compare the response of fathead minnows (FHM; *Pimephales promelas*) exposed to a mixture of estrogenic chemicals under hypoxic vs. normoxic conditions, using an *in vivo* assay that is based on the induction of egg yolk protein (vitellogenin; VTG) synthesis in male fish (*15*). There is already evidence to suggest that hypoxia is associated with altered VTG levels in wild estuarine fish, as well as those maintained under laboratory conditions (*16*). However, here, we will consider the influence of hypoxia on the VTG response of male fish stimulated by exposure to a defined mixture of estrogenic chemicals. The data generated will contribute to our understanding of the risks that exist in multiple stress exposure situations, which is of relevance with regard to the development of risk assessment methodology.

2. MATERIALS AND METHODS

2.1 Experimental Design

The design of this investigation is based on that of a previous study by Brian et al. (*17*), in which the response of male FHM to a defined mixture of estrogenic chemicals was characterised, using the induction of plasma VTG as an endpoint, following an exposure period of two weeks. The mixture consisted of the endogenous steroidal

estrogen, 17β-estradiol (E2) and the synthetic steroidal estrogen, 17α–ethinylestradiol (EE2), as well as three other 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). Stocks of E2 (98% purity), EE2 (98% purity), OP (97% purity) and BPA (99% purity) were purchased from Sigma Aldrich, Dorset, UK. NP (99% purity) was obtained from ACROS Organics, Leicestershire, UK. Each of the chemicals was combined at a fixed ratio, based on their potency with regard to the induction of VTG. The joint action of these chemicals is known to be consistent with predictions based on concentration additivity (CA; *17*).

A master stock, containing each component of the mixture at a concentration that was known to elicit a 50% response with regard to the induction of VTG (i.e. its EC50), was prepared in dimethylformamide (DMF; VWR International, Leicestershire, UK). This master stock, which comprised 13.5 μg/l EE2, 375 μg/l E2, 105 mg/l NP, 675 mg/l OP and 2.25 g/l BPA, was then diluted in DMF to produce five further stocks that were 0.5, 0.3, 0.2, 0.1 and 0.05 of the original mixture concentration. The stock solutions were diluted by 1:15000 with de-chlorinated tap water (pre-heated to 25 ºC) prior to delivery to the experimental tanks. This flow-through exposure system is described in more detail in an earlier publication (*17*).

The resulting mixture concentrations in the fish tanks were sufficient to cover the full extent of the concentration response curve in fish maintained under normal oxygen conditions (7 mg/l DO ± 1 mg/l; *17*). A solvent control tank was run alongside those containing each of the various dilutions of the mixture. This was dosed with a stock of pure DMF, which was delivered at the same rate as the mixture-treated tanks.

141 The chemical dosing commenced one week before the start of each exposure study.
142 This conditioning process ensured that the chemical concentrations in the tanks were
143 accurate. Analytical chemistry was used to verify the water concentrations in samples
144 collected immediately prior to the addition of the fish and after one week of exposure.
145 A third and final set of samples was collected on the day that the exposure study was
146 terminated. The phenolic compounds (NP, OP and BPA) were measured by direct
147 injection onto a reverse phase HPLC column, according to the methods described by
148 Pojana et al. (*18*). The steroids (E2 and EE2) were analysed by RIA, using the
149 technique outlined by Länge et al. (*19*).

150 *2.2 Protocol*

151 Two exposure studies were set up, in parallel, according to the design outlined above.
152 One set of tanks was maintained under hypoxic conditions (<2 mgO_2/l). This was
153 achieved by bubbling nitrogen gas through the tanks, which displaced the oxygen in
154 the water. Each tank was supplied with a close fitting glass lid with a hole at either
155 end to allow the delivery of water and pressurised nitrogen to the tanks, via silicone
156 tubing. The nitrogen, which was fed by a series of nitrogen cylinders with individual
157 flow controls to each tank, was then diffused into the water using a 15 cm ceramic air
158 stone (PlanetRena, Charlotte, NC, USA). The second set of tanks was set up in an
159 identical manner, except that they were supplied with pressurised air, as opposed to
160 nitrogen. It was expected that the oxygen conditions in these tanks would be close to
161 100% saturation (7-8 mg/l in our system), thereby representing normoxic conditions.

