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Measurement of vitellogenin gene expression by RT-PCR as a tool to identify endocrine disruption in Japanese medaka (*Oryzias latipes*)

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In order to monitor vitellogenin gene expression in the Japanese medaka (*Oryzias latipes*), a reverse transcription-polymerase chain reaction (RT-PCR) system was developed. To date cDNA for medaka vitellogenin has not been published; therefore, initially a sequence fragment had to be obtained and compared with other known vertebrate vitellogenins. For this, a 1.2 kb cDNA of medaka vitellogenin (M-Vg1.2) was amplified by RT-PCR and cloned into a pCR® II-TOPO bacterial vector. On Northern blot analysis, the antisense cRNA of M-Vg1.2 stained a 5.5 kb gene product found exclusively in female fish, but not in males. Additionally, the 5′-end of medaka vitellogenin cDNA was amplified by 5′-RACE-PCR. The analysed nucleotide sequence of 1.6 kb shared significant similarities with vitellogenins known from other fish species: approximately 72% similarity with mummichog (*Fundulus heteroclitus*) vitellogenin I and approximately 62% with fathead minnow (*Pimephales promelas*) vitellogenin. To develop a semiquantitative RT-PCR for the measurement of vitellogenin gene expression, primers specific to a 500 bp sequence of the vitellogenin cDNA (M-Vg0.5) were constructed using the gene product of elongation factor 1α as internal standard. Induction of vitellogenin gene expression was measured in male medaka exposed to 0, 2, 20 and 50 $\mu g\,l^{-1}$ nonylphenol and 0, 2.5, 25 and 100 $ng\,l^{-1}$ 17α-ethinyloestradiol for 7 days. The LOECs for vitellogenin induction in male medaka were 20 $\mu g\,l^{-1}$ and 25 $ng\,l^{-1}$ for nonylphenol and 17α-ethinyloestradiol, respectively.

Keywords: vitellogenin, gene expression, medaka, RT-PCR, nonylphenol, ethinyloestradiol

Introduction

The presence of oestrogen-mimicking chemicals in the aquatic environment has been reported from most industrialized countries of the northern hemisphere (Pirie *et al*. 1996, Stumpf *et al*. 1996, Fytianos *et al*. 1997, Bennett and Metcalfe 1998, Ding and Tzing 1998). These so-called 'endocrine disrupters' belong to various classes of industrial products, including nonylphenol, bisphenol-A, phthalate esters and pesticides, as well as natural and synthetic oestrogens such as 17β-oestradiol, oestrone, 17α-ethinyloestradiol and phyto-oestrogens. Alterations in reproduction and development have not only been reported in wildlife (mammals: Facemire *et al*. 1995; birds: Birnbaum 1995; reptiles: Guillette *et al*. 1994; amphibians: Carey and Bryant 1995; fish: Leatherland 1992, Jobling *et al*. 1996; invertebrates: Fiorini *et al*. 1991, Matthiessen and Gibbs, 1998, Sangalang and

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Jones 1997), but also in laboratory experiments, which have shown that oestrogens or oestrogen mimics have the potential to affect the development of reproductive and nervous systems, behaviour and immune response in higher organisms (Colborn and Clement 1992, McCarthy 1994, Gimeno *et al.* 1997, Ogawa *et al.* 1997, Gray and Ostby 1998). The frequently weak yet undoubtable oestrogenic effect of many substances released into the environment has been demonstrated using numerous *in vitro* systems, including reporter gene assays in recombinant cells systems (Klein *et al.* 1994, Flouriot *et al.* 1995, Routledge and Sumpter 1996), oestrogen-responsive cell lines (Soto *et al.* 1991, Krishnan *et al.* 1993, Ren *et al.* 1997) and primary cultures of hepatocytes (Vaillant *et al.* 1988, Pelissero *et al.* 1993). These systems are capable of acting as sensitive bioassays for the detection of oestrogenic compounds in contaminated effluents as well as for the evaluation of the oestrogenic potential of a known xeno-oestrogen; however, *in vitro* systems cannot simulate the complex physiological endocrine network of an intact organism, especially with respect to bioaccumulation and biotransformation of xeno-oestrogens. Thus, the impact of endocrine-disrupting chemicals on the developmental and reproductive physiology of whole organisms has to be examined by means of *in vivo* experiments at different stages of development and over extended periods of time.

In oviparous vertebrates, the induction of vitellogenin gene expression is a commonly accepted biomarker of oestrogenic activity by xenobiotics or effluents from sewage treatment plants both *in vivo* (Purdom *et al.* 1994, Lech *et al.* 1996, Ren *et al.* 1996a, Harries *et al.* 1997) and *in vitro* in cultured hepatocytes (White *et al.* 1994, Flouriot *et al.* 1995, Peyon *et al.* 1996, Petit *et al.* 1997, Kloas *et al.* 1999). Vitellogenin is specifically synthesized in the liver of females under the control of 17β-oestradiol (E_2) via activation of its cognate receptor. Vitellogenin is secreted into the blood stream and finally taken up by the oocytes via receptor-mediated endocytosis (see Wallace 1985 for review). Normally, vitellogenin cannot be identified in male and immature female fish; however, its synthesis can be induced by exogenous stimulation by oestrogenic compounds. Vitellogenin can be measured both at the protein (Purdom *et al.* 1994, Harries *et al.* 1997) and the mRNA level (Flouriot *et al.* 1995, Ren *et al.* 1996a, Islinger *et al.* 1999). However, there is little information about the correlation between vitellogenin induction in male oviparous vertebrates and the formation of reproductive dysfunction or malformations affecting sexual differentiation.

