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Environmental Toxicology

KINETIC DETERMINATION OF VITELLOGENIN INDUCTION IN THE EPIDERMIS OF CYPRINID AND PERCIFORM FISHES: EVALUATION OF SENSITIVE ENZYME-LINKED IMMUNOSORBENT ASSAYS

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Abstract: Induction of vitellogenin (VTG) in male and immature fish is a standardized endpoint in endocrine-disruption testing. To establish a nondestructive swab sampling method, VTG induction in the epidermis of Cypriniformes and Perciformes species was investigated. Both VTG and estrogen receptor genes are expressed in epidermal cells. Immunoaffinity and mass fingerprint analyses show induction of identical VTG peptides in liver and epidermis. Induction of VTG by estradiol (E2) and bisphenol A (BPA) in the epidermis was quantified with homolog enzyme-linked immunosorbent assays. Initial values in juveniles and males were below 1 ng VTG/mL extraction buffer. Exposure to E2 led to values between 200 ng/mL and 4600 ng/mL in cyprinids and between 10 ng/mL and 81 ng/mL in perciforms. Exposure to BPA increased VTG amounts to 250 ng/mL in fathead minnows, 1360 ng/mL in goldfish, 100 ng/mL in zebrafish, and 12 ng/mL in bluegills. Serum VTG contents demonstrated a similar dose–response pattern in the epidermis and the blood. These results show that VTG induction may be reliably assessed in the skin mucus of fishes, demonstrating the suitability of this biological sample for investigating estrogenic activity in compliance with Organisation for Economic Co-operation and Development standard protocols. This broadens the perspectives in toxicological screening and environmental monitoring, reducing the number of tested animals and minimizing harmful effects for animals, allowing for follow-up of individual induction profiles. *Environ Toxicol Chem* 2016;9999:1–15. © 2016 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals, Inc. on behalf of SETAC.

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INTRODUCTION

Over the last decades, researchers and regulatory agencies have been increasingly concerned with the discharge of endocrine disrupting chemicals (EDCs) in aquatic environments and the potential consequences of xenoestrogens to the reproductive health of wildlife populations [1–3]. Despite large investments and enormous technological progress in wastewater-treatment facilities in recent years, EDCs may still be encountered above no-observed-effect concentrations in treated effluents [4,5].

Many endpoints have been employed in assessing the hazard of EDCs to individuals and populations, including the gonadosomatic index, sex ratio, fecundity and/or reproductive success, maturity index, and egg size [6–8]. Particularly, induction of the egg yolk precursor protein vitellogenin (VTG) in juvenile and male fish has long been established as a reliable biomarker for the presence of EDCs in aquatic environments [9–14]. As one of the most sensitive endpoints in drug and chemical testing, VTG content shows an excellent correlation to different modes of action of EDCs and, thus, has become a mandatory criterion [8,11–13]. In recent years, many authors

have also highlighted the advantages of including data from screening assays in risk-assessment analyses, expediting decisions concerning potential EDCs by regulatory agencies [14–16].

Given the high sensitivity and reliability of enzyme-linked immunosorbent assays (ELISAs), they have become the method of choice for assessing VTG induction [11,12], with several species-specific systems having been developed in recent years [17–20]. These systems rely either on serum/plasma or on whole-body, liver, and head/tail homogenates for VTG measurement, introducing difficulties with regard to matrix-associated affinity discrepancies [21]. Moreover, sampling procedures require large initial test groups for parallel studies to be performed. Collecting plasma from small fish species, as is the case in the models recommended by the Organisation for Economic Co-operation and Development (OECD), requires euthanizing individuals [11,12]. Induction of VTG is estimated by statistical comparison, disregarding interindividual differences [22]. To overcome these limitations, partial success has been achieved with in vitro systems, which have potential in providing consistent information of estrogenicity for chemical screening [23–25]. Nevertheless, such methods are obviously unable to appraise uptake and distribution issues, overestimating the in vivo potency of xenoestrogens. Valid pass/fail criteria (i.e., acceptable coefficient of variance, limit of detection, controls) have yet to be established [26–28].

Detection of VTG in the skin mucus of females was first reported over 3 decades ago [29], and VTG has been shown to occur naturally in the epidermis of females as well as that of males of species with paternal brooding [30,31]. It has been

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shown that EDCs induce VTG in the epidermis in a dose-dependent function, allowing for the evaluation of VTG induction in a minimally invasive manner, broadening the alternatives for biomonitoring and chemical testing [32–34].

Sound inferences concerning endocrine-disrupting potential and risk assessment require data from multiple biological tiers [14,35]. A nondestructive alternative for assessing VTG induction in fish provides toxicologists with a remarkable tool for performing parallel studies and investigating different endpoints in single specimens. Repeated sampling of individuals enables recording of the induction kinetic and more precise comparisons.

Sensitive immunoassays for VTG in perciform fishes have been developed and the effects of EDCs consequently documented for several species of the order [36–38]. To enable environmental monitoring of all aquatic environments as well as targeted chemical testing, special emphasis was put on the inclusion of perciforms in the approach of noninvasive (xeno) estrogenicity assessment via VTG induction measurement. The majority of VTG induction studies thus far are related to cyprinid and salmonid species. Induction of VTG in these groups constitutes an important (eco)toxicological endpoint [18,19,23,31,39–41]. The purpose of the present study was to evaluate 2 sandwich ELISAs sensitive to the variation of VTG in the skin mucus of perciform and cyprinid fish used as test organisms, both in chemical testing and as indicator species in environmental monitoring.

Several different methods besides VTG quantification through the presented ELISAs have been employed to investigate the skin mucus as a suitable biological sample for the assessment of VTG induction. Gene expression analyses were performed to demonstrate that VTG production in the epidermis may be induced in situ. Immunofluorescence staining and western blot analysis served to show the recognition of VTG by the antisera employed in the immunosorbent assays. Matrix-associated laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis demonstrated the identity of the immunoreactive proteins.

MATERIALS AND METHODS

Test organisms

The following cyprinid species were investigated for validation of the Cyprinidae ELISA: the goldfish *Carassius auratus auratus* (Linnaeus, 1758), the common carp *Cyprinus carpio* (Linnaeus, 1758), the zebrafish *Danio rerio* (Hamilton, 1822), and the fathead minnow *Pimephales promelas* (Rafinesque, 1820). In addition, a member of another teleost order (i.e., Beloniformes) was included for validating this assay for noncyprinids, namely, the Japanese medaka *Oryzias latipes* (Temminck and Schlegel, 1846). For the Perciformes assay, 3 species were selected: the bluegill *Lepomis macrochirus* (Rafinesque, 1819), the Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758), and the European perch *Perca fluviatilis* (Linnaeus, 1758).

The OECD test fishes were obtained from GLP/ISO 17025–certified rearing facilities. Perch and tilapia were purchased from commercial fish farms. Fishes were bred, maintained, and exposed at the Gobio laboratory.

Fish were not euthanized at the end of most experiments. In the zebrafish case study (see *Exposure experiments* section), fish were dissected for sexing and blood sampling. In all other experiments, only males or confirmed juveniles were used. Subadult zebrafish lacking secondary sexual characteristics

were randomly selected for the experiments. A complete overview of the characteristics of tested animals is given in Supplemental Data, Table S1.

Exposure experiments

Under semistatic (static renewal) conditions, 5 to 7 specimens of each species were exposed to waterborne estradiol (E2) and bisphenol A (BPA), as well as to solvent (ethanol) controls, with daily water changes and considering a carrying capacity (loading) of 1 g/L. Fish were maintained at a 16:8-h light:dark photoperiod with daily water quality control (temperature, pH, O_2, electric conductivity) after water exchange and fed twice a day (at 10:00 h and 18:00 h) ad libitum with TetraMin Flakes (Tetra, Spectrum Brands). Experiments were conducted with dechlorinated tap water. A chemical analysis of random samples of water immediately after dosing (at 0 h) and before the daily water change (after 24 h) was performed to ensure that actual amounts of each substance (i.e., E2 and BPA) corresponded to the nominal concentrations within acceptable limits (Supplemental Data, Table S2). It should be noted that BPA ($\approx 20.5\,\mu$g/L) was detected in the random control sample at 0 h, as well as traces of E2 (3 ng/L) before the water exchange at 24 h. In the complete set of experiments, a mortality rate of 5.7% was the result of accidental injury during handling.

Nominal E2 concentrations were 500 ng/L for the cyprinid species and 1000 ng/L for the species of Perciformes, with exposure periods of 96 and 168 h, respectively. Selection of E2 concentrations and exposure durations was based on preliminary data (data not shown) with the objective of inducing high VTG levels in the skin mucus, hence the observed differences between exposure concentrations for Cypriniformes (and medaka) and Perciformes species. Nominal BPA concentrations were 100 μg/L, 300 μg/L, 600 μg/L, 800 μg/L, and 1000 μg/L, with an exposure period of 168 h (7 d), in goldfish, zebrafish, fathead minnow, and Nile tilapia.

The exposure of zebrafish to BPA was intended as a case study, addressing the normalization of VTG amounts in the skin mucus by the total protein content of the swab samples. The objective of the exposure to E2 in the present experiment was to compare VTG levels in the serum and in the skin mucus at the end of exposure (168 h). Because blood sampling required the euthanizing of fish, histological investigation of gonads was possible. The highest obtained values at 0 h and 96 h were assigned a posteriori to females in equal numbers as detected at 168 h.

