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Quantification of vitellogenin mRNA induction in mosquitofish (*Gambusia affinis*) by reverse transcription real-time polymerase chain reaction (RT-PCR)

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Abstract

A method to quantify induction of vitellogenin (Vtg) mRNA in adult male mosquitofish was developed. Male mosquitofish were exposed to 0, 1, 20 and 250 ng l^{-1} 17β-oestradiol (E_2) for 4 and 8 days in static exposures, and liver Vtg mRNA and 18S rRNA expression were quantified in duplex RT-PCR. Liver 18S rRNA expression was very consistent among individuals, and there was a highly significant increase in Vtg mRNA expression after exposure of mosquitofish for just 4 days at 250 ng l^{-1} E_2. Lower doses did not induce Vtg mRNA expression even at 4 or 8 days. This method could be used as a rapid test to detect exposure of mosquitofish to oestrogenic chemicals. Further work is needed to determine if increased Vtg mRNA levels in male mosquitofish induce Vtg synthesis, and to determine the usefulness of the method in field sampling.

Keywords: *18S rRNA, LUX® primers, one-step RT-PCR, Vtg mRNA*

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Introduction

Vitellogenin (Vtg) is a glycolipophosphoprotein precursor to egg yolk produced in the liver of mature female fish under oestrogenic stimulation. Although it is not normally synthesized in males, the gene is present in both sexes and Vtg expression can be induced in males exposed to exogenous oestrogens (Denslow et al. 1999). This abnormal production of a female-specific protein in male fish has been used as a sensitive biochemical indicator of exposure to oestrogenic chemicals in several fish species (Folmar et al. 1996, Harries et al. 1996, Porter & Janz 2003, Nakari 2004).

Mosquitofish (*Gambusia affinis*, Baird and Girard 1853) are sexually dimorphic fish. Males have an elongated anal fin, the gonopodium, which is used during

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reproduction. Elongation of the anal fin in developing males is under androgenic stimulation from the maturing testes (Turner 1941), and can be stimulated in juveniles of both sexes by exposure to androgens or inhibited by exposure to oestrogens (Angus et al. 2001, Doyle & Lim 2002). This simple indicator of exposure to oestrogenic or androgenic stimulation, along with their restricted home range, abundance and near pan-global distribution (Bortone & Davis 1994, Overstreet et al. 1996, FishBase 2004), has made them a very popular species for diagnosing exposure to hormonally active chemicals in the environment (Batty & Lim 1999, Bortone & Cody 1999, Parks et al. 2001, Angus et al. 2002, Toft et al. 2003). Development of the gonopodium occurs in the first 40–60 days of life (Angus et al. 2001). Once fully developed, as indicated by the appearance of terminal hooks, it remains a permanent structure and does not grow or regress further under hormonal stimulation. This long duration and irreversibility make it impractical to use as a morphological indicator of exposure to oestrogens or androgens in caging studies. Alternative short-term indicators of exposure to hormones need to be developed if mosquitofish are to be used in such studies.

A method to measure plasma Vtg in mosquitofish using an immunoblot assay has been recently developed (Tolar et al. 2001), but this technique requires drawing blood, a difficult procedure in such a small fish species (adult males are on average 2 cm long). In this study, method development to measure liver Vtg mRNA induction in mosquitofish using RT-PCR is described.

Materials and methods

Fish

Mosquitofish were captured from Lake Tarawera, Rotorua, New Zealand, with a beach seine net. Examination of the tip of the gonopodium of adult males confirmed this species as *G. affinis* (i.e. the presence of internal spines on the distal end of ray 3; Rauchenberger 1989). Mosquitofish were transported back to the laboratory and kept in well-aerated 80-litre glass aquaria in water collected from the Tarawera River supplemented with 2‰ NaCl as a disease preventative and to reduce osmotic stress and maintained at approximately 25°C.

Exposure of fish to 17β-oestradiol

Eighty adult male mosquitofish were chosen at random and allocated in groups of 20 to four aerated 10-litre glass aquaria containing 8 litres of Lake Tarawera water supplemented with 0.2‰ NaCl. The water temperature was kept constant at approximately 25°C. After a 1-day acclimatization period, each aquarium received 50 µl ethanol containing 17β-oestradiol (E_2; Sigma-Aldrich, Sydney, Australia) at final concentrations of 0 (control), 1, 20 and 250 ng l^{-1}. Dissolved oxygen and temperature were measured daily with a YSI 55 meter (YSI, Inc., Yellowsprings, OH, USA). Half the water in each aquarium was replaced and a new dose of E_2 in 50 µl ethanol added daily for 8 days. Therefore, the concentrations reported are nominal. Fish were fed standard flake food (Nutrafin, Hagen, Canada) every second day.