The Japanese medaka (*Oryzias latipes*) is a small fish species (up to 4 cm in length) with a growth period of 3–4 months from fertilization to sexual maturity. The medaka is a differentiated gonochorist, i.e. indifferent gonads develop directly into either testis or ovary and no ovary-like stages exist in juvenile male fish (Yamamoto 1969). In the medaka, real sex chromosomes have been identified (Matsuda *et al.* 1998), and there is no evidence of spontaneously occurring intersexes or sex reversal (Yamamoto 1975). The medaka is a widely used model organism in reproductive biology and ecotoxicology, since it is easy to breed and maintain under laboratory conditions.

To date, however, vitellogenin gene expression in medaka has never been measured at the mRNA level. The measurement of vitellogenin at the protein level in serum by a Western blot technique has recently been published (Gronen *et al.* 1999). However, the measurement of vitellogenin in blood serum of male fish by enzyme-linked immunosorbent assay (ELISA) or Western blot depicts an

accumulation of this protein in the blood, which may lead to an overestimation of an oestrogenic response if compared with females. Since males are not able to incorporate vitellogenin into their gonads, a long half-life of 4 weeks in the blood stream has been reported, which is significantly different in females, where vitellogenin is rapidly taken up by the oocytes within 2 days (Wallace 1985). With respect to these findings, the measurement of vitellogenin at the mRNA level is a more sophisticated way to represent the actual time-dependent oestrogenic status of an organism or tissue, which is difficult for vitellogenin at the protein level because of its rapid export into the bloodstream (Ng and Idler 1983, Selman and Wallace 1983, Mañanos *et al.* 1994). Thus, the purpose of the present study was to clone and analyse a section of medaka vitellogenin cDNA for the development of a semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) assay. RT-PCR has successfully been used as a very sensitive technique for the detection of oestrogen-induced vitellogenin mRNA expression in rainbow trout (*Oncorhynchus mykiss*) and *Xenopus laevis* (Lech *et al.* 1996, Ren *et al.* 1996b, Kloas *et al.* 1999). Since only very small amounts of RNA are required, RT-PCR represents a sophisticated technique for the detection of vitellogenin gene expression in the liver of individual animals, even in small species such as the medaka. In order to validate the assay, induction of vitellogenin gene expression was measured after exposure of mature male medaka to nonylphenol and 17α-ethinyloestradiol, two chemicals that are known as oestrogen mimics and have already been tested in several *in vivo* and *in vitro* systems.

Materials and methods

Fish

Wild-type medaka (*Oryzias latipes*) were kept in well-aerated 50 l aquaria for routine culture at a temperature of $25 \pm 1^{\circ}C$ with a 12 h:12 h light cycle. Fish were fed with Tetramin^{TM} (Tetra, Melle, FRG) once daily. The dry food was supplemented with *Artemia* nauplii every second day. Carbonate concentrations (320 mg l^{-1}) and pH (7.7) were adjusted by automatic mixing of tap water and deionized water; oxygen concentrations were kept at $\geq$97% by aerating the water.

Exposure of fish to nonylphenol and 17α-ethinyloestradiol

Nonylphenol (techn.; Aldrich, Deisenhofen, FRG) and 17α-ethinyloestradiol (purity $\geq$ 98%; Sigma, Deisenhofen, FRG) were tested in 7-day exposure experiments at nominal concentrations of 2, 20 and 50 $\mu g\,l^{-1}$ and 2.5, 25 and 100 $ng\,l^{-1}$, respectively. Dimethyl sulphoxide (DMSO) was used as a solvent at a concentration of 0.001%. Solvent controls received 0.001% DMSO only. Since in untreated males medaka vitellogenin mRNA was found in at least trace amounts and DMSO is not reported to have any oestrogenic effects in vertebrates, a dilution water control was not included. In both experiments, five adult male medaka ($\sim$3.5 cm in length) per concentration were kept in 2 l aquaria with a daily water change of 1 l. Faeces and debris were removed with each water change. The aquaria were aerated with fresh air to keep the oxygen concentration $\geq$ 97% and the temperature was kept at 25°C using commercially available heating mats.