The experiment with bluegill exposed to BPA focused on the feasibility of repeated VTG measurements in the course of long-term exposures (i.e., 672 h, or 28 d); therefore, only 2 concentrations (600 μg/L and 1000 μg/L) were studied. This experiment was also intended as a comparison between parallel samples (each side of every specimen) to be measured independently at 2 different laboratories, which was performed up to 504 h (day 21). Samples from 672 h (day 28) exposure were measured only at lab 1.

Sampling procedures

Skin mucus was collected with validated extraction swabs following the instructions of the TECO Mucus Collection Set (TE1034). Blood was collected from cervical dislocating cuts and diluted in tris(hydroxymethyl)aminomethane buffer with a protease inhibitor (1:1). After centrifugation of the samples, the supernatant was employed in the assays. Head and tail homogenates were prepared according to Annex 5 of the OECD test guideline 234 (fish sexual development test).

Gene expression

Ribonucleic acid (RNA) was extracted from skin and liver samples with Trizol Reagent (Thermo Fischer Scientific), according to the manufacturer's instructions. A 2-step protocol was employed for reverse-transcription polymerase chain reaction, with reverse transcription of total RNA performed with Superscript II Reverse Transcriptase (Thermo Fischer Scientific) following the recommended protocol. Endpoint polymerase chain reaction was performed with the JumpStart REDTaq ReadyMix Reaction Mix following the standard protocol, and amplification was performed in a Robocycler Gradient 96 Thermal Cycler (Stratagene). Messenger RNA (mRNA) sequences (GenBank accession numbers) for primer designing were as follows: estrogen receptor α (*Cyprinus carpio esr1*, AB334722.1), estrogen receptor ß (*Cyprinus carpio esr2*, AB083064.1), G protein–coupled estrogen receptor 1 (*D. rerio gper1*, NM_001128723.1), vitellogenin (*Carassius auratus vtg*, DQ641252.1; *Cyprinus carpio vtg*, AB331884.1; *D. rerio vtg*, BC094995.1; *Oryzias latipes vtg*, AB064320.1; *Pimephales promelas vtg*, AF130354.1). Because no sequence for bluegill *vtg* mRNA was published at the time of the present study, the primer pair employed for this species was that designed for the *vtg* mRNA sequence of *Oreochromis niloticus* (FJ231887.1), which should also amplify a 265-bp product in the corresponding *vtg* sequence of *Oreochromis aureus* (AF072686.1) and *Micropterus salmoides* (AF169287.1). All reactions were performed with initial denaturation for 5 min at 95 °C and final elongation for 10 min at 72 °C. For the investigation of the *vtg* gene of carp, medaka, and bluegill, 36 cycles with the following parameters were applied: denaturation = 30 s at 95 °C, annealing = 30 s at 58 °C, extension = 30 s at 72 °C. For the *vtg* sequences of the other species and for all 3 estrogen receptor sequences, the annealing temperature was set at 60 °C, whereas the other parameters remained unchanged. A complete description of primer pairs and expected product sizes is included in Supplemental Data, Table S3.

Immunohistochemical staining

Paraffin-embedded, 5-μm cross sections of ovaries fixed in Bouin Hollande's solution were produced using standard histological techniques. Immunofluorescence staining followed permeabilization of slices with 0.05% Triton-X (in phosphate-buffered saline) and washing with phosphate-buffered saline. Slices were incubated for 2 h in blocking solution (Roti-Block; Carl Roth) and incubated in the respective antisera as primary "antibodies" (dilution 1:500 in phosphate-buffered saline) for 24 h. After rinsing, slices were incubated for 2 h in antirabbit fluorescein isothiocyanate–labeled secondary antibodies (Carl Roth) for fluorescent detection of bound antisera in the samples.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blot

Reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blots were performed with skin mucus and serum samples of goldfish exposed to BPA (1000 μg/L for 168 h), according to the method described by Hennies et al. [18]. The corresponding primary antisera were used in dilutions of 1:20 000 with the blotting buffer.

Mass spectrometry

Tryptic in-gel digestion followed the method of Shevchenko et al. [42] with minor modifications. Samples were mixed in a 1:1 proportion with a saturated sinapinic acid (Carl Roth) solution of 0.1% trifluoroacetic acid in water/acetonitrile (2:1) and spotted in the target. Peptide measurement was performed in a MALDI-TOF mass spectrometer (Autoflex Speed; Bruker) in reflector mode. For the peptide mass fingerprint analysis, the Mascot search engine was employed (see *Data availability*); and peptide sequences, including in silico digest peptides, were acquired from the databases at UniProt KB and Expasy. Despite not having been stained by Coomassie after the electrophoretic run, bands at 140 kDa and 160 kDa of the skin mucus sample were identified in the western blot analysis as containing immunoreactive proteins. These regions of the polyacrylamide gels were therefore extracted and submitted to in-gel digestion along with the serum sample bands. In the mass fingerprint analysis, databases were restricted for "Chordata" for the 120-kDa serum band and for "other Actinopterygii" in the skin mucus 160-kDa band.

ELISAs

The ELISAs for cyprinids (TECO Cyprinid Vitellogenin ELISA, TE1037) and for perciforms (TECO Perch [Perciformes] Vitellogenin ELISA, TE1035) were performed according to the manufactureŕs protocol. The VTG contents are expressed in nanograms (or micrograms, in the case of serum data) VTG per milliliter extraction buffer, except for the normalized data of *D. rerio*, which correspond to the percentage of VTG in relation to the total protein content of the swabs. The total protein content of the samples was quantified with the Roti-Nanoquant Assay (Carl Roth) following the kit instructions. Mucus samples of controls and initial values were employed without further dilution, except in specific cases (i.e., *Carassius auratus*, *D. rerio*) where a dilution of 1:10 (or 1:100) was employed in a second measurement of females. Mucus samples from groups exposed to high EDC concentrations (particularly of E2) were usually diluted 1:100 and/or 1:500 times after extraction. Serum samples were diluted according to the manufacturer's instructions, with dilutions ranging from $1:10^3$ to $1:10^5$.

Statistics

A one-way analysis of variance (ANOVA) in the R environment [43] was applied to estimate the statistical significance of induction profiles within groups during the exposure and between groups at specific instants of the exposure, as recommended in the OECD guidelines [11,12]. A paired samples *t* test with unequal variances (Welch's *t* test) was also performed when only 2 groups were compared (data

Table 1. Expression of genes in the skin and/or mucus of fish exposed to estradiol

Gene (species)	Expected product	Unexpected products
gper1 (*Danio rerio*)	478 bp	—
esr1 (*Cyprinus carpio*)	216 bp	—
esr2 (*Cyprinus carpio*)	160 bp	≈750 bp
vtg (*Carassius auratus*)	602 bp	≈1200 bp
vtg (*Cyprinus carpio*)	176 bp	≈400 bp
vtg (*D. rerio*)	—	—
vtg (*Lepomis macrochirus*)	265 bp	—
vtg (*Oreochromis niloticus*)	—	≈400 bp
vtg (*Oryzias latipes*)	—	≈280 bp
vtg (*Pimephales promelas*)	—	≈500 bp

esr1 = estrogen receptor α; *esr2* = estrogen receptor ß; *gper1* = G protein–coupled estrogen receptor; *vtg* = vitellogenin (gene).
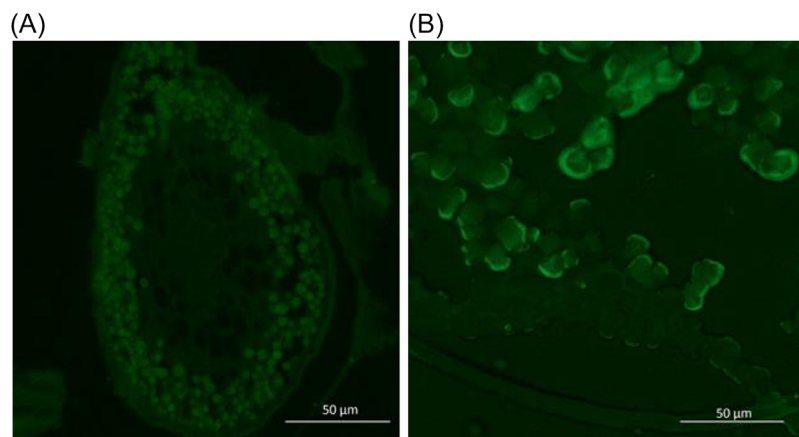
Figure 1. Immunofluorescence staining of vitellogenin in oocytes of *Lepomis macrochirus* (**A**) and in ovulated eggs of *Cyprinus carpio* (**B**).

Table 2. Peptide mass fingerprinting[a] against the National Center for Biotechnology Information nonredundant database

Sample	Taxonomy	Missed cleavage sites	Score	Expectation value p	Top protein identified
Serum (160 kDa)	All sequences	1	107	0.0014	Vitellogenin (*Carassius auratus*)
Serum (140 kDa)	All sequences	1	382	4.5e-31	Vitellogenin (*C. auratus*)
Serum (120 kDa)	Chordata	1	86	0.017	Vitellogenin (*C. auratus*)
Mucus (160 kDa)	Other Actinopterygii	2	63	0.45	Vitellogenin (*C. auratus*)

[a]Search engine, MASCOT; enzyme, trypsin.

not shown), but significance values included in the tables correspond to those obtained by the ANOVA. Because normal distribution of the data could be assumed given the physiological context of endocrine reactions in individuals of the same species to the same stimuli, the results were considered relevant despite the formal statistical difficulties in normality testing because of small sample sizes.