162 One week prior to exposure, whilst the experimental tanks were being conditioned,
163 male fathead minnows were selected from our laboratory-reared stocks. These fish
164 were split into two groups before being transferred to two sets of holding tanks. In

one set of holding tanks, the fish were equilibrated to hypoxic conditions. This was achieved by increasing the flow of nitrogen into the tanks such that the oxygen levels were reduced by approximately 1mg/l each day. In the other set of tanks, the flow rate of air was increased in a similar manner. At the end of the week the fish in each group were randomly allocated to the treated tanks (8 per tank).

During the equilibration period and the experiment itself, the fish in each treatment group were fed twice daily: once with frozen brine shrimp and once with flaked fish food. The photoperiod was maintained on a 16 hr light/8 hr dark cycle with 20 minute dawn and dusk transition periods. The DO concentrations in each tank were recorded several times daily using an Oxi 340i digital meter and CellOx® 325 probe (WTW; Weilheim, Germany). Water temperature was also measured daily. Various other water quality parameters (i.e. ammonia, nitrite and nitrate) were analysed at routine intervals to ensure that there were no differences between the two sets of fish tanks, aside from the oxygen availability.

2.3 Sampling and Analysis

At the end of the experiment, the fish were sacrificed by overdose with anaesthetic (MS222; Sigma Aldrich). Their lengths and weights were recorded. Blood samples were then collected from the caudal peduncle using heparinised capillary tubes. The blood samples were centrifuged at 4000 g for 5 minutes and the plasma drawn off and snap frozen on dry ice. The plasma samples were then stored at –20 ºC until required for the determination of VTG protein levels. This was carried out using a FHM VTG ELISA kit, which was supplied by Biosense Laboratories AS (Bergen, Norway).

2.4 Statistical Analysis

A series of statistical tests were performed on the physicochemical and biological data sets. Whilst there was a clear difference between the DO levels in each of the parallel exposures, the levels measured within each set of tanks were compared statistically to determine their variability. This was achieved using the ANOVA procedure, followed by Tukey's pairwise comparisons. The chemical concentrations measured in each set of tanks at the start of the experiment were also compared statistically to ensure that there were no differences between the exposure levels. The measurements were first converted into proportions by dividing by the nominal values and comparisons were made between tanks with the same nominal exposure levels using paired t-tests. The VTG levels were also analysed, using t-tests, to compare the mean response of fish in each treatment group across the parallel exposures. Where necessary, these data were log transformed prior to analysis in order to achieve normality. The statistical testing was carried out using Minitab version 13.1 (Minitab Inc. State College, PA, USA).

3. RESULTS

3.1. Oxygen Conditions

There was a clear difference between the oxygen conditions in the parallel exposure studies (Figure 1). The mean daily DO concentration in the normoxic tanks ranged between 6.58 and 7.18 mg/l. Some of these values were slightly lower than the target of 100% oxygen saturation. This can be attributed to the fact that these tanks suffered from a slight bacterial build up towards the end of the exposure period. The bacterial levels tended to be higher in the tanks that contained more of the mixture, which is reflected by the trend of reducing DO with increasing level of exposure. However, despite of this, there were no significant differences between the DO levels in this set

211 of tanks and the DO concentrations were well within the range of encountered under
212 normoxic conditions.

213 In contrast, the mean daily DO levels in the hypoxic tanks during the exposure were
214 between 1.44 and 1.75 mg/l. These levels were less variable than those recorded in
215 the normoxic tanks, probably due to the lower levels of bacteria in the hypoxia tanks
216 (these factors may have been related). However, there was a statistically significant
217 difference ($p<0.01$) between the levels measured in the tanks containing the mixture
218 at a 0.05 and 1.0 dilution, which had the lowest and highest mean DO concentrations,
219 respectively. Nevertheless, the oxygen conditions within this set of tanks fell below
220 the hypoxic threshold of 2 mg/l throughout the period of exposure, thereby satisfying
221 the experimental criteria.

222 The differential growth of bacteria in each of the parallel exposures, which became
223 apparent at the beginning of the second week of exposure, raised concerns regarding
224 the chemical concentrations in each set of tanks. This was based on prior experience
225 indicating that bacterial blooms may be associated with increased rates of chemical
226 biodegradation, potentially lead to a reduction in the exposure levels in the affected
227 tanks (*20*). Hence, as a precaution, the decision was taken to terminate the experiment
228 early and, as a result, the duration of the exposure was reduced from 14 to 10 days.