Preparation of RNA

For RNA preparation, kits based on either phenolic extraction or RNA adsorbance to spin columns were used. The fish were anaesthetized in benzocaine and opened ventrally. The liver was dissected, immediately frozen in liquid nitrogen and directly homogenized. For this, each liver was homogenized in 600 µl of the guanicline isothiocyanate-based lysis buffer supplied by the manufacturer using a rotor-stator homogenizer (Polytron, Kinematica, Littau/Luzern, Switzerland). To avoid RNAse degradation, a time-consuming weight determination of the dissected liver was not performed. For isolation and sequencing of a vitellogenin cDNA, total RNA was prepared from liver samples of 14 male and 14 female fish of the wild-type medaka stock. Total RNA was isolated by modified phenolic extraction with

the Roti Quick Kit (Roth, Karlsruhe, FRG). In both exposure experiments, liver RNA was prepared from the livers of individual fish with RNeasy Mini-Kit spin columns (Qiagen, Hilden, FRG). RNA contents and purity were determined by photometry at 260 and 280 nm. All RNA samples were stored in RNAse-free water at $< -20^{\circ}C$ until further use.

Amplification and cloning of a partial sequence of medaka vitellogenin

For amplification of a cDNA of medaka vitellogenin, primers for two short conserved regions of fish vitellogenin spanning a region of approximately 1.2 kb were constructed using the gapped Blast2.0 and Primer3 software and synthesized by MWG-Biotech, Ebersberg, FRG (M-Vg1.2+, 5′-GCT GAA CAT CAA GAA GAC CCA-3′; M-Vg1.2−, 5′-ACA ATG CAG GAC AGC ATA CG-3′). RT-PCR was performed with Gene Amp® RNA PCR Kit (Perkin Elmer, Branchburg, USA) using 1 µg of total RNA and oligo-dT_{16} primers for reverse transcription. Reverse transcription was accomplished with MulV reverse transcriptase (2.5 U μl^{-1}) for 15 min at 42°C, with a final 5 min denaturation step at 99°C.

PCR amplification was conducted over 30 cycles with 1.5 mM $MgCl_2$ using the following programme: initial denaturation step for 2 min at 95°C, 30 s at 95°C, 30 s at 60°C, 1 min at 72°C and a final extension step for 10 min at 70°C. The resulting 1.2 kb PCR product was cut out of a 1% agarose gel (Biozym, Hess. Oldendorf, FRG) and extracted with QIAquick Gel Extraction Kit (Qiagen) for further subcloning. The extracted M-Vg1.2 cDNA was directly cloned into a 3.9 kb pCR®II-TOPO vector (Invitrogen, Carlsbad, USA) in a 5 min ligation reaction at room temperature.

The ligation products were transformed by heat shock into competent Top10F′ *Escherichia coli* host cells (Invitrogen) and applied to IPTG/X-gal coated LB plates with ampicillin (50 µg ml^{-1}) and grown overnight at 37°C. Five colonies were selected and grown for an additional 24 h in 5 ml of LB medium (50 µg ml^{-1} ampicillin) at 37°C with orbital shaking at 250 r.p.m. The plasmid preparation was achieved using QiaPrep Spin Columns (Qiagen). Successful ligation was checked by EcoRI digestion (Stratagene, Heidelberg, FRG) of a small plasmid sample for 1.5 h.

5′-RACE-PCR was achieved using 5′-RACE System Version 2.0 (GIBCO Life Technologies, Karlsruhe, FRG). First-strand cDNA synthesis was performed with 500 ng of total RNA according to the user manual. The nucleotide sequence of the first gene-specific primer was 5′-TCT GAT TGG CAG AGA TGC AG-3′. The resulting cDNA was isolated by GlassMAX spin cartridges (GIBCO) and eluted in 50 µl of sterilized water. For TdT tailing, 10 µl of the purified cDNA sample were used. Amplification of target cDNA was achieved by a second gene-specific primer (5′-CCG TAA CGC TCA GAC ACA GG-3′) and the Abridged Anchor Primers provided by the kit. Cycle parameters were: 2 min at 94°C, 30 s at 94°C, 30 s at 62°C, 1 min at 72°C, and 7 min at 72°C for 35 cycles. After visualization of the PCR products in ethidium bromide-stained agarose gels, the band of interest was excised and purified using the QIAquick Gel Extraction Kit (Qiagen).

Vg1.2 and the 5′-RACE product were sequenced at Seqlab laboratories (Göttingen, FRG).

Northern blots

For Northern blots, digoxigenin-labelled antisense cRNA of M-Vg1.2 was produced using T7 polymerase and DIG-11-UTPs (Roche Diagnostics, Mannheim, FRG). Total RNA of 2 µg and 4 µg, respectively, from female and male liver were separated by 1.2% agarose formaldehyde gel electrophoresis; the integrity of the 28 s and 18 s RNAs were controlled by ethidium bromide staining of the gel. Blotting of RNA on positively charged nylon membranes (Qiagen) was performed by capillary elution according to Sambrook *et al.* (1989). RNA immobilization was accomplished by ultraviolet crosslinking with 120 mJ per side of the membrane (UV-Stratalinker, Stratagene). After crosslinking, the blots were incubated in hybridization buffer (5 × SSC, 50% formamide, 2% blocking reagent [Roche Diagnostics], 0.1% *N*-lauryl sarcosine, 0.02% sodium dodecyl sulphate [SDS]) for 2 h at 68°C. Then, vitellogenin mRNA was hybridized at 68°C with a digoxigenin-labelled M-Vg1.2 antisense cRNA overnight (100 ng ml^{-1} hybridization buffer). Filter membranes were rinsed twice at room temperature in 2 × SSC, 0.1% SDS, followed by three washes at 68°C in 0.1 × SSC, 0.1% SDS. One half of the membrane was subjected to additional RNAse digestion to distinguish between specific and unspecific staining. For this end, the blot was equilibrated for 10 min in buffer R (10 mM Tris, 300 mM NaCl, 5 mM ethylene diamine tetra-acetic acid [EDTA], pH 7.5), digested with 1 µg ml^{-1} RNAse A (Roche Diagnostics) in buffer R and washed twice with buffer R, once with 2 × SSC, 0.1% SDS. Detection was performed on both parts of the blot with anti-digoxigenin Fab fragments coupled to alkaline phosphatase and CDP-Star™ luminescence reagent (Roche Diagnostics). Hybridization signals were recorded on BioMax Light film (Eastman Kodak, Rochester, New York, USA). Size determination of staining products was achieved using a digoxigenin-labelled RNA molecular weight standard (Roche Diagnostics) in the same gels.