RESULTS

Induction of VTG in the epidermis

To evaluate the skin mucus of fish as a potential matrix for investigating VTG induction by EDCs, the expression of several genes was investigated in the epidermis of specimens exposed to estrogens. The detection of bands corresponding to expected and unexpected complementary DNA products is displayed in Table 1.

Both forms of the nuclear estrogen receptor (ERα and ERß) as well as the G protein–coupled estrogen receptor are transcribed in epidermal cells of fish, clearly indicating that these cells are sensitive to activation in situ. Thus, transcription of the *vtg* gene may be directly regulated in epidermal cells. Expression of the *vtg* gene in the skin of several species (i.e., goldfish, carp, bluegill) conclusively demonstrates *vtg* transcription modulated by estrogen activation in epidermal cells, since no control specimens (not exposed to E2) showed expression of this gene in the skin. Relevant portions of the agarose gels are shown in Supplemental Data, Figure S1. The recurrence of unexpected bands approximately twice the size of the expected products (with *vtg* primers designed for different parts of the coding sequences) suggests the presence of a specific target similar to the *vtg* gene in several species (goldfish, carp, fathead minnow, and tilapia). Sequencing of the obtained products will be performed to investigate the nature of the amplicons (e.g., homology, pseudogenes).

A competitive ELISA was initially developed for the proof of concept of mucus VTG assessment in carp. A significant 5-fold increase in skin mucus VTG could be measured with this competitive assay after 96-h estrogen exposure (Supplemental

Table 3. Mean vitellogenin content in the skin mucus of fish exposed to estradiol

		0 h	96 h	168 h
Estradiol (500 ng/L)				
Carassius auratus	Mean (ng/mL)	0.3	730.4	NA
	SD (ng/mL)	0.2	468.1	
	Significance	$p = 0.021^*$		
Cyprinus carpio	Mean (ng/mL)	0.1	215.2	NA
	SD (ng/mL)	0.1	196.6	
	Significance	$p = 0.040^*$		
Danio rerio[a]	Mean (ng/mL)	0.2	986.0	NA
	SD (ng/mL)	0.1	363.3	
	Significance	$p = 0.001^*$		
Oryzias latipes	Mean (ng/mL)	0.2	3.3	NA
	SD (ng/mL)	0.1	3.3	
	Significance	$p = 0.045^*$		
Pimephales promelas	Mean (ng/mL)	0.5	213.7	NA
	SD (ng/mL)	0.9	218.8	
	Significance	$p = 0.039^*$		
Estradiol (1000 ng/L)				
Lepomis macrochirus	Mean (ng/mL)	3.2	9.0	11.3
	SD (ng/mL)	3.5	1.7	2.6
	Significance	$p = 0.001^*$		
Oreochromis niloticus	Mean (ng/mL)	2.4	14.5	80.6
	SD (ng/mL)	1.4	11.0	67.6
	Significance	$p = 0.004^*$		
Perca fluviatilis	Mean (ng/mL)	2.8	7.4	10.2
	SD (ng/mL)	1.0	1.6	4.4
	Significance	$p = 0.001^*$		

[a]Values from presumptive males only. See Supplemental Data, Table S7 for complete data set.
*Statistically significant difference.
NA = not analyzed; SD = standard deviation.

Data, Figure S2). Because sandwich technology is more practical in terms of routine performance, a second set of adequate antibodies was generated to establish a sandwich assay suitable for investigating VTG in the skin mucus of 2 important teleost orders (Cypriniformes and Perciformes).

Antigen recognition

The antisera for VTG of both Perciformes and Cyprinidae were tested for antigen recognition through immunofluorescence staining and western blot techniques. Immunofluorescence staining of histological sections of ovaries of bluegill (Figure 1A) and ovulated eggs of carp (Figure 1B), employing the respective antisera, displayed fluorescence structured in vesicles in the cytoplasm of oocytes/eggs.

These vesicles and/or granules are thoroughly described in the literature (see Parenti and Grier [44] for a review) as storing the yolk protein, conclusively demonstrating the successful binding of each antiserum with VTG components in ovaries/eggs.

Furthermore, samples of skin mucus, serum, and head and tail homogenates were employed in SDS-PAGE and western blots (Supplemental Data, Figure S3). The antisera did not cross-react with any protein in the skin mucus or blood of male control fish. In samples of mature females and of males exposed to E2, the antiserum against cyprinid VTG clearly recognized 2 bands approximately 140 kDa to 150 kDa and 160 kDa to 170 kDa. Similarly, in mature females of tilapia 2 bands were identified by the antiserum against Perciformes VTG, namely
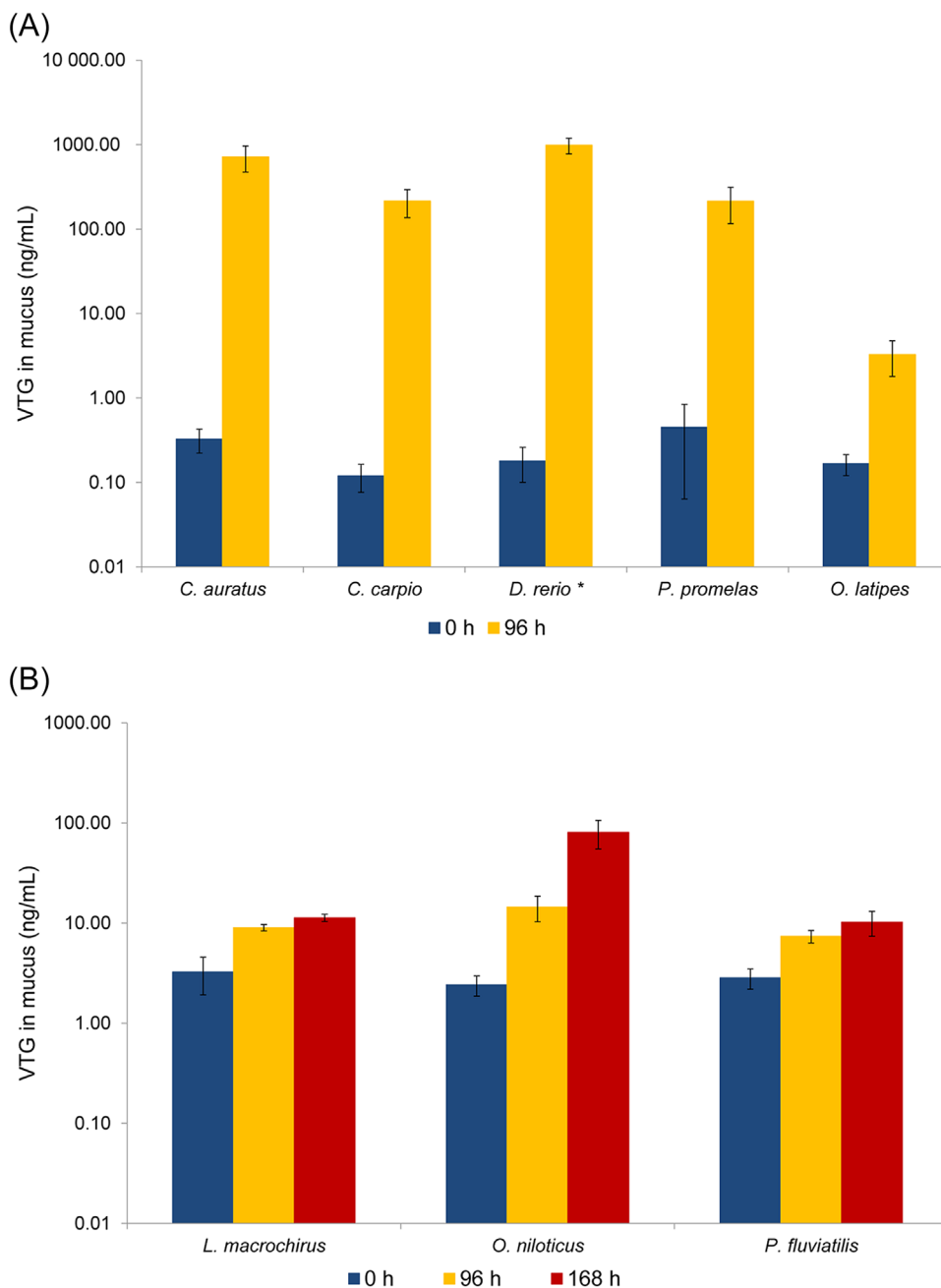

Figure 2. Vitellogenin (VTG) content in the skin mucus of several species (**A**) exposed to estradiol (E2) at 500 ng/L and measured by the TECO Cyprinid Vitellogenin enzyme-linked immunosorbent assay (ELISA), (*Carassius auratus, Cyprinus carpio, Danio rerio, Pimephales promelas*, and *Oryzias latipes*) at 0 h and 96 h and (**B**) exposed to E2 at 1000 ng/L and measured by the TECO Perch (Perciformes) VTG ELISA (*Lepomis macrochirus, Oreochromis niloticus*, and *Perca fluviatilis*) at 0 h, 96 h, and 168 h. *Values for *D. rerio* correspond to data from presumptive males only. Error bars correspond to the standard error of the mean.

approximately 150 kDa and 190 kDa. These results clearly show that the antisera recognize VTG in the blood and the skin mucus of the examined fish species.

Mass spectrometric identification of VTG

Specific VTG-immunoreactive bands of goldfish skin mucus and serum samples (i.e., 120 kDa, 140 kDa, and 160 kDa) recognized in the western blot analysis were subjected to tryptic digestion to identify VTG using peptide mass fingerprinting and analysis by MALDI-TOF MS. The VTG content in the skin mucus samples was below 5 ng, since no band in these samples was stained by Coomassie [45].