On days 4 and 8, eight mosquitofish were removed from each of the four treatment tanks and anaesthetized with tricaine methanesulfonate (MS222, 0.1 g l^{-1}; Acros Organics, Belgium). Fish were killed by spinal severance and whole livers excised and

stored in 200 µl RNAlater® (Qiagen BioLab Ltd., Albany, New Zealand) in cryovials (Griener; Raylab, Auckland, New Zealand) at 4°C for 24 h, then at −80°C for long-term storage. All fish manipulations were done pursuant to the New Zealand Animal Welfare Act (1999).

RNA extractions

All work surfaces and tools were wiped with RNase AWAY® (Molecular BioProducts, CA, USA) immediately before extractions. Total RNA was extracted from five livers (selected at random from the eight available) from each treatment group (0, 1, 10 and 250 ng l^{-1} E_2) at each time point (4 and 8 days) with RNeasy® MinElute™ spin columns following the protocol for tissues described in the RNeasy Micro Handbook (Qiagen).

Total RNA was quantified using a RiboGreen® kit (RediPlate™ 96 RiboGreen RNA quantitation kit; Molecular Probes, Alphatech, New Zealand) as per the manufacturer's instructions. In short, serial dilutions of the samples and an RNA standard curve were incubated with RiboGreen reagent in a 96-well plate for 10 min at room temperature. After incubation, fluorescence was read with a fluorometer (FLUOStar model 403, BMG Lab Technologies, Victoria, Australia) and compared with the RNA standard curve to determine the amount of total RNA in each sample.

Primer design

Mosquitofish Vtg mRNA was sequenced using the protocol described in Laurie (2004) (GenBank Accession Number DQ190844). Based on this sequence, LUX® primers were constructed using the LUX Designer software (Invitrogen, Victoria, Australia). The primer sequences are shown in Table I. The forward primer was labelled with a FAM fluorophore, while the reverse primer was unlabelled. The resulting Vtg mRNA amplicon had a design size of 92 bp.

Preliminary studies indicated that the β-actin gene was not stably expressed in mosquitofish livers (data not shown), and 18S rRNA was therefore selected as the reference housekeeping gene. The mosquitofish gene was not sequenced as a 250-bp as a conserved region of the 18S rRNA sequence was identified with GenBank (2004) sequences from several teleosts (*Oncorhynchus mykiss*, *Acanthopagrus latus*, *Chrysophrys major*, *Dentex dentex*, *Lateolabrax japonicus*, *Megalaspis cordyla*, *Pampus argenteus*, *Rastrelliger kanagurta*, *Siniperca chuatsi* and *Trichiurus haumela*) using DNAMAN (Lynnon Corp., Vaudreuil, QC, Canada). Again, LUX primers were designed using the LUX Designer software (Table I). The forward primer was labelled

Table I. Primer sequences and melting temperatures (T_m) for vitellogenin mRNA and 18S rRNA in the western mosquitofish (*Gambusia affinis*).

Primer	Sequence*	T_m (°C)
Forward mRNA	Vtg cactgg AGG GAT GGT ATC CAA GAA CCA GtG	64.5
Reverse mRNA	Vtg TTG CTC GCT ACG AAG ATT TGG A	64.2
Forward rRNA	18S catgc TGT GGG TGG TGG TGC AtG	63.8
Reverse rRNA	18S TGC CGG AGT CTC GTT CGT TA	64.1

*Lower-case letters indicate LUX-specific bases required for the hairpin structure of the primer in the unbound form.

with a JOE fluorophore, and the reverse primer was unlabelled. The resulting amplicon had a design size of 85 bp.