Semiquantitative RT-PCR of M-Vg0.5

For semiquantitative RT-PCR of vitellogenin mRNA, primers specific to a 500 bp region of M-Vg1.2 (M-Vg0.5+, 5′-GAC CTG GAA GCA TTC TGG AG-3′; M-Vg0.5−, 5′-TCT GAT TGG

CAG AGA TGC AG-3′) were constructed. In addition, primers specific to a 0.3 kb region of medaka elongation factor 1α (M-ELF0.3+, 5′-AGC GAC AAG ATG AGC TGG TT-3′; M-ELF0.3−, 5′-GGG CAC AGC TTC TGG TAA AG-3′) were selected as an internal standard (GenBank Acc No. AB013606). Reverse transcription of RNA samples was accomplished as described above for M-Vg1.2, and PCR amplification was performed in a 30 cycle programme using 30 s at 95°C for denaturation, 30 s at 57°C for primer annealing and 1 min at 72°C for DNA amplification using 1.5 mM $MgCl_2$ and 2.5 U per 100 µl Taq-polymerase. PCR samples were separated on 1.5% agarose gels. The ethidium bromide-stained gels were analysed densitometrically using the software EASY Win 32 (Herolab, Wiesloch, FRG).

Results

Preparation and identification of a medaka vitellogenin cDNA

In order to obtain information about the nucleotide sequence of medaka vitellogenin, a cDNA was generated by RT-PCR using hepatic RNA from mature female medaka and primer pairs directed to conserved regions among vertebrate vitellogenins (figure 1). The PCR product was purified to give a single band that was shown to comprise 1218 bp. Importantly, no such product could be prepared from hepatic RNA derived from male fish. The cDNA generated was transcribed to a digoxigenin-labelled cRNA, which was raised as a probe for Northern blot analysis of liver vitellogenin mRNA recovered from untreated mature male and spawning female medaka. As expected, an intensive signal was detected in the female liver, yet not in the sample of the male tissue (figure 2). The molecular size of this band was determined to be approximately 5.5 kb.

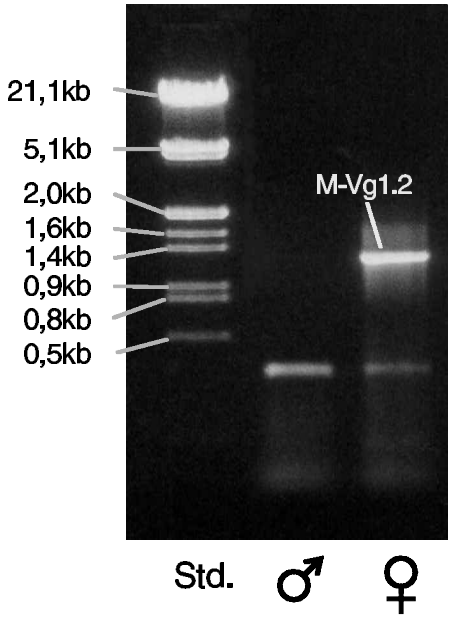

Figure 1. Amplification of Vg1.2 in male and female medaka (*Oryzias latipes*). A prominent band at 1.2 kb appeared only in the liver homogenates of females. The < 0.5 kb byproduct, amplified in both sexes, was not identified. Lane 1, standard (Std.) 1 kb DNA ladder (Peqlab); lane 2, RT-PCR with M-Vg1.2 primers and male hepatic total RNA; lane 3, RT-PCR with MVg1.2 primers and female hepatic total RNA.