The peptide signals corresponding to sinapinic acid (matrix) and to the in silico tryptic digestion of *Sus scrofa* trypsin (Uniprot KB: P00761) were disregarded as background in the obtained spectra. Masses of the remaining peaks were searched against the National Center for Biotechnology Information's nonredundant database (NCBInr 20150823) via peptide mass fingerprinting using the MASCOT search engine. In the serum samples, VTG was identified with significant scores and probabilities, $p < 0.05$ (Table 2). Vitellogenin was also considered to be the likeliest protein present in the 160-kDa band of the skin mucus sample. Due to the low content of VTG in this sample, only a few peaks could be detected; thus, the match was not statistically significant.

In addition to peptide mass fingerprinting, the detected masses were compared with theoretical peptides of VTG from goldfish (UniProtKB Q156A2) obtained by in silico tryptic digestion. Several peptides corresponding to digested goldfish VTG were observed (Supplemental Data, Tables S4–S6) in the 3 bands of the serum sample, particularly in the 140-kDa band. Three peptides were detected in all 3 serum bands (namely, the peaks around the mass-to-charge [*m/z*] ratios 1129.700,

1139.650, and 1463.900), with very high accuracy levels (differences ≤ 14 ppm). In the skin mucus sample, VTG-corresponding peptides (Supplemental Data, Table S7) could be detected in the 160-kDa range. Six peptides correlated to VTG could be observed in this band, including 2 that were also found in serum samples, namely in the 140-kDa (*m/z* 749.4) and 160-kDa (*m/z* 1708.0) bands. The skin mucus contained the lowest amount of protein among the analyzed samples so that a low yield of assignable peptides is plausible.

VTG quantification

For the quantification of VTG in the skin mucus, environmental monitoring species and test organisms recommended in OECD guidelines were exposed to natural estrogen (i.e., E2) in concentrations expected to cause a strong VTG induction (Table 3). Exposure experiments with the model xenoestrogen BPA at sublethal concentrations above the lowest-observed-effect concentration addressed the question of dose dependency of the epidermal estrogen response.

All values obtained after VTG induction by E2 (Table 3) significantly differed from the initial values after 96 h (for cyprinid species and medaka) and 168 h (for perciform species) of exposure. Figure 2 illustrates the VTG induction in the epidermis after exposure to E2, allowing for a comparison between the species assessed by each assay (complete datasets in Supplemental Data, Tables S8 and S9).

Mean VTG values in the skin mucus of cyprinids (Figure 2A) were below 1 ng/mL before exposure to the estrogen, with the exception of zebrafish, for which the value was 1.3 ng/mL when only males were considered (i.e., excluding the 4 high pre-exposure values; see Supplemental Data, Table S8). After 96-h exposure to E2 (at 500 ng/L), average VTG values in the skin mucus varied considerably among species, ranging

Table 4. Mean vitellogenin content in the skin mucus of *Carassius auratus* and *Pimephales promelas* exposed to bisphenol A

	C. auratus			*P. promelas*		
	0 h	96 h	168 h	0 h	96 h	168 h
Control (ethanol)						
Mean (ng/mL)	0	0	0	1.3	2.2	6.1
SD (ng/mL)	0	0	0	2.4	4.9	13.3
Significance		Not significant ($p = 0.376$)			Not significant ($p = 0.627$)	
Bisphenol A						
100 µg/L						
Mean (ng/mL)	0	0.4	3.9	0.2	0.6	2.3
SD (ng/mL)	0	0.5	5.0	0.2	0.9	3.8
Significance		Not significant ($p = 0.108$)			Not significant ($p = 0.243$)	
300 µg/L						
Mean (ng/mL)	0.1	151.5	283.4	0.1	4.3	23.5
SD (ng/mL)	0	292.1	293.5	0.1	8.7	47.8
Significance		Not significant ($p = 0.214$)			Not significant ($p = 0.355$)	
600 µg/L						
Mean (ng/mL)	0.2	67.3	104.4	0.2	0.9	16.9
SD (ng/mL)	0.1	45.5	34.0	0.1	1.6	19.9
Significance		Significant ($p = 0.001$)*			Significant ($p = 0.039$)*	
800 µg/L						
Mean (ng/mL)	1.0	314.9	550.8	0.4	16.5	125.9
SD (ng/mL)	1.8	77.3	359.5	0.5	17.6	99.1
Significance		Significant ($p = 0.005$)*			Significant ($p = 0.003$)*	
1000 µg/L						
Mean (ng/mL)	0.2	667.8	1361.3	0.1	15.1	312.7
SD (ng/mL)	0.1	925.6	1764.9	0.1	27.1	342.2
Significance		Significant ($p = 0.002$)*[a]			Significant ($p = 0.023$)*[b]	

[a]Two outliers left out in the analysis of variance, respectively, at d 4 and d 7 (see Supplemental Data, Table S10).
[b]One outlier left out in the analysis of variance, at d 7 (see Supplemental Data, Table S11).
*Statistically significant difference.
SD = standard deviation.

from approximately 3 ng/mL in medaka and approximately 200 ng/mL in carp and fathead minnow to close to 1 μg/mL in zebrafish (excluding the 4 highest values after 96 h; see Supplemental Data, Table S8). In an experiment with stunted pubertal carps (see Supplemental Data, Table S10), mean pre-exposure values already showed a distinct elevation (138.8 ng/mL).

Mean pre-exposure values obtained in the Perciformes assay (Figure 2B) were also below 1 ng/mL. A significant increase ($p = 0.002$ for bluegill, $p = 0.014$ for tilapia, and $p = 0.001$ for perch; values not included in tables) was observed after 96-h exposure to E2 (1000 ng/L). The VTG values further increased after 168-h exposure, with corresponding higher significance values for the differences (Supplemental Data, Table S9).

Vitellogenin was also induced by the reference environmental estrogen BPA in 3 cyprinid (goldfish, zebrafish, and fathead minnow) and in 1 perciform (bluegill) species. Individual values and significance levels regarding the differences between test groups in goldfish and fathead minnow are displayed in Supplemental Data, Tables S11 and S12, whereas averages and *p* values for the comparison between time points are displayed in Table 4.

For an overview of induction profiles in goldfish and fathead minnow, Figure 3 shows the average VTG mucus values in these species at different concentrations of BPA after 0 h, 96 h, and 168 h.

Zebrafish and bluegill as case studies

The zebrafish case study addressed the influence of sample size (i.e., amount of mucus on swabs) on VTG quantification and the comparison of VTG induction in blood and mucus. The mean VTG values of the exposure of zebrafish to BPA and E2,
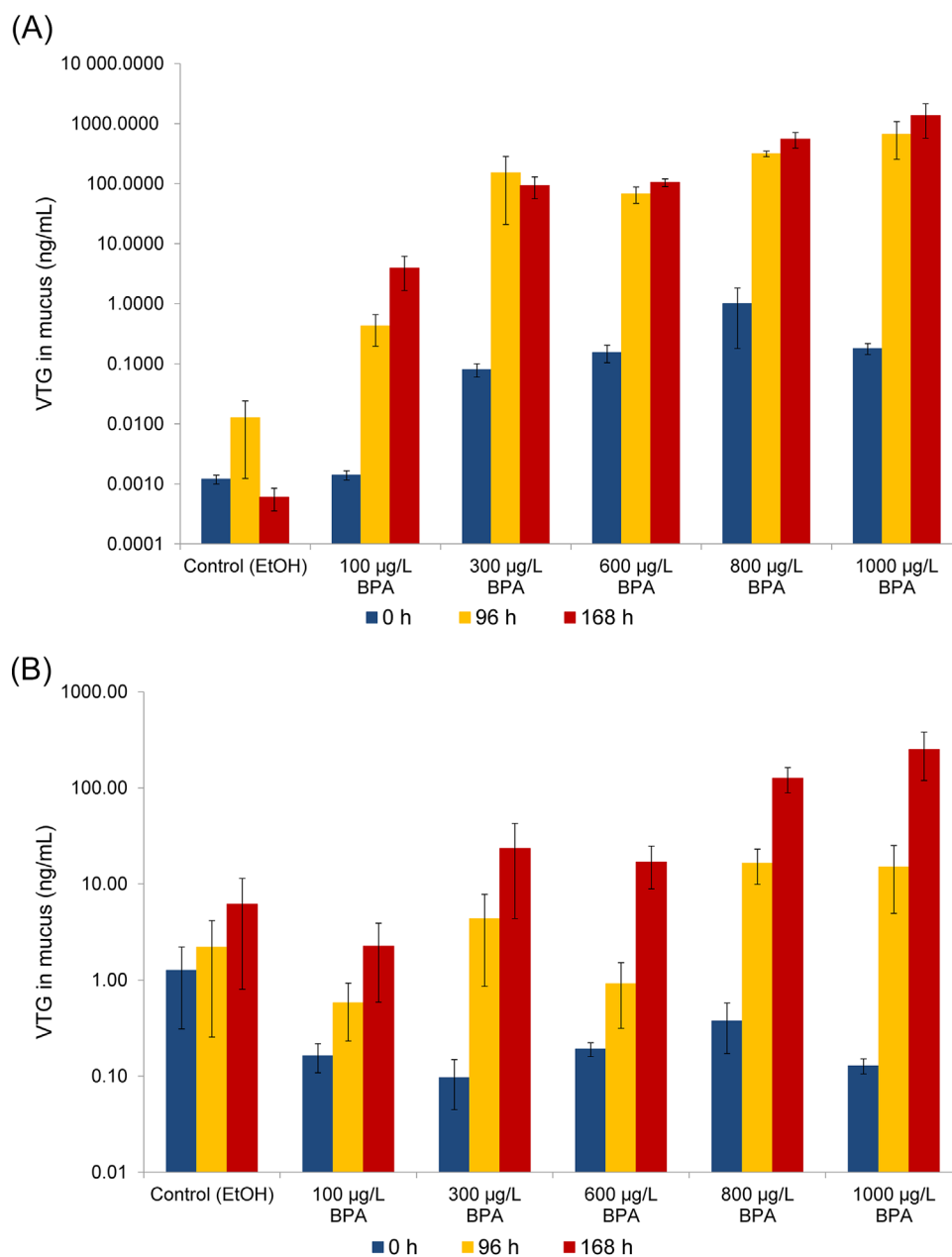

Figure 3. Vitellogenin (VTG) content in the skin mucus exposed to bisphenol A (BPA; at 100 μg/L, 300 μg/L, 600 μg/L, 800 μg/L, and 1000 μg/L) and measured by the TECO Cyprinid VTG enzyme-linked immunosorbent assay at 0 h, 96 h, and 168 h in (**A**) *Carassius auratus* and (**B**) *Pimephales promelas*. Error bars correspond to the standard error of the mean. EtOH = ethanol.