Reverse transcription and amplification of vitellogenin mRNA and 18S rRNA

Vtg mRNA and 18S rRNA were quantified in duplex using a one-step quantitative RT-PCR enzyme mix (SuperScript™ III Platinum® one-step quantitative RT-PCR system, Invitrogen) in an iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The reactions were performed in 96-well PCR plates sealed with PCR sealing tape (Bio-Rad) to prevent evaporation of the 50-µl reaction mix (1 µl SuperScript III/Taq polymerase enzyme mix, 25 µl 2 × reaction buffer, 1 µl RNase OUT, 1 µl Vtg forward primer (final concentration of 300 nM), 1 µl Vtg reverse primer (600 nM), 1 µl 18S forward primer (100 nM), 1 µl 18S reverse primer (100 nM), 9 µl RNase-free water, and 10 µl sample diluted for a final concentration of 5 ng hepatic total RNA in each well) during the reaction. The thermal cycler was programmed for one cycle at 50°C for 20 min (RT step), one cycle at 95°C for 2 min for denaturation of the reverse transcriptase (SuperScript III) and activation of the polymerase (Taq), 45 cycles of 95°C for 15 s followed by 60°C for 30 s for the PCR reaction, concluded by melting curve analysis from 55 to 91°C (180 cycles of 10 s, ramping of 0.2°C/cycle). The real-time fluorescence detector was set for JOE-530 (18S rRNA primers) and FAM-490 (Vtg mRNA primers).

Threshold cycles (C_t values) for Vtg mRNA and 18S rRNA were estimated by analysis of the amplification curves with iQ 3.0a (Bio-Rad). ΔC_t, a measure of the relative induction of Vtg mRNA, was calculated by subtracting the 18S rRNA C_t values from the Vtg mRNA C_t values.

Agarose gel electrophoresis

Several amplification products of the RT-PCR were run on agarose gel electrophoresis: (1) Vtg mRNA primers and 5 ng hepatic total RNA from an adult male exposed to 250 ng l^{-1} E_2 for 8 days; (2) Vtg mRNA primers and 5 ng hepatic total RNA from a gravid female; (c) Vtg mRNA primers and 5 ng hepatic total RNA from an adult male exposed to 0 ng l^{-1} E_2 (control) for 8 days; and (d) 18S rRNA primers and 5 ng hepatic total RNA from an adult male. The RT-PCR products (10 µl) were loaded with 5 µl loading dye and run for 1 h at 100 V in 3.5% agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0). The gel was stained with ethidium bromide for 10 min, destained for 2 min, and a digital photograph of the gel was taken with GelDoc 2000 (BioRad).

Statistical analysis

All statistical tests were undertaken with SPSS 10.0 for Windows (SPSS, Inc., Chicago, IL, USA). The critical level of significance was set at $p = 0.05$ for all tests. A one-way analysis of variance (ANOVA) was used to test for differences in temperature and dissolved oxygen (DO) among the different exposure tanks. Differences in 18S rRNA C_t, Vtg mRNA C_t and ΔC_t values were tested separately for each exposure duration (4 and 8 days) with a one-way ANOVA followed by Bonferroni's test for multiple comparisons.

Results

Exposure conditions

The average temperature over the 8 days of exposure was 25.0°C (range 23.3–27.5°C). The average DO was 7.86 mg l^{-1} (range 7.48–8.40 mg l^{-1}). There were no significant differences among any of the tanks in either temperature or DO.

Amplification products

RT-PCR with Vtg mRNA primers and RNA from an adult male exposed to 250 ng l^{-1} E_2 for 8 days produced a single amplicon with a melting temperature (T_m) of 80.6°C, a $C_t = 25.9$, and a size slightly less than 100 bp (Figure 1, lane 2). With Vtg mRNA primers and RNA from a gravid female, an amplicon with the same T_m and size was obtained (Figure 1, lane 3), but with a slightly lower $C_t = 25.3$. With Vtg mRNA primers and RNA from an adult male exposed to 0 ng l^{-1} E_2 for 8 days (control), an amplicon with a similar $T_m = 80.8$°C was obtained, but with a much higher $C_t = 37.3$ it was barely visible on the gel (Figure 1, lane 5). Finally, with 18S rRNA primers and RNA from an adult male, a single amplicon with a melting temperature of 83.0°C, a $C_t = 10.5$, and a clear band at slightly more than 75 bp was produced (Figure 1, lane 4).

C_t and ΔC_t values

There were no significant differences in 18S rRNA C_t values among any of the treatment groups after either 4 or 8 days of exposure (one-way ANOVA, $p = 0.201$ and 0.763, respectively; Table II). Melting curve analysis revealed a single peak with a T_m of 82.9 ± 0.03°C (mode = 83.0°C).