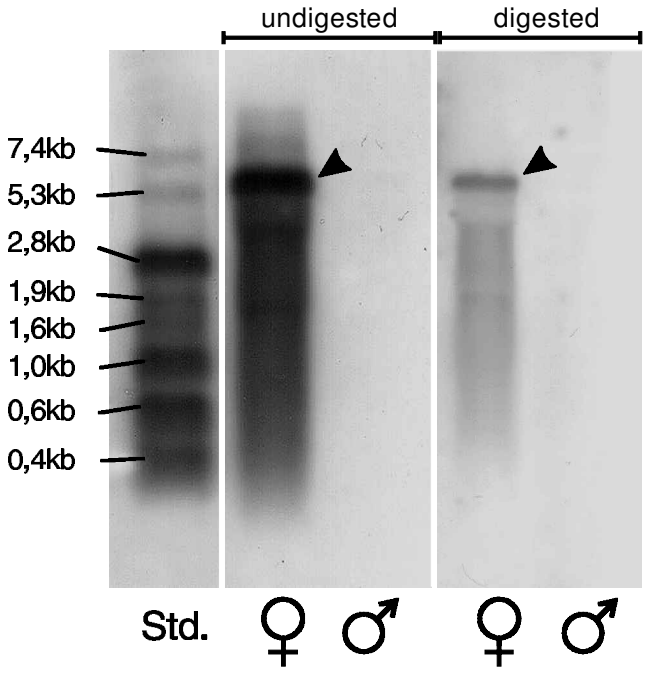

Figure 2. Northern blots of liver homogenates from male and female medaka (*Oryzias latipes*) with and without RNAse digestion. Male and female liver total RNA were applied to the gel in amounts of 4 and 2 µg, respectively. A single band of ~5.5 kb (arrowheads) was stained by the M-Vg1.2 cRNA, and was visible after RNAse digestion. Std., RNA molecular weight standard.

M-Vg1.2 could be aligned to the PCR product M-Vg0.7 derived from RACE PCR, resulting in a continuous sequence of 1620 bp (Acc No. AF268284). According to a PROSITE search (Bairoch *et al*. 1997), the sequence bears one potential cAMP- and cGMP-dependent protein kinase phosphorylation site, seven potential protein kinase C phosphorylation sites, three potential casein kinase II phosphorylation sites and one potential *N*-myristoylation site. The partial sequence of medaka vitellogenin revealed significant similarities to several other vertebrate vitellogenins (figure 3). Using the ClustalW multiple sequence alignment program (Thompson *et al*. 1994), 72% sequence similarity to the corresponding region of mummichog (*Fundulus heteroclitus*) vitellogenin I cDNA and 62% similarity to fathead minnow (*Pimephales promelas*) vitellogenin cDNA was detected. Maxhom multiple sequence alignment of the deduced amino acid sequence revealed 60% identity to *Fundulus heteroclitus*, 51% to rainbow trout (*Oncorhynchus mykiss*), 42% to white sturgeon (*Acipenser transmontanus*), 37% to *Xenopus laevis* and 35% to chicken vitellogenin, documenting the evolutionary relationship among vertebrate vitellogenins.

Application of RT-PCR to monitor vitellogenin induction in medaka by xeno-oestrogens

To measure vitellogenin gene expression by means of RT-PCR, primers were constructed corresponding to 500 bp and 300 bp sequences of medaka Vg1.2 as well as elongation factor 1α, respectively. As expected, RT-PCR revealed the same