Table 5. Mean vitellogenin content in the skin mucus in the case study with *Danio rerio*

			Vitellogenin (ng/mL)			Protein (µg/mL)			% Vitellogenin		
			0 h	96 h	168 h	0 h	96 h	168 h	0 h	96 h	168 h
Control (ethanol)											
	♂ + ♀	Mean	19.4	32.7	27.2	50.0	41.8	64.6	0.044	0.101	0.045
		SD	22.8	30.2	26.4	18.2	14.6	17.2	0.046	0.103	0.046
		Significance	Not significant (p = 0.652)			Not significant (p = 0.074)			Not significant (p = 0.272)		
	♂	Mean	1.7	0.5	3.2	53.4	49.1	68.0	0.003	0.001	0.005
		SD	0.9	0.4	1.4	12.1	11.7	23.1	0.001	0.001	0.001
		Significance	Significant (p = 0.038)*			Not significant (p = 0.395)			Significant (p = 0.015)*		
	♀	Mean	32.7	56.8	51.2	47.4	36.4	61.1	0.075	0.176	0.086
		SD	22.2	2.2	1.9	23.4	15.5	13.1	0.037	0.061	0.016
		Significance	Not significant (p = 0.087)			Not significant (pp = 0.269)			Significant (pp = 0.026)*		
Estradiol											
	♂ + ♀	Mean	8.6	139.0	592.8	50.6	47.2	78.3	0.022	0.316	0.757
		SD	20.4	117.4	388.0	8.6	13.9	24.6	0.051	0.275	0.407
		Significance	Significant (p = 0.001)*			Significant (p = 0.006)*			Significant (p = 0.001)*		
100 ng/L	♂	Mean	0.3	100.6	475.9	52.4	48.1	72.1	0.001	0.223	0.696
		SD	0.3	64.7	256.7	7.8	15.0	20.2	0.001	0.133	0.409
		Significance	Significant (p = 0.001)*			Significant (p = 0.035)*			Significant (p = 0.001)*		
	♀	Mean	50.3	369.1	1293.9	39.9	42.2	115.3	0.126	0.875	1.123
		SD	n.a.			n.a.			n.a.		
		Significance	n.a.			n.a.			n.a.		
	♂ + ♀	Mean	48.4	1577.7	3073.3	63.0	59.8	107.5	0.135	2.518	2.706
		SD	24.7	977.9	1336.7	79.9	19.5	31.2	0.107	1.016	0.519
		Significance	Significant (p = 0.001)*			Not significant (p = 0.166)*			Significant (p = 0.001)*		
500 ng/L	♂	Mean	n.a.			n.a.			n.a.		
		SD									
		Significance									
	♀	Mean	48.4	1577.7	3073.3	63.0	59.8	107.5	0.135	2.518	2.706
		SD	24.7	977.9	1336.7	79.9	19.5	31.8	0.107	1.016	0.519
		Significance	Significant (p = 0.001)*			Not significant (p = 0.166)			Significant (p = 0.001)*		
Bisphenol A											
	♂ + ♀	Mean	13.7	27.2	42.3	35.6	27.1	39.4	0.084	0.127	0.133
		SD	21.6	25.0	33.1	32.1	13.7	17.2	0.128	0.119	0.128
		Significance	Not significant (p = 0.168)			Not significant (p = 0.586)			Not significant (p = 0.728)		
100 µg/L	♂	Mean	0.1	10.6	22.2	47.8	31.4	44.2	0.001	0.049	0.057
		SD	0.1	19.7	30.6	37.7	17.7	20.1	0.001	0.094	0.088
		Significance	Not significant (p = 0.371)			Not significant (p = 0.672)			Not significant (p = 0.540)		
	♀	Mean	31.7	49.3	69.0	19.3	21.4	33.1	0.194	0.232	0.235
		SD	23.3	0.7	0.4	15.9	2.1	13.1	0.131	0.026	0.102
		Significance	Significant (p = 0.040)*			Not significant (p = 0.377)			Not significant (p = 0.855)		
	♂ + ♀	Mean	6.1	7.1	11.4	27.2	30.5	24.2	0.050	0.058	0.042
		SD	15.1	17.8	26.1	19.0	16.5	6.2	0.129	0.148	0.093
		Significance	Not significant (p = 0.876)			Not significant (p = 0.306)			Not significant (p = 0.973)		
300 µg/L	♂	Mean	0.5	0.4	1.5	29.8	33.5	23.6	0.002	0.002	0.007
		SD	1.0	0.2	1.6	19.4	15.8	6.6	0.004	0.001	0.008
		Significance	Not significant (p = 0.163)			Not significant (p = 0.403)			Not significant (p = 0.168)		
	♀	Mean	40.2	47.5	70.5	11.7	12.1	28.0	0.343	0.393	0.25
		SD	n.a.			n.a.			n.a.		
		Significance	n.a.			n.a.			n.a.		
	♂ + ♀	Mean	14.2	23.0	107.8	29.2	32.8	33.6	0.092	0.139	0.407
		SD	14.8	25.0	139.9	22.7	15.9	9.7	0.111	0.246	0.542
		Significance	Not significant (p = 0.090)			Not significant (p = 0.878)			Not significant (p = 0.218)		
600 µg/L	♂	Mean	0.3	0.5	1.8	37.4	31.7	34.2	0.001	0.001	0.005
		SD	0.3	0.4	2.1	30.1	14.6	4.9	0.001	0.001	0.006
		Significance	Not significant (p = 0.315)			Not significant (p = 0.881)			Not significant (p = 0.362)		
	♀	Mean	24.6	39.9	187.2	23.1	33.6	33.2	0.159	0.242	0.709
		SD	9.9	19.0	139.6	17.7	19.0	13.0	0.101	0.296	0.552
		Significance	Significant (p = 0.037)*			Not significant (p = 0.623)			Not significant (p = 0.127)		
	♂ + ♀	Mean	0.9	31.6	102.5	27.7	32.2	42.2	0.004	0.107	0.345
		SD	1.8	83.5	260.1	18.9	11.9	19.2	0.009	0.282	0.896
		Significance	Not significant (p = 0.480)			Not significant (p = 0.287)			Not significant (p = 0.496)		
800 µg/L	♂	Mean	0.2	0	4.2	28.9	32.7	44.4	0.001	0	0.007
		SD	0.2	0.1	8.3	20.3	12.9	20.0	0.001	0	0.013
		Significance	Not significant (p = 0.258)			Not significant (p = 0.332)			Not significant (p = 0.290)		
	♀	Mean	4.9	220.9	692.1	20.0	29.6	29.1	0.024	0.745	2.377
		SD	n.a.			n.a.			n.a.		
		Significance	n.a.			n.a.			n.a.		

Table 5. (*Continued*)

			Vitellogenin (ng/mL)			Protein (µg/mL)			% Vitellogenin		
			0 h	96 h	168 h	0 h	96 h	168 h	0 h	96 h	168 h
1000 µg/L	♂ + ♀	Mean	12.1	111.2	112.1	30.5	42.0	46.4	0.036	0.370	0.328
		SD	21.8	186.7	162.1	9.8	16.9	14.6	0.064	0.658	0.485
		Significance	Not significant ($p = 0.346$)			Not significant ($p = 0.121$)			Not significant ($p = 0.377$)		
	♂	Mean	0.2	2.2	19.6	29.4	45.7	51.5	0	0.003	0.050
		SD	0.2	3.5	35.4	11.8	17.7	14.3	0	0.005	0.099
		Significance	Not significant ($p = 0.295$)			Not significant ($p = 0.087$)			Not significant ($p = 0.343$)		
	♀	Mean	42.0	383.9	343.6	33.2	32.8	33.5	0.125	1.286	1.023
		SD	18.7	30.0	51.3	1.3	15.1	0.6	0.051	0.500	0.134
		Significance	Significant ($p = 0.004$)*			Not significant ($p = 0.996$)			Not significant ($p = 0.061$)		

*Statistically significant difference.
n.a. = not analyzed; SD = standard deviation.

along with the mean protein contents and the normalized values (nanograms of VTG per microgram of protein, expressed as a percentage of VTG), are shown in Table 5. For individual values and the statistical comparison between groups, see Supplemental Data, Tables S13 to S15. The lack of secondary sexual characteristics did not allow for an all-male setup for this experiment. The high VTG contents in the skin mucus of several specimens before exposure (Supplemental Data, Table S13) points to females among the tested individuals, as confirmed through histological evaluation of gonads at the end of the experiment (data not shown). Thus, separate statistical analyses were performed with 1) all specimens of each group and 2) presumptive females (corresponding to the highest values at 0 h and 96 h in equal numbers as those identified histologically at 168 h) and males separately.