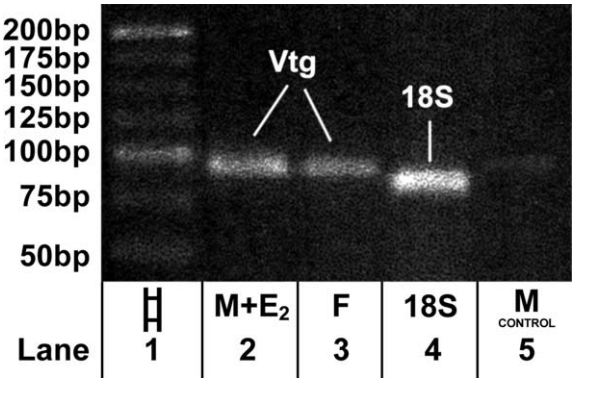

Figure 1. Agarose gel image of the reverse transcription-polymerase chain reaction (RT-PCR) amplification products in exposed and control mosquitofish (*Gambusia affinis*). Lanes 1, DNA ladder; 2, single amplicon of approximately 90 bp after RT-PCR with vitellogenin (Vtg) primers and total RNA isolated from the liver of an adult male exposed to 250 ng l^{-1} of 17β-oestradiol (E_2) for 8 days ($M+E_2$); 3, single amplicon of approximately 90 bp after RT-PCR with Vtg primers and total RNA isolated from the liver of a gravid female (F); 4, single amplicon of approximately 80 bp after RT-PCR with 18S primers and total RNA isolated from the liver of an adult male; 5, no amplification products after RT-PCR with Vtg primers and total RNA isolated from the liver of an adult male from the control group (after 8 days of exposure to 0 ng l^{-1} of E_2).

Table II. Vitellogenin (Vtg) mRNA and 18S rRNA expression in adult male mosquitofish after 4 and 8 days of exposure to 17β-oestradiol (E_2).

Oestradiol (ng l^{-1})	18S rRNA $C_t \pm$SE	Vtg mRNA $C_t \pm$SE	$\Delta C_t \pm$SE*	$-\Delta\Delta C_t$	Vtg mRNA induction†
Day 4:					
0 (control)	9.6 ± 0.3	38.6 ± 0.7	29.0 ± 0.6	0.0	1.0 ×
1	10.2 ± 0.3	38.8 ± 0.5	28.5 ± 0.5	0.5	1.4 ×
20	10.3 ± 0.3	38.9 ± 0.5	28.6 ± 0.4	0.5	1.4 ×
250	9.8 ± 0.2	29.4 ± 1.4‡	19.6 ± 1.4‡	9.4	690 ×
Day 8:					
0 (control)	10.3 ± 0.2	38.8 ± 0.9	28.5 ± 1.0	0.0	1.0 ×
1	10.3 ± 0.2	39.5 ± 0.5	29.2 ± 0.5	−0.7	0.6 ×
20	10.3 ± 0.3	38.0 ± 0.9	27.6 ± 0.9	0.8	1.8 ×
250	10.0 ± 0.3	25.4 ± 0.8‡	15.4 ± 0.7‡	13.1	8500 ×

*Vtg mRNA expression relative to the housekeeping gene 18S rRNA is calculated as: ΔC_t = (Vtg mRNA C_t) − (18S rRNA C_t).
†Vtg mRNA induction relative to the control group (0 ng l^{-1}) is computed as $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t$ = (treatment ΔC_t) − (control ΔC_t).
‡Significant deviation from the respective control group (Bonferroni's, $p < 0.001$, $n = 5$).

There was a significant effect of E_2 treatment on Vtg mRNA C_t after both 4 and 8 days of exposure (one-way ANOVA, $p < 0.001$ in both cases). However, only mosquitofish exposed to the highest E_2 concentration (250 ng l^{-1}) had significantly lower C_t values than the control group after both 4 and 8 days (Bonferroni's, $p < 0.001$ in both cases; Table II). Melting curve analysis produced a single peak with a T_m of 80.4 ± 0.05°C (mode = 80.6°C).