229 *3.2. Chemical Concentrations*

230 The analysis of the chemical concentrations in each fish tank revealed that, in general,
231 there was good agreement between the nominal and actual exposure concentrations
232 measured at the start of the experiment (Table 1). There was also good agreement
233 between the concentrations measured across each of the parallel exposures. However,

234 BPA was an exception to this rule. Whilst the actual concentrations of this chemical
235 were close to nominal in the hypoxic tanks, these measurements were considerably
236 lower in the tanks maintained under normoxic conditions and, hence, a significant
237 difference ($p<0.05$) between the levels measured in the parallel studies was detected.
238 However, the agreement between the nominal and actual exposure concentrations in
239 the normoxic tanks improved at the second and third time points (see the supporting
240 information, S1 and S2, respectively), suggesting that the inconsistency between the
241 levels measured at the start of the experiment may have been an analytical anomaly,
242 although the values were consistently lower than those measured in the hypoxic tanks.
243 This may reflect the ease with which BPA is biodegraded in the presence of bacteria,
244 which was more prevalent in the normoxic tanks. In contrast, the other components
245 of the mixture (E2, EE2, NP and OP) appeared to be unaffected by the presence of the
246 bacteria, with their concentrations remaining consistent throughout the experimental
247 period.

248 *3.2. VTG Protein Induction*

249 The analysis of VTG induction levels at the end of the experiment (Figure 2) revealed
250 that there was a clear and consistent concentration-response to the mixture in each of
251 the parallel exposures. The potency was similar to that reported in previous work by
252 the same authors (*17, 20*) in that a 50% VTG response was induced by the 0.2 mixture
253 dilution, which contained each chemical at a fifth of its individual EC50. There was
254 no evidence that the VTG response of fish differed between the normoxic and hypoxic
255 conditions, as reflected by the fact that there were no significant differences detected
256 between the mean VTG levels within each treatment group.

257 **4. DISCUSSION**

The results of this study clearly demonstrate that the VTG response of FHM exposed to a mixture of estrogenic chemicals is similar under hypoxic vs. normoxic conditions. Hence, the data refute the hypothesis that the estrogenic response may be elevated at low oxygen levels, either as a result of increased chemical uptake or due to changes in the rate of physiological processing under varying physicochemical conditions. These findings contrast with evidence from similar studies involving the exposure of fish to micro-pollutants and metals. For example, recent research by Hattink et al. (*8*) revealed that common carp (*Cyprinus carpio)* are around three times more sensitive to the effects of cadmium under hypoxia (at 25% oxygen saturation) in relation to those maintained under normal oxygen conditions, although it was not possible to identify the mechanism responsible. The lack of response to hypoxia in the present study indicates that EDCs may not behave in the same way as other toxicants under hypoxic conditions and that the rate at which they are taken up and metabolised remains constant, regardless of oxygen availability. However, this theory is not consistent with *in vitro* data, which shows that hypoxia and estrogen treatment act together to affect molecular-level responses, leading to significant effects on gene expression profiles (*14*).

Whilst we would normally expect molecular responses, such as those reported by Seifeddine et al. (*14*), to be reflected at higher levels of biological organisation, it is possible that effects on VTG induction were not observed *in vivo* due to the influence of negative feedback processes. As a result, we cannot exclude the possibility that hypoxia was associated with effects on rates of chemical uptake and metabolism: these alterations may have acted against one another, thereby countering any overall effect. The potential for physiological interactions of this nature is highlighted by recent evidence that the expression of the estrogen receptor, ERα, is three-fold lower

283 in wild fish inhabiting hypoxic sites, compared to those at normoxic locations (*16*).
284 Presumably, differences in receptor activity have the capacity to affect the rate and
285 efficiency with which molecular responses are transcribed and subsequently translated
286 at the proteomic level, thereby altering the magnitude of the response to estrogenic
287 stimuli. It is therefore possible that changes in the expression of ERα could,
288 potentially, have masked any effects arising as a result of changes in the rate of
289 chemical uptake, although this hypothesis requires further investigation.