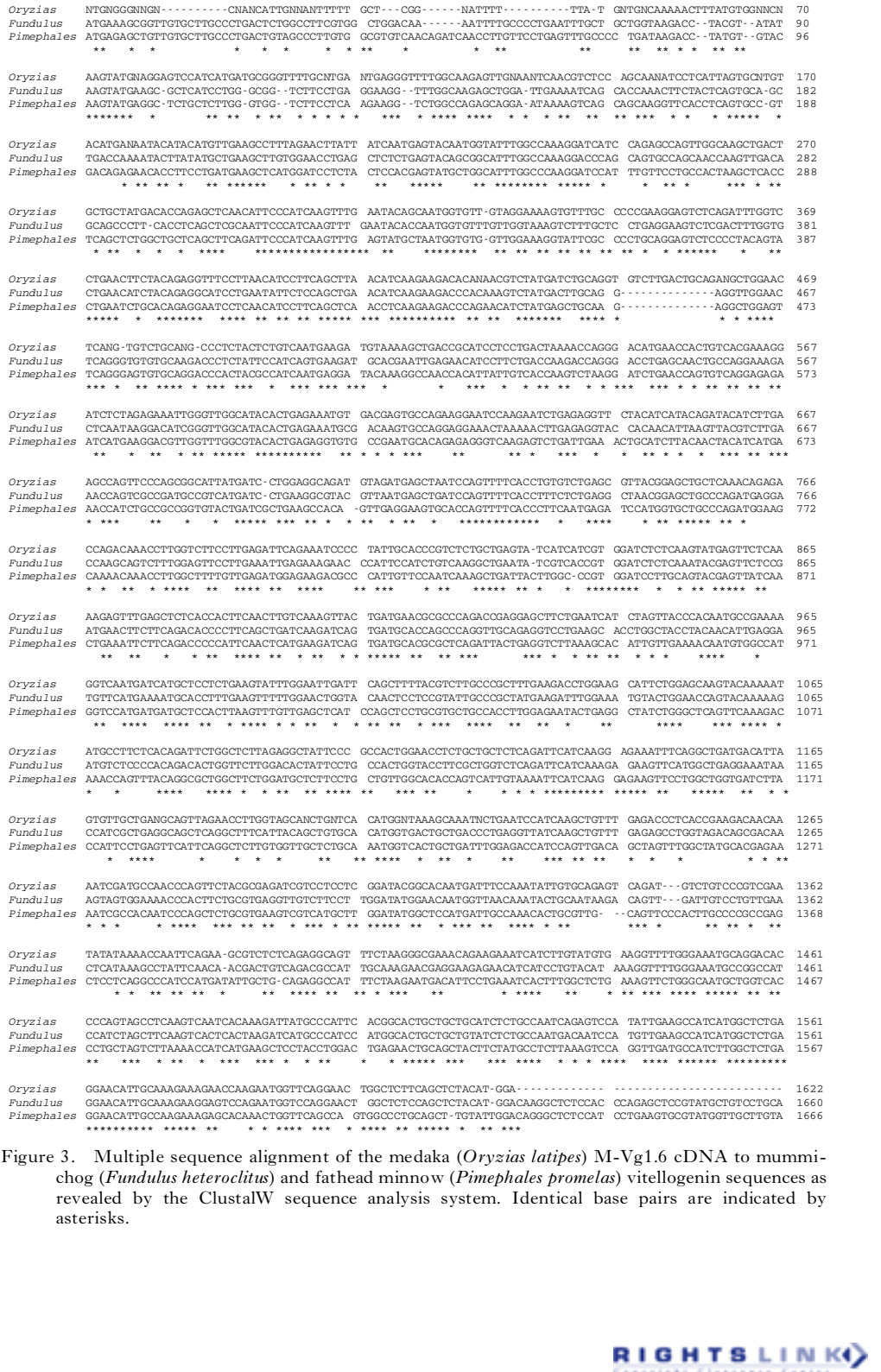

Figure 3. Multiple sequence alignment of the medaka (*Oryzias latipes*) M-Vg1.6 cDNA to mummichog (*Fundulus heteroclitus*) and fathead minnow (*Pimephales promelas*) vitellogenin sequences as revealed by the ClustalW sequence analysis system. Identical base pairs are indicated by asterisks.

bands of elongation factor 1α in both female and male liver RNA samples, in contrast to the vitellogenin band, which could only be demonstrated at high levels in mature female fish (data not shown). For further verification, bands of the female samples were extracted from the gel and sequenced. The band at ~800 bp that was produced in un-induced samples can be attributed to trace amounts of DNA contamination, which resulted in the amplification of the corresponding vitellogenin gene fragment bearing an intron sequence.

Remarkably, however, after densitometric analysis of the RT-PCR products visualized by ethidium bromide staining, induction of vitellogenin gene expression could be detected in mature male medaka exposed for a period of 7 days to either 20 or 50 $\mu g\,l^{-1}$ nonylphenol or 25 or 100 $ng\,l^{-1}$ 17α-ethinyloestradiol (figure 4). Exposure to 17α-ethinyloestradiol resulted in a massive induction of vitellogenin at a concentration of 25 $ng\,l^{-1}$, while only a slight induction was observed in three out of four fish exposed to 20 $\mu g\,l^{-1}$ nonylphenol. Increasing the nonylphenol concentration to 50 $\mu g\,l^{-1}$ resulted in massive vitellogenin expression in all the fish investigated. Almost no vitellogenin was detectable at concentrations below these LOECs, or in the solvent controls incubated in 0.001% DMSO.

Discussion

In order to establish a specific RT-PCR assay for medaka vitellogenin, the mRNA had first to be identified and partially sequenced, since no sequence data

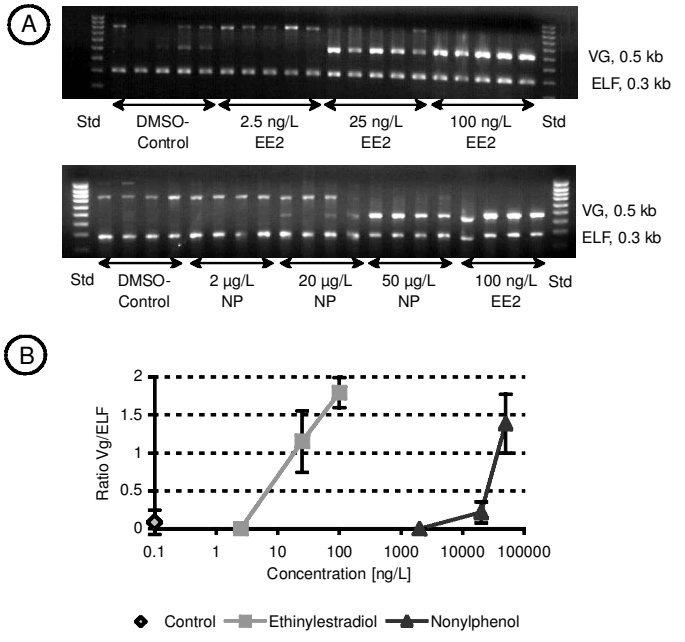

Figure 4. (A) RT-PCR of vitellogenin (VG) expression in male medaka (*Oryzias latipes*) exposed to nonylphenol (NP) or ethinyloestradiol (EE2) for 7 days. Total RNA of four individuals exposed to 100 $ng\,l^{-1}$ 17α-ethinyloestradiol were coamplified with the nonylphenol-exposed samples to ensure comparability of both PCR runs. ELF, elongation factor 1α; Std, 100 bp DNA ladder (Peqlab). (B) Densitometric analysis of the gel represented as the ratio between vitellogenin (Vg) and elongation factor 1α (ELF).