Figure 4 displays the absolute (nanograms per milliliter) and relative (percentage of VTG) increases in VTG in the skin mucus of specimens after exposure to E2 (Figure 4A) and BPA (Figure 4B). Induction profiles remained largely unaltered by the normalization through total protein amount (percentage of VTG). The VTG amounts in unexposed zebrafish correspond to approximately 0.05% to 0.1% of the total protein content in the mucus, increasing in 3 exposure groups after 96 h, to values ranging from 0.3% (in the 100 ng/L E2 and the 1000 µg/L BPA exposures) up to 2.5% (in the 500 ng/L E2 exposure). After 168 h, the VTG percentage in the skin mucus of fish exposed to 100 ng/L E2 further increased to 0.75% and that of fish exposed to 500 ng/L E2, to 2.7%. Higher VTG percentages (0.4% and 0.35%) in comparison with pre-exposure values were also observed in the skin mucus after 168 h in the 600 µg/L and 800 µg/L BPA concentrations, respectively. Both absolute and normalized values showed a significant increase of VTG content in the skin mucus when exposed to E2 at 100 ng/L and 500 ng/L (Table 5), for each sex separately (i.e., only males and only females) and for all specimens taken together.

No significant differences in the total protein amount in samples were related to EDC concentration (Table 5). Thus, normalization of VTG levels by protein content is not necessary in routine investigations.

This experiment also addressed a comparison of VTG induction by E2 in the epidermis and in the blood at 168 h, as shown in Table 6 (full data set in Supplemental Data, Table S16). Skin mucus VTG values reached 3073 ng/mL after 168 h, corresponding to 2.7% of the total protein content. Serum VTG values reached 869 µg/mL, corresponding to 28.9% of the total serum protein amount. Despite the considerable differences in VTG amounts, dose dependency is quite similar in blood and skin mucus, in terms of both absolute (Figure 5A) and normalized (Figure 5B) values.

A preliminary experiment assessing VTG induction in the blood and in the skin mucus was also performed with perch, showing similar induction profiles in both matrices (Figure 6).

The case study with bluegill exposed to BPA (at 600 µg/L and 1000 µg/L) for 672 h (28 d) included measurements performed in 2 different laboratories of samples taken at 0 h, 96 h, 168 h, 336 h, and 504 h (i.e., at days 0, 4, 7, 14, and 21; see Table 7). This experiment addressed 1) the interlaboratory comparison of measurements of parallel swabs (i.e., 1 from each side of the specimens), and 2) recording of kinetic changes for longer exposure periods, showing that VTG assessment might be performed in parallel with other long-term tests, such as the juvenile fish growth test (data not shown, OECD test guideline 215). The complete data set of the present experiment is included in Supplemental Data, Table S17. The highest VTG values in the skin mucus were observed after 168 h (except for the measurement of lab 2 of the 600 µg/L group, which presented a slightly higher VTG content at 96 h; see Table 7 and Supplemental Data, Table S17). This suggests that longer exposures to BPA may not be needed for full epidermal induction of the protein, at least for high concentrations of the xenoestrogen (i.e., ≥ 600 µg/L).

Figure 7 illustrates how sampling from different sides of specimens led to very close results even when measured in different facilities, as may be noted in the linear regression of data obtained in the 2 laboratories (Supplemental Data, Figure S4). The coefficients of determination at $R^2 = 0.91$ and $R^2 = 0.98$ (for the 600 µg/L and 1000 µg/L, respectively) indicate a very high correlation between determined VTG values, especially given the small number of specimens of each test group ($n = 5$). In the fish exposed to 1000 µg/L, particularly, a slope very close to 1 (1.0372) clearly indicates that the values correlate closely between parallel samples.

DISCUSSION

The induction of VTG is a decision-driving parameter in terms of the execution of chemical laws (acts) as well as of environmental protection measures. The nondestructive VTG determination in the skin mucus of fish aims to 1) facilitate VTG measurement in the frame of OECD guidelines related to endocrine disruption (test guidelines 229, 230, and 234); 2) supplement non–mode of action toxicity testing strategies with an additional endpoint reflecting estrogen activity; and 3) enable nondestructive field monitoring of the biomarker
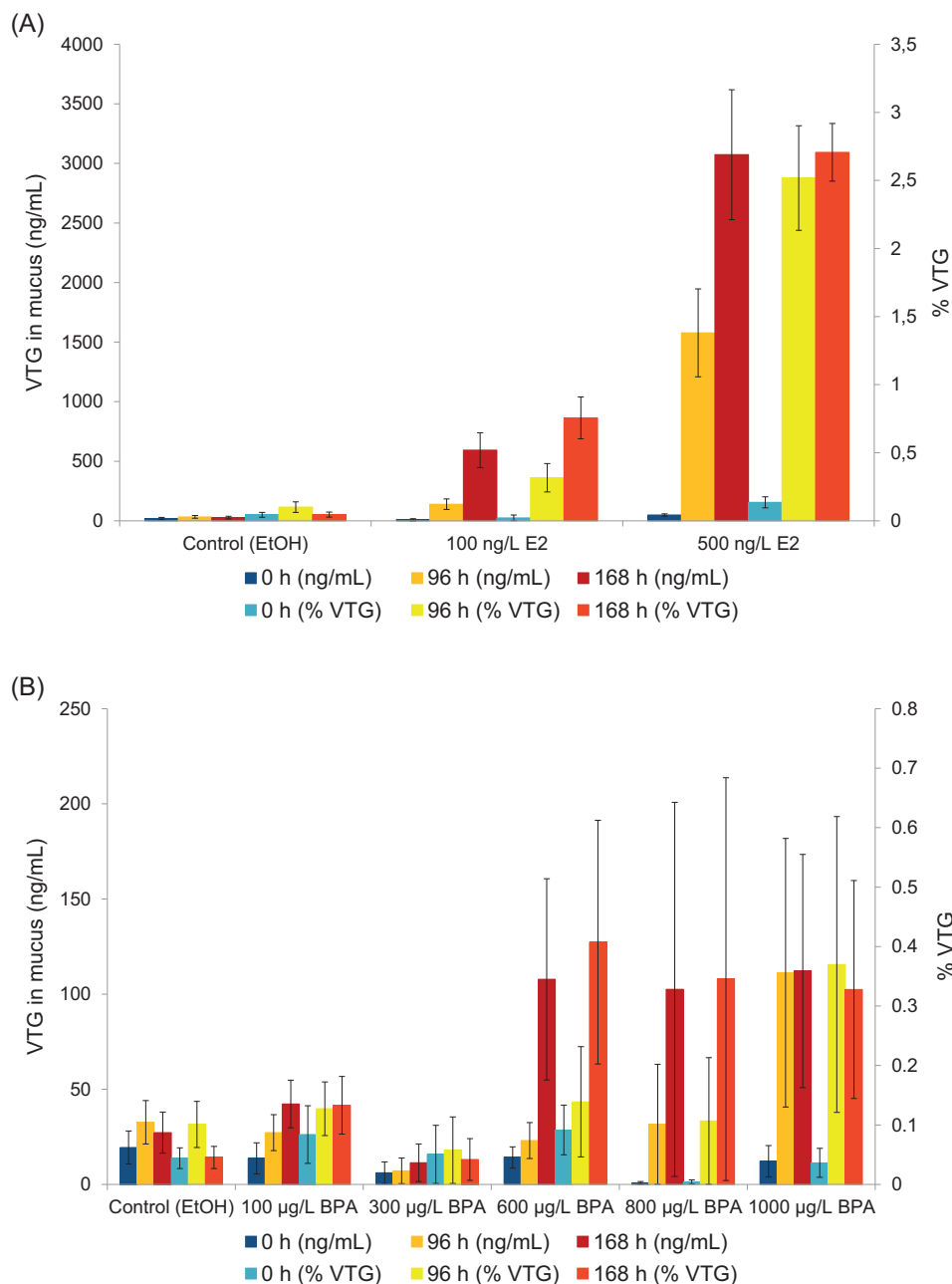
Figure 4. Average absolute (nanograms per milliliter) and normalized (percentage) vitellogenin (VTG) content measured by the TECO Cyprinid VTG enzyme-linked immunosorbent assay in the skin mucus of *Danio rerio* specimens exposed to (**A**) estradiol (at 100 ng/L and 500 ng/L) and (**B**) bisphenol A (BPA; at 100 μg/L, 300 μg/L, 600 μg/L, 800 μg/L, and 1000 μg/L) for 0 h, 96 h, and 168 h. Error bars correspond to the standard error of the mean. EtOH = ethanol.

VTG. For these purposes, the chemical and functional identity of the addressed protein and the reliability of its quantification need to be confirmed.

The expression of both classes of estrogen receptors (i.e., estrogen receptors and G protein–coupled estrogen receptor) in the skin of carp and zebrafish indicates that estrogen-dependent proteins (such as VTG) may be induced in situ. Expression of the *vtg* gene in response to E2 could be demonstrated in the skin of both perciform and cyprinid species, confirming that biosynthesis of VTG may take place in the epidermis of fish [32,33].