Consequently, there was a significant effect of E_2 exposure on relative Vtg mRNA expression (ΔC_t) at both 4 and 8 days (one-way ANOVA, $p < 0.001$ in both cases), but only mosquitofish exposed to the highest concentration had a significantly higher Vtg mRNA expression relative to 18S rRNA expression (as indicated by the lower ΔC_t; Bonferroni's, $p < 0.001$; Table II and Figure 2). This relative expression translated into a 690-fold induction of Vtg mRNA in mosquitofish exposed for 4 days to 250 ng l^{-1} E_2, and a 8500-fold induction after 8 days of the same exposure (Table II).

Discussion

The RT-PCR method described here allowed quantification of a significant Vtg mRNA induction in adult male mosquitofish after just 4 days of static waterborne exposure to 250 ng l^{-1} E_2 (Figure 2). This is similar to the results reported by Denslow et al. (2001), where male sheepshead minnows (*Cyprinidon variegatus*) exposed to 100 ng l^{-1} E_2 in a flow-through system showed a significant induction of the Vtg mRNA after just 2 days. Due to their relatively quick disappearance from the water phase, the potency of the tested chemicals is often underestimated in static exposures. This may explain the apparent lower sensitivity of mosquitofish to E_2 compared with sheepshead minnows. Most other studies on Vtg mRNA induction have been performed with injections of E_2 or waterborne exposure to the more potent, synthetic oestrogen, ethinylestradiol (EE_2). Lattier et al. (2001) measured a significant Vtg mRNA induction in male carp (*Cyprinus carpio*) after 24 and 48 h of an injection

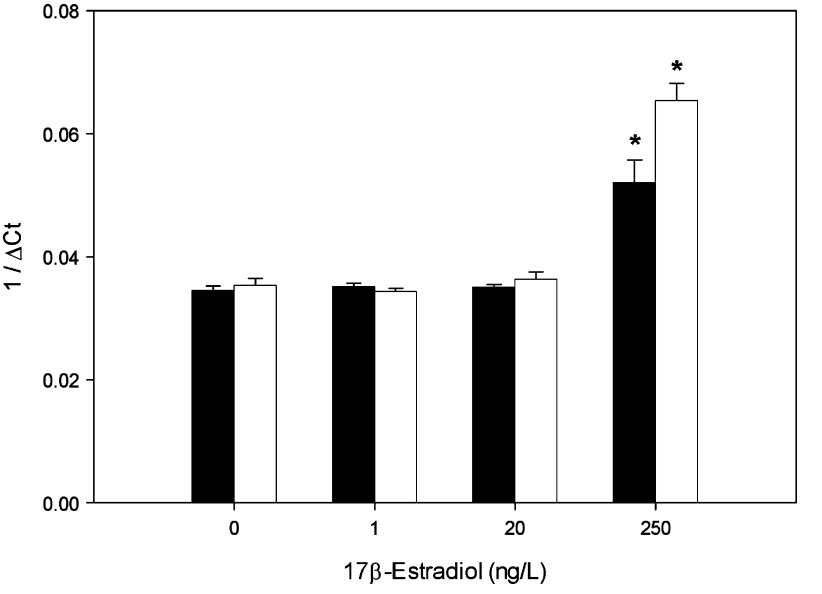

Figure 2. Induction of vitellogenin (Vtg) mRNA relative to the 18S rRNA housekeeping gene in adult male mosquitofish exposed to 0, 1, 20 and 250 ng l^{-1} 17β-oestradiol for 4 (■) and 8 days (□). Induction is expressed as $1/\Delta C_t$, where ΔC_t is the difference between the amplification threshold cycle (C_t) for Vtg mRNA and the C_t for 18S rRNA. *Statistically significance deviation from the control value (0 ng l^{-1}) at that sampling time.

of 0.033 mg E_2 kg^{-1} body weight using an RT-PCR protocol similar to that described in the present study. Juvenile fathead minnows (*Pimephales promelas*) injected with a high dose of 5 mg E_2 kg^{-1} body weight showed a significant induction of Vtg mRNA within 24 h (Thomas-Jones et al. 2003). A significant Vtg mRNA induction was detectable in mature male Japanese medaka (*Oryzias latipes*) exposed to 25 ng l^{-1} EE_2 for 7 days (Islinger et al. 2002). In this study, static exposure of adult male mosquitofish to 20 ng l^{-1} E_2 for 8 days did not significantly induce Vtg mRNA. However, EE_2 is more potent than E_2 *in vivo* (Islinger et al. 2002), and exposure to EE_2 is about twice as potent as the same dose of E_2 at inducing Vtg production in sheepshead minnows (Folmar et al. 2002). Therefore, it is not clear if mosquitofish are less sensitive than other species to induce Vtg mRNA, or if the differences are due to different exposure methods (static versus flow through) and/or chemical (E_2 versus EE_2). A more complete gradient of E_2 exposure concentrations between 20 and 250 ng l^{-1} and exposure to EE_2 would help determine the sensitivity of mosquitofish relative to other species.