for medaka vitellogenin have been published so far. According to the data presented, M-Vg1.6 cDNA could be identified as the 5′-end of medaka vitellogenin. As revealed by the sequencing data and Northern blots, medaka vitellogenin is most closely related to the vitellogenin of *Fundulus heteroclitus*. The sequence similarity between *Oryzias* and *Fundulus*, both belonging to the order of Cyprinodontiformes, is 72%, and the size of the medaka full-length mRNA transcript is approximately 5500 bp as revealed by Northern blot. This is comparable to the length of the *Fundulus* vitellogenin mRNA of 5197 bp (LaFleur *et al.* 1995). With regard to the deduced amino acid sequence, the sequence similarities to other vertebrate species declined with decreasing systematic relationship; whereas alignment to other euteleostei, such as mummichog (*Fundulus heteroclitus*) or rainbow trout (Mouchel *et al.* 1996), resulted in more than 50% identical amino acids, other vertebrates such as sturgeon (*Acipenser transmontanus*; Bidwel and Carlson 1995), *Xenopus laevis* (Gerber-Huber *et al.* 1987) and lamprey (*Ichthyomyzon unicuspis*; Sharrock *et al.* 1992) shared only 30–40% identity to M-Vg1.6 in declining order of similarity. Nevertheless, with respect to ProDom computer analysis (Corpet *et al.* 1998), M-Vg1.6 shares the typical Lv1 domain in the N-terminal region, which is characteristic of vertebrate vitellogenin as well as apolipoprotein B100 and microsomal triglyceride transfer protein (Anderson *et al.* 1998).

Using the new sequence information, primers specific to medaka vitellogenin were constructed for a semiquantitative RT-PCR assay, which was further evaluated in two exposure experiments. The induction of vitellogenin expression by exposure to nonylphenol and 17α-ethinyloestradiol was clearly dose-related, revealing a dose–response curve with a steep gradient from the LOEC for the enhanced vitellogenin gene expression. Occasionally, slight vitellogenin bands were obtained in control samples, which can be explained by the high sensitivity of RT-PCR in detecting even trace amounts of vitellogenin mRNA.

The LOEC of 20 $\mu g\,l^{-1}$ for nonylphenol is comparable to data obtained for rainbow trout (Jobling *et al.* 1996, Lech *et al.* 1996). The former reported a concentration of 20.3 $\mu g\,l^{-1}$ by measurement of vitellogenin accumulation in the plasma, while the latter detected an induction of vitellogenin expression at $\geq 10\,\mu g\,l^{-1}$ by measurement of mRNA in liver by RT-PCR. Gronen *et al.* (1999) detected an LOEC of 41 $\mu g\,l^{-1}$ nonylphenol for medaka by the measurement of vitellogenin in the serum using a monoclonal antibody-based Western blot. However, 4-tert-octylphenol was reported to be an about one magnitude more potent oestrogen mimic than nonylphenol, whether they are tested in mammalian, avian or piscine-based systems (White *et al.* 1994, Jobling *et al.* 1996). With regard to this data, the sensitivity of this antibody-based system is considerably low, which may be caused by the limited availability of serum in this small fish species.

In rainbow trout, vitellogenin synthesis was stimulated at concentrations as low as 0.1–0.3 $ng\,l^{-1}$ of 17α-ethinyloestradiol (Purdom *et al.* 1994, Sheahan *et al.* 1994). Since *in vivo* data for vitellogenin induction by 17α-ethinyloestradiol in other fish species are limited, only comparisons to the *in vivo* inductive potential of the natural oestrogen 17β-oestradiol can be made. These revealed a similar oestrogenic potential *in vitro* in primary hepatocyte cultures (Pelissero *et al.* 1993, Islinger *et al.* 1999), which are capable of metabolizing both 17α-ethinyloestradiol and 17β-oestradiol (Tenniswood *et al.* 1983, Standeven *et al.* 1990). However, it has to be

considered that 17α-ethinyloestradiol usually shows a higher oestrogenic potential than 17β-oestradiol *in vivo*, because the natural oestrogen is more effectively bound by steroid-binding proteins in the blood and thus inactivated (Toppari *et al*. 1996); additionally, 17α-ethinyloestradiol was found to be more slowly metabolized than 17β-oestradiol *in vivo* as well as *in vitro* (Ball *et al*. 1973, Fotherby 1982, Tenniswood *et al*. 1983). Tyler *et al*. (1999) reported elevated levels of plasma vitellogenin in juvenile fathead minnow down to the lowest concentration tested (25 $ng\,l^{-1}$), which is comparable to the LOEC of 25 $ng\,l^{-1}$ 17α-ethinyloestradiol observed for medaka. In male roach (*Rutilus rutilus*), the LOEC for vitellogenin synthesis was 100 $ng\,l^{-1}$ 17β-oestradiol when tested by ELISA (Routledge *et al*. 1998). With regard to these data, 17α-ethinyloestradiol showed a remarkably weak oestrogenic potency in the male. Thus, without further data for vitellogenin induction in non-salmonid fish species, it remains to be clarified if the weak sensitivity to 17α-ethinyloestradiol is unique for medaka or if there is indeed a broad range of effective concentrations among fish, potentially caused by considerable differences in the metabolic activity of biotransformation enzymes.