Antigen recognition by the antisera employed in the ELISAs has been conclusively demonstrated both in western blot analyses and by means of immunostaining of oocytes and ovulated eggs. The mass spectra obtained in the MALDI-TOF analyses of the 160-kDa band of the skin mucus and the 140-kDa and 160-kDa bands of the serum extracted from polyacrylamide gels showed several identical VTG peptides. Combined with the highly significant score of the mass fingerprint analysis, particularly for the serum bands at 140 kDa and 160 kDa, these results clearly indicate that VTG is being addressed by the antisera in both matrices. The 2 bands observed between 130 kDa and 190 kDa in the western blots represent the 2 paralogous isoforms of VTG (VTG A and VTG B) recurrently observed in teleosts (see Matsubara et al. [46] for a thorough description of VTG components). Taken together, the lines of investigation followed in the present study point to a contribution of exogenously triggered epidermal synthesis of VTG detected in skin mucus swab samples.

A clear estrogen-dependent induction of VTG in the epidermis of all investigated species was observed after 96 h.

Table 6. Mean vitellogenin (VTG), total protein, and normalized VTG content in skin mucus and serum samples of *Danio rerio* for 168 h

	Vitellogenin (ng/mL)		Protein (µg/mL)		% Vitellogenin	
	Mucus	Serum	Mucus	Serum	Mucus	Serum
Control (ethanol)						
Mean	27.2	5654.3	64.6	720.9	0.05	1.92
SD	26.4	4930.7	17.2	447.9	0.05	2.60
Estradiol (100 ng/L)						
Mean	592.8	67 354.4	78.3	901.4	0.76	7.02
SD	388.0	72 469.6	24.6	614.0	0.41	3.81
Estradiol (500 ng/L)						
Mean	3073.3	869 224.7	111.9	2911.5	2.71	28.85
SD	1336.7	591 658.4	31.8	1610.8	0.52	6.10

SD = standard deviation.
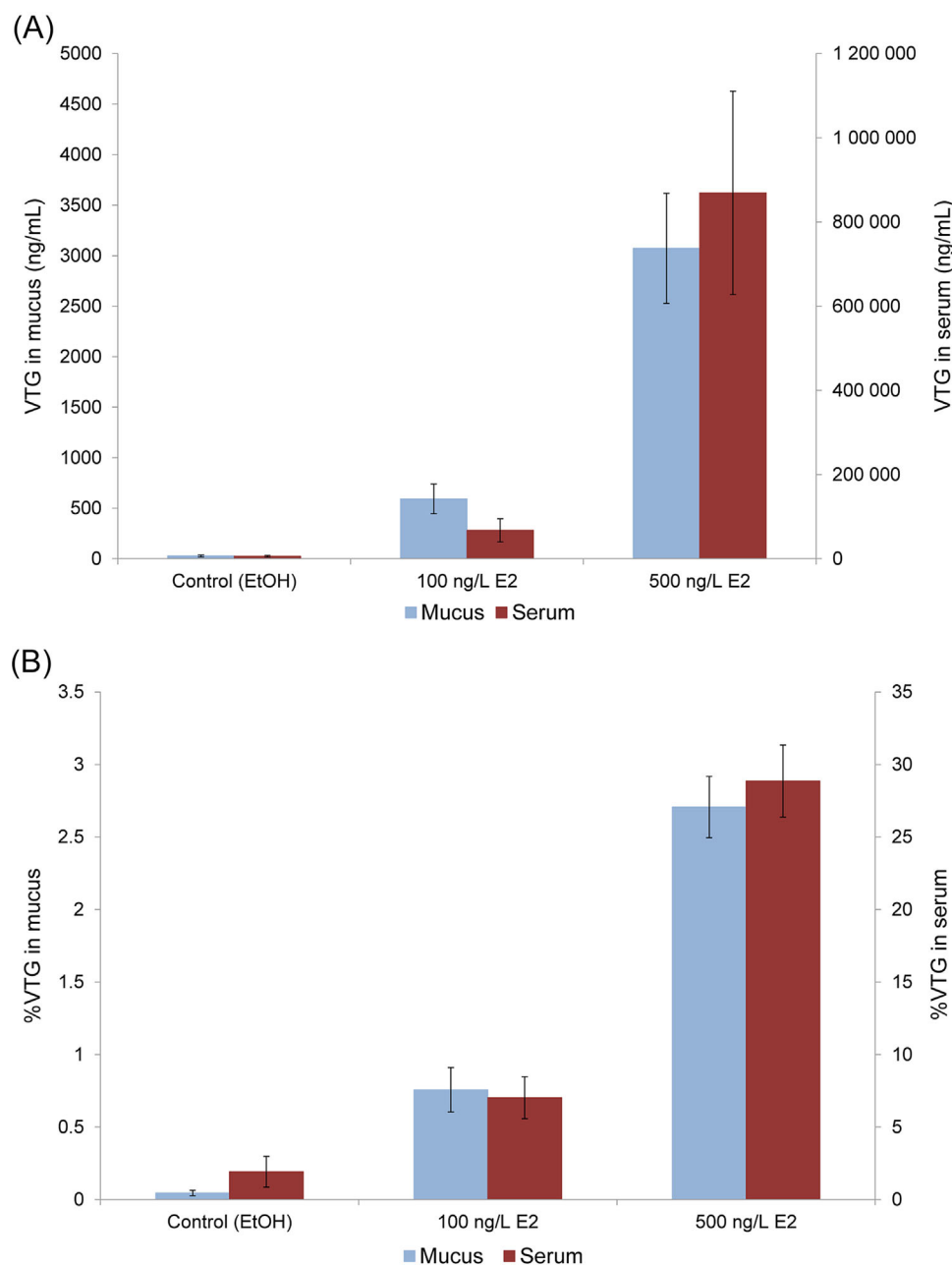
Figure 5. Comparison of serum and skin mucus vitellogenin (VTG) content measured by the TECO Cyprinid VTG enzyme-linked immunosorbent assay in *Danio rerio* specimens exposed to estradiol for 168 h. (**A**) Average absolute (nanograms per milliliter) VTG contents in the skin mucus and blood. (**B**) Average normalized VTG values in the skin mucus and blood. Error bars correspond to the standard error of the mean. EtOH = ethanol.
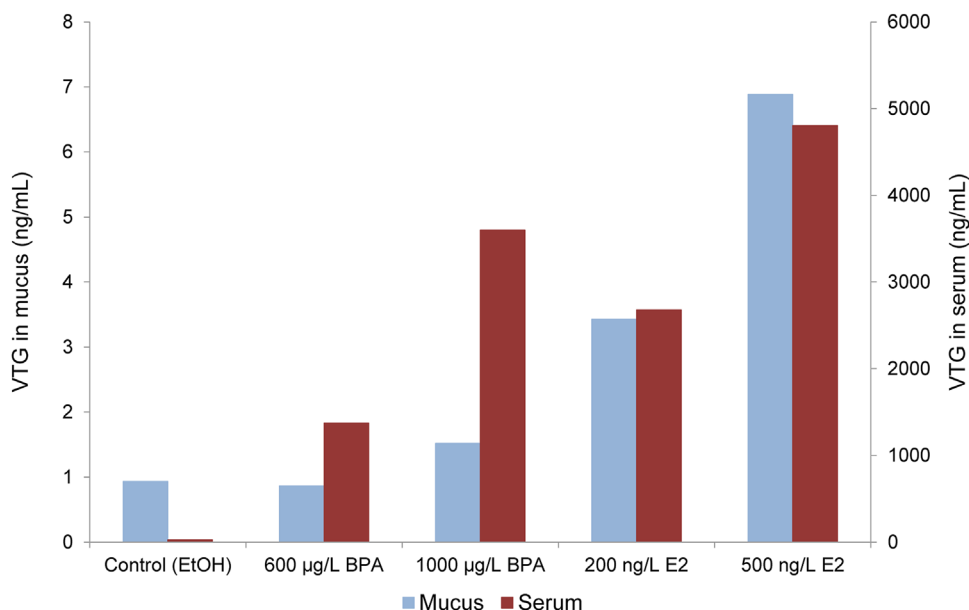
Figure 6. Comparison of individual serum and skin mucus vitellogenin (VTG) content measured by the TECO Perch (Perciformes) VTG enzyme-linked immunosorbent assay in *Perca fluviatilis* specimens exposed to estradiol (E2) and bisphenol A (BPA) for 168 h. Error bars correspond to the standard error of the mean. EtOH = ethanol.

Exposure to BPA also showed a slight increment in VTG content in the epidermis already after 96 h, even at low concentrations in some species (e.g., 100 μg/L and 300 μg/L in goldfish). Between 96 h and 168 h, these values further increased in goldfish and fathead minnow, especially in the 600 μg/L to 1000 μg/L range. Similarly, induction of VTG in the epidermis of bluegills was observed after 96-h exposure to BPA at 600 μg/L, and in the exposure to BPA at 1000 μg/L a vast increase could be detected between 96 h and 168 h.

A partially flattened dose–response curve of blood VTG concentration for this model xenoestrogen was already postulated in previous studies. The higher resolution of VTG measurement in the skin mucus in the present study enables reliable evaluation of low-level induction (e.g., Allner and Schaat [47]).