Real-time RT-PCR reactions are so sensitive to minute quantities of RNA that variations in RNA extraction efficiencies or minor pipetting inconsistencies can have a significant effect on the resulting C_t values (Bustin 2002). This problem was overcome in the present study by the use of an internal RNA standard. Ribosomal RNA has been suggested as a stable internal RNA normalizer (Zhong & Simmons 1999), and 18S rRNA was chosen as the housekeeping gene in this study. However, expression of 18S rRNA in mosquitofish liver was very high and the amount of total RNA added to each well had to be diluted to 5 ng per microplate well to obtain a sufficient baseline for accurate estimation of C_t. Unfortunately, this dilution also decreased the number

of copies of the Vtg mRNA template and hence the sensitivity of the Vtg mRNA amplification. Use of a relatively less expressed housekeeping gene (such as β-actin) that would require less dilution of the samples may increase the sensitivity of this method. Nevertheless, expression of 18S rRNA was remarkably stable, with C_t values for all exposure groups around ten cycles (Table II) when 5 ng total hepatic RNA were added to the reaction well. This stable expression suggests that it is a reliable housekeeping gene in *G. affinis* liver.

Compared with other assays to measure Vtg in mosquitofish, this RT-PCR method was sensitive and a significant induction was measurable after a very short period. No other study has measured the effect of waterborne steroids on Vtg in mosquitofish, but an increase in plasma Vtg (measured by immunoblot assay) after 7 days of a dietary exposure was observed at 10 μg ethinylestradiol (EE_2) g^{-1} food (Tolar et al. 2001). No data are available on the comparability of dietary and waterborne exposures to steroids in mosquitofish, so an extrapolation is difficult to evaluate. In studies using rainbow trout, juvenile fish exposed for 7 days to 100 ng l^{-1} EE_2 had about half the plasma Vtg levels of trout exposed to 10 μg EE_2 g^{-1} food for the same period (Verslycke et al. 2002). Without taking into account potential species differences, a similar relationship (i.e. 10 μg g^{-1} food is equivalent to 200 ng l^{-1}) for mosquitofish would roughly correspond to the equivalent waterborne threshold for the immunoblot assay after 7 days of exposure to 200 ng l^{-1} EE_2, or the equivalent of 400 ng l^{-1} E_2. This speculative exercise helps justify the 250 ng l^{-1} E_2 used in this study. From a practical point of view, however, the RT-PCR method is easier to carry out than the immunoblot method, as extraction of whole livers from mosquitofish carcasses is relatively simple and could potentially be carried out in the field (with RNA adequately preserved in RNAlater solution).

Gonopodium development in mosquitofish juvenile males was significantly affected after 84 days of flow-through exposure to 100 ng l^{-1} E_2 (Doyle & Lim 2002). A significant oestrogenic effect could be measured within 4 days of exposure to a slightly higher concentration (250 ng l^{-1} E_2) with the RT-PCR method described here. The shorter period required before a significant effect was measurable means that the RT-PCR method could be used in short-term field-caging studies to measure oestrogenic induction at the caging site. It may also be used to determine if wild mosquitofish populations are being exposed to oestrogenic chemicals, although the effect of long-term exposure to oestrogenic stimulation on Vtg mRNA is not known. Studies with male sheepshead minnows suggest that Vtg mRNA levels remain high even after 3 weeks of a continued waterborne exposure to low levels of oestradiol (100 ng l^{-1} E_2) (Denslow et al. 2001). This suggests that male mosquitofish chronically exposed to oestrogenic chemicals would continue to have higher levels of Vtg mRNA than reference male fish.

More research is needed to link the increase in Vtg mRNA levels in male mosquitofish with increased Vtg synthesis, and to determine the usefulness of the method in field monitoring.

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