290 In addition, whilst there was no effect of hypoxia on the VTG response measured after
291 10 days of exposure, it is possible that differences may have been detected at earlier
292 time points (e.g. after 24 hours or 7 days). This response pattern has previously been
293 reported for FHM exposed to the same estrogenic mixture at different temperatures
294 (*21*). The transient nature of this response was attributed to an increase in the rate of
295 induction of the VTG response at higher temperatures, which was mediated via both
296 transcriptional and translational effects. After two weeks, however, these differences
297 were no longer apparent and the VTG response was identical for fish maintained at 20
298 and 30 °C. The same temporal pattern may have been apparent in the present study,
299 with fish maintained under hypoxic conditions exhibiting an elevated response to the
300 mixture at earlier time points due to an increased rate of chemical uptake under these
301 conditions. However, the potential for short-term effects on the estrogenic response
302 were not considered because the relevance of such transient alterations with regard to
303 chemical risk assessment remains unclear (*21*).

304 To conclude, the results of this study reiterate that mixtures of estrogenic chemicals,
305 or indeed any chemicals that act via a common mechanism, have the capacity to act
306 together to exert combined effects *in vivo*, as previously reported by Brian et al. (*17*,

20), thereby highlighting the need to take account of their joint effects. However, there was no evidence to suggest that the estrogenic response was confounded by the effects of an additional physicochemical variable, which was, in this case, represented by hypoxia, contrary to expectations based on previous studies. The lack of response indicates that the VTG response is robust and relatively insensitive to the effects of additional challenges that arise in multiple stress exposure situations. Hence, it would appear that existing safety factors are sufficient to protect against the effects of interactions with confounding factors, such as low oxygen conditions. Nevertheless, this conclusion should be interpreted with caution, as the response may vary, depending both on the nature of the physicochemical challenge and the characteristics of the toxicant in question. It is also possible that the response becomes more plastic at higher levels of biological organisation, which means that confounding factors may have a greater impact on endpoints that relate to survival and reproduction. Hence, despite the negative conclusion of the present study, there may be a need for greater stringency when assessing the risk posed by chemicals in the "real world", in which multiple stress exposure situations are the norm.

5. ACKNOWLEDGEMENTS

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6. SUPPORTING INFORMATION

The nominal and actual exposure concentrations in each set of tanks at time points 1 (after 7 days) and 2 (on the final day of exposure) are presented in tabular form in S1 and S2.

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Table 1: Nominal and actual chemical concentrations in each tank at the beginning of the parallel exposure studies. The water samples were collected immediately prior to the addition of the fish.

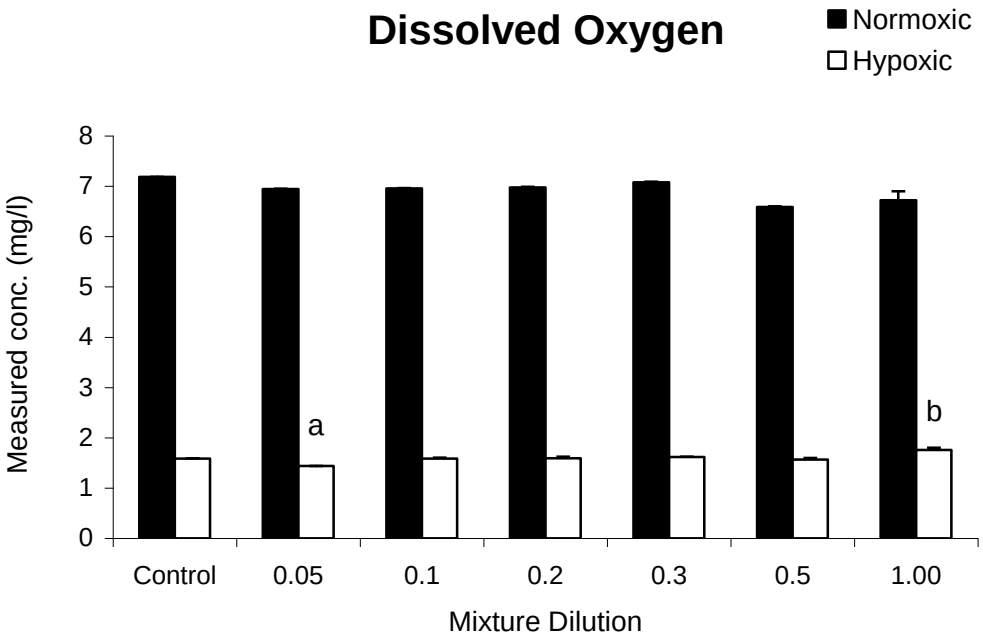
	Nominal					Hypoxic					Normoxic				
Treatment	EE2	E2	NP	OP	BPA	EE2	E2	NP	OP	BPA	EE2	E2	NP	OP	BPA
	ng/l	ng/l	μg/l	μg/l	μg/l	ng/l	ng/l	μg/l	μg/l	μg/l	ng/l	ng/l	μg/l	μg/l	μg/l
Tank 1: Control	0	0	0	0	0	0.0	0.3	0.3	0.0	0.0	0.0	0.1	0.3	0.0	0.2
Tank 2: 0.05 dilution	0.05	1.25	0.35	2.25	7.5	0.07	1.19	0.46	5.22	0.5	0.06	0.93	0.50	1.34	1.0
Tank 3: 0.1 dilution	0.09	2.5	0.7	4.5	15	0.14	2.39	0.65	2.38	16.6	0.13	1.82	0.69	2.55	9.8
Tank 4: 0.2 dilution	0.18	5	1.4	9	30	0.24	3.94	1.12	14.2	36.1	0.24	4.17	1.51	8.80	0.0
Tank 5: 0.3 dilution	0.27	7.5	2.7	13.5	45	0.39	6.36	1.59	13.6	52.3	0.38	6.86	1.86	15.0	0.3
Tank 6: 0.5 dilution	0.45	12.5	3.7	22.5	75	0.63	9.34	3.36	32.9	87.7	0.69	10.3	3.38	27.8	0.0
Tank 7: 1:0 dilution	0.9	25	7	45	150	1.68	19.2	5.15	57.6	169	1.26	21.5	6.12	59.4	12