In males of species with a clear sexual dimorphism (i.e., fathead minnow, medaka) or for specimens in confirmed prepubertal status (as in the case of the investigated goldfish, carp, and bluegill), the increase in VTG concentration in the

Table 7. Average vitellogenin in the skin mucus of *Lepomis macrochirus* exposed in bisphenol A

	0 h	96 h	168 h	336 h	504 h	672 h
Lab 1						
Control (ethanol)						
Mean	0.3	0.2	0.6	0.5	0.4	0.2
SD	0.4	0.1	0.3	0.3	0.1	0.1
Significance			Not significant ($p = 0.077$)			
Bisphenol A						
600 μg/L						
Mean	0.2	2.4	2.8	1.4	0.7	0.4
SD	0.1	1.9	2.4	1.9	0.4	0.1
Significance			Not significant ($p = 0.094$)			
1000 μg/L						
Mean	0.4	2.7	10.5	7.7	6.1	3.0
SD	0.3	1.8	2.0	5.5	3.3	1.3
Significance			Significant ($p = 0.001$)*			
Lab 2						
Control (ethanol)						
Mean	0	0.1	0	0	0.0	n.a.
SD	0	0.1	0.1	0	0.0	n.a.
Significance			Not significant ($p = 0.322$)			
Bisphenol A						
600 μg/L						
Mean	0	1.8	1.6	0.5	0.2	n.a.
SD	0	0.8	2.0	1.0	0.5	n.a.
Significance			Not significant ($p = 0.061$)			
1000 μg/L						
Mean	0	4.0	11.3	7.7	6.4	n.a.
SD	0	3.8	7.0	3.9	4.3	n.a.
Significance			Significant ($p = 0.009$)*			

*Statistically significant difference.
n.a. = not analyzed; SD = standard deviation.

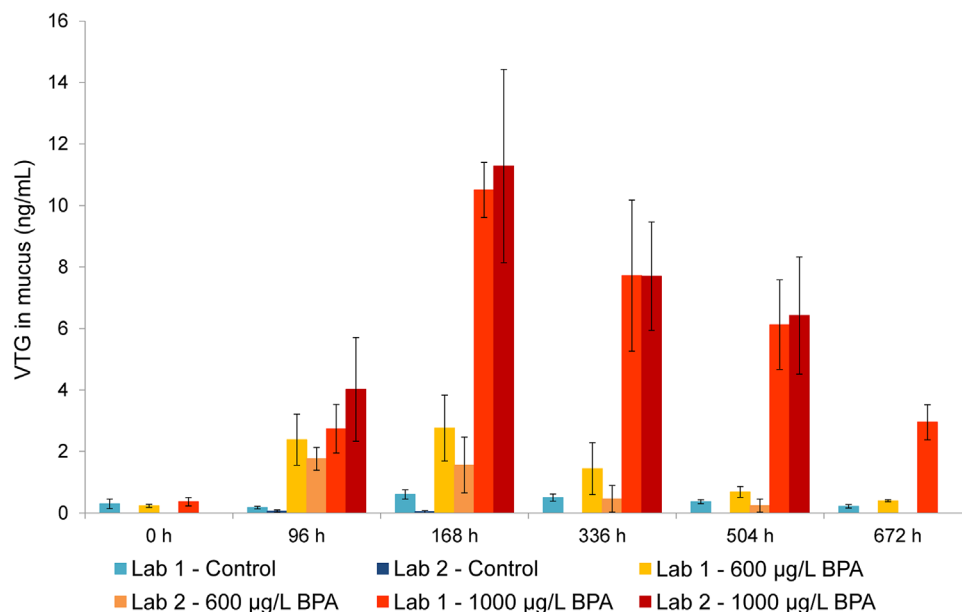Figure 7. Laboratory comparison of vitellogenin (VTG) content in skin mucus samples ($n = 5$) from different sides of *Lepomis macrochirus* specimens exposed to bisphenol A (BPA; at 600 µg/L and 1000 µg/L) for 672 h (lab 2 only measured up to 504 h) as measured by the TECO Perch (Perciformes) VTG enzyme-linked immunosorbent assay. Error bars correspond to the standard error of the mean.

skin mucus in response to estrogen/xenoestrogen exposure was at least 1 order of magnitude after 96 h.

Given the sensitivity of the ELISAs evaluated in the present study, it is possible to record changes in trace amounts of VTG in the skin mucus. Thus, in contrast to systemic measurements (e.g., blood, homogenates) skin mucus testing enables recording of estrogen receptor activation in vivo independently from bioaccumulation and liver function. Testing VTG in the skin mucus of individuals exposed to sublethal concentrations of EDCs in short-term experiments can provide basic information on the estrogenic potential of test compounds. Decisive in vivo data—triggering higher-tier estrogen effect assessment—can be obtained with low experimental effort at an earlier stage of chemical testing. For this purpose, also OECD test species (e.g., carp, goldfish, bluegill) employed in tests not explicitly addressing an estrogen mode of action (e.g., OECD test guideline 203, test guideline 212, and test guideline 215) were included in the present study. In addition, perch were investigated even though they are not suited to be maintained under laboratory conditions and consequently are not studied in chemical testing. The proof of their sensitivity and their wide distribution, however, endorse the usage of this species for monitoring purposes.

The similar VTG amounts in parallel swab samples of bluegill (taken at each time point from different sides of single specimens) points to a consistently reproducible sampling procedure. The strong correlation between values measured in 2 different laboratories demonstrates the reliability of the ELISA.

Repeated swab samplings are feasible along with the measurement of other toxicological endpoints (e.g., recording weight and length in compliance with OECD test guideline 215) over several weeks. The significant decline in VTG levels after several days (particularly after 336 h, day 14) shows that intermediary sampling can avoid underestimating the estrogenic effect of substances after longer exposures (e.g., OECD test guideline 230).

The zebrafish employed in the exposure experiment described herein were approximately 100 d old. Sexing of these individuals on the basis of secondary characteristics was unreliable, thus confirming that maturing and nonbreeding adults present only nonquantifiable, low-degree secondary sexual characteristics [11,48,49]. Pre-exposure and control fish VTG skin mucus values were 10-fold higher in females than in males. Thus, sex identification based on skin mucus VTG becomes an excellent tool for assembling experimental groups of young adults in estrogen assessment assays (e.g., OECD test guideline 229), as well as for forecasting appropriate sample dilutions for systemic ELISA VTG measurements of maturing 60-d-old individuals to be investigated in the frame of OECD test guideline 234.

Measurement of VTG in serum samples has been shown to be possible with the evaluated assays, given an appropriate dilution of samples. Induction profiles in the epidermis closely followed those observed in the blood, with a similar dose–response relationship, albeit differences in VTG concentration.

Although a strong binding of the cyprinid antiserum to medaka VTG was observed in the western blot analysis of skin mucus samples, the VTG values estimated by the cyprinid ELISA in the skin mucus of this species were 1 order of magnitude lower than the lowest induced values in the investigated cyprinid species. This suggests that the quantification data for the medaka might have to be reevaluated, even though significant results ($p = 0.045$) in terms of VTG induction in the epidermis were obtained.

The ANOVA estimated the induction of VTG in the epidermis as statistically significant for all species exposed to E2. A paired samples t test with unequal variances (Welch's t test), however, did not recognize the significance of the differences in the exposure experiments with carp, goldfish, and fathead minnow. The statistical analysis must be critically interpreted in a physiological context because a delayed endocrine response of a single individual in such small data sets leads to a high variance. Considering the strong responses observed in these species in absolute terms (corresponding to increases between 2 and 3 orders of magnitude), the results of the t test were interpreted as false negatives (type II errors).

The induction of VTG in the epidermis by BPA was less pronounced. Several groups which empirically show increased VTG values (e.g., goldfish and fathead minnow groups exposed to 300 μg/L and most of the zebrafish groups exposed to BPA) were not evaluated as significantly different in the ANOVA. Given the intensity of responses, these results were also interpreted as type II errors due to high variances. Conversely, the comparisons between some control values at different sampling events and the pre-exposure values of a few groups (e.g., in the exposure experiment with zebrafish) were considered significant in the ANOVA even though no induction took place. These results were interpreted as false positives (type I errors). Considering that the endocrine system is subjected to several positive and negative feedback loops, a strong variation between individual responses is not unusual.

Generally, the statistical power of the analyses was severely reduced by the small sample sizes and the high interindividual variability of responses. Even though the strong effects measured partially overcome these difficulties, some responses, particularly those induced by low concentrations of weak estrogenic substances, require sophisticated interdisciplinary approaches. Monitoring individual induction profiles results in more expressive data sets, allowing for more robust statistical analyses.

In terms of the 3 principles of animal welfare in experimental testing (i.e., replacement, reduction, and refinement), the advantage of nondestructive sampling of the skin mucus for assessing EDC effects is evident. This technique has the potential to drastically reduce the number of animals and/or experiments to be used in drug screening, enabling information about endocrine disruption potential to be derived from lower-tier assays. Moreover, the procedure constitutes a clear refinement of currently employed methods in terms of minimizing pain and distress (and ultimately death) of tested animals. This particularly applies to monitoring studies, allowing for animals to be assessed in a minimally invasive manner that avoids injury and lasting harm to sampled specimens. Thus, environmental monitoring for EDCs could be performed without impact on investigated populations.

The present study has shown the validity of assessing VTG induction by EDCs in the skin mucus of fish based on the evidence of gene expression, immunoreactivity, and VTG quantification. We have shown that the induction of VTG may be quantified and monitored by the evaluated ELISAs in an external matrix that does not require invasive sampling. The data presented show that the estrogen-dependent protein induced in the epidermis is identical to that recommended by the OECD for assessing endocrine disruption. The lines of evidence in the present study strongly support the inclusion of the skin mucus of fish as a valid biological sample for EDC effect assessment, providing a refined alternative to the techniques currently employed in chemical testing and environmental monitoring for endocrine disruption.

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