With regard to other physiological aspects of endocrine disruption, effects on testicular growth and development were reported at concentrations of nonylphenol similar to those inducing vitellogenin expression in this test system. Testicular growth was significantly inhibited at a concentration of 30 $\mu g\,l^{-1}$ nonylphenol (Jobling *et al*. 1996). In medaka, significant rates of sex reversal and production of testis-ova were reported at concentrations of 50 $\mu g\,l^{-1}$ (Gray and Metcalfe 1997). In fathead minnow, changes in number and size of Sertoli cells were reported at concentrations of 1.1 $\mu g\,l^{-1}$, but no significant differences in secondary sex characteristics or size of gonads were observed, thus suggesting an early stage of oestrogenic action in these fish (Miles-Richardson *et al*. 1999b). Miles-Richardson *et al*. (1999a) reported degenerative changes in the testis of fathead minnow at concentrations of 135 $ng\,l^{-1}$ 17β-oestradiol. In medaka, changes in the sex ratio towards feminization were observed at 10 $ng\,l^{-1}$ 17β-oestradiol (Nimrod and Benson 1998) and 100 $ng\,l^{-1}$ 17α-ethinyloestradiol (Scholz and Gutzeit 2000). At concentrations of 10 $ng\,l^{-1}$ 17α-ethinyloestradiol, Scholz and Gutzeit (2000) observed a decline in spawning success in female medaka and elevated aromatase gene expression in male testes. Thus, ranges of vitellogenin induction in male fish seem to correlate with the formation of severe sexual disorders. This means that the observation of massive vitellogenin induction in male medaka at the level of gene expression may act as a biomarker of severe endocrine effects in this species. Because of the small size and high temperature tolerance of medaka, exemplary *in vivo* experiments in bypass systems at sewage treatment works could act as a monitoring system for endocrine contamination of aquatic systems. In contrast to European freshwater fish species, medaka has no seasonal breeding period. Thus, vitellogenesis should not be modulated by seasonal variations in endogenous steroid hormone concentrations. Indigenous fish species belong predominantly to the order of Cypriniformes, which are highly different to Aterinomorpha in evolutionary terms. For this reason, it has to be kept in mind that species differences in oestrogenic sensitivity have to be considered and investigated in future experiments.

In order to get an impression of the relevance of the observed effects, analytical data of nonylphenol in the environment have to be considered. Nonylphenol,

nonylphenolethoxylates and nonylphenoxycarboxilic acids (with reported oestrogenic potencies slightly weaker than nonylphenol; Jobling *et al.* 1996, Routledge and Sumpter 1996) were measured in concentrations from 0.7–37 $\mu g\,l^{-1}$ and 1.8–65 $\mu g\,l^{-1}$ in Scottish sewage plant effluents (Pirie *et al.* 1996). Studies at sewage treatment works in Italy reported effluent concentrations of up to 4 $\mu g\,l^{-1}$ nonylphenol, 27 $\mu g\,l^{-1}$ nonylphenolethoxylates and 145 $\mu g\,l^{-1}$ nonylphenoxycarboxilic acids (Dicorcia *et al.* 1994). Elevated concentrations of nonylphenolethoxylates and their degradation products up to 1100 $\mu g\,l^{-1}$ were reported from Taiwan (Ding *et al.* 1999), thus showing significant regional variability in environmental concentrations. 17α-ethinyloestradiol was detected in concentrations of 4.5 $ng\,l^{-1}$ in effluent water of domestic sewage treatment works (Larsson *et al.* 1999). Desbrow *et al.* (1998) measured concentrations between 0.2 and 7 $ng\,l^{-1}$ in some of the waste waters investigated. Thus, taking into consideration the high dilution capacity of large rivers, no elevated vitellogenin gene expression should occur in freshwater fish, except for individuals caught near discharges of waste water treatment plants or in some highly polluted locations.

Nevertheless, it has to be taken into account that lipophilic xeno-oestrogens may accumulate in sediments as well as in invertebrates, the predominate nutrition of fish. For this reason, it is important to investigate the impact of a whole life-time exposure on vitellogenin gene expression as well as more population-relevant parameters such as sex ratio and fertilization rates after exposure. In addition, oestrogens may also have an impact on neuronal development during embryogenesis (Gray and Ostby 1998, Beyer 1999); as yet no information is available about the regulative processes and effective concentrations involved. For this reason, small and fast-reproducing fish species such as medaka or zebrafish (*Danio rerio*) may serve as model organisms for putative endocrine disruption in wildlife. In this context, the results of the present study, illustrating the suitability of RT-PCR for the detection of the oestrogenic effects of industrial compounds in small fish species used in *in vivo* experiments under laboratory conditions might be of relevance.

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