LEGENDS

Figure 1: Mean of the daily dissolved oxygen (DO) concentrations in each tank throughout each of the parallel exposure studies. Daily DO concentrations were taken to be the average value recorded based on 6-10 measurements that were made on each day. Error bars represent one standard error of the mean. The letters (a and b) denote that there was a significant difference between the mean DO level in tanks containing the 0.05 and 1.0 mixture dilution under hypoxia. No differences were detected within the remaining tanks maintained under each set of oxygen conditions.

Figure 2: Mean of the plasma VTG concentrations in each tank following each of the parallel exposure studies. Error bars represent one standard error of the mean, which was calculated on the basis of measurements made from eight fish in each tank.

415 Figure 1:



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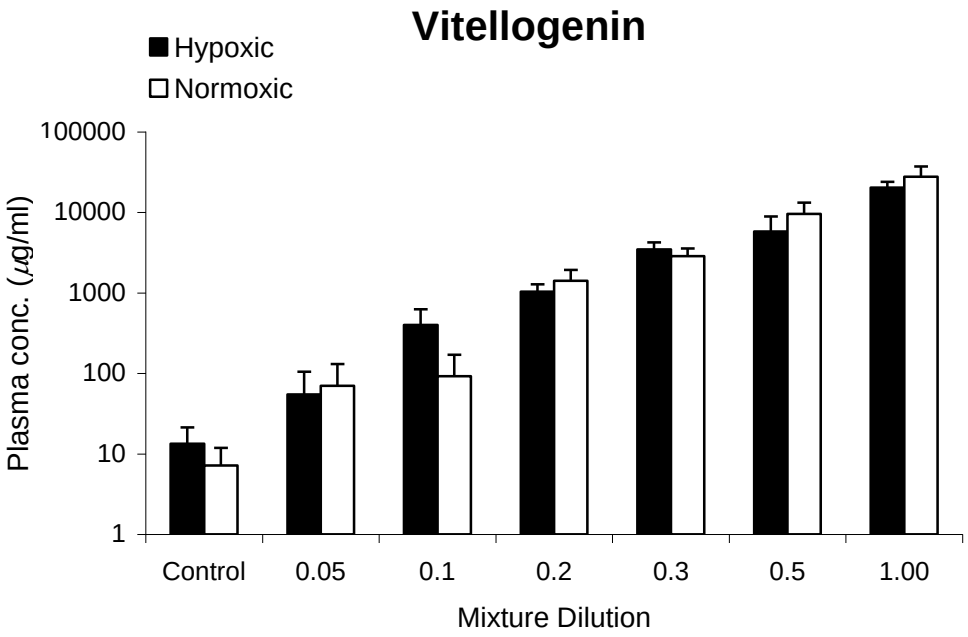
418 Figure 2:



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Brief:

The response of fish to a mixture of estrogenic chemicals is not affected by concomitant exposure to hypoxia.