DEVELOPMENT OF A SPECIFIC ELISA FOR SHORTFIN EEL (*ANGUILLA AUSTRALIS*) VITELLOGENIN

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ABSTRACT

Vitellogenin (Vtg) was purified from the plasma of immature shortfin eels (*Anguilla australis*) treated with 17β-oestradiol (E_2). Fast protein liquid chromatography was used to isolate Vtg. Fractions corresponding to an E_2-induced protein peak were dialysed, concentrated and characterised by SDS-PAGE to reveal an E_2-treatment-dependent band (197kDa). Polyclonal antisera directed against this protein preparation were raised in New Zealand white rabbits and characterised by Western blot analysis. The primary antisera (1°Ab) consistently recognised the purified protein and E_2-injected plasma but also exhibited non-specific binding with control and E_2-treated plasma samples. Preliminary ELISA analyses and Western blotting confirmed non-specific binding. Immunoelectrophoresis analysis revealed two major antibody species: one recognised an epitope specific to both the purified and E_2-injected plasma, and the other recognised an epitope shared between E_2 and control plasma. Non-specific binding was negated by adsorbing 1°Ab with control plasma. Elimination of the non-specific binding was confirmed by Western blot analysis, but adsorption of the 1°Ab resulted in high background readings for ELISA. This high background was removed by substituting PBS-T 0.1% gelatin assay buffer for TBS-T BSA. The optimal ELISA conditions for Vtg well coating concentration and 1°Ab dilution were determined to be 4 ng/well and 1:80,000 respectively. Serial dilution of purified eel Vtg and E_2-treated eel plasma displayed parallelism indicating antisera specificity for eel Vtg. Using this ELISA, Vtg induction was measured in eels exposed to E_2 and 4-nonylphenol.

Key words: shortfinned eel, *Anguilla australis*, vitellogenin, ELISA, assay development

INTRODUCTION

Vitellogenin (Vtg) is a large glycolipophosphoprotein synthesised in the liver of fish and other oviparous vertebrates. Under natural conditions Vtg production is induced after activation of oestrogen-responsive Vtg genes by the sex steroid 17β-oestradiol (E_2). Therefore, plasma Vtg concentrations rise steadily during sexual maturation of female fish, concomitant with increasing E_2 levels, to a point where Vtg can constitute the major protein in plasma (Sumpter and Jobling 1995). From the liver, Vtg is carried to the maturing oocytes via the circulatory system, where it is cleaved into smaller proteins that provide nutrition to the developing embryo. Both male and female teleost species possess the cellular and genetic machinery to produce Vtg. However, very low plasma concentrations of E_2 in males and immature animals produce only low levels of Vtg.

The recognition of compounds that mimic the actions of the steroid hormone E_2 has initiated concerns about their impact on the reproductive fitness of exposed wildlife and human populations. The potential for these compounds to induce significant Vtg production in males and immature fish provides the underlying rationale for the use of sensitive *in vivo* and *in vitro* bioassays to investigate the impact of anthropogenic effluents on aquatic organisms (Folmar *et al.* 1996; Harries *et al.* 1997; Denslow *et al.* 1999).

Despite considerable overseas studies, relatively little is known of the potential impacts of estrogenic compounds on the aquatic environment in New Zealand (Jones 1998). The shortfin eel (*Anguilla australis*) provides a unique opportunity to assess chemical contaminant impact on aquatic life. This catadromous fish species is widely distributed in inland and coastal freshwater environments, where males can live for up to 14 years and females for 23 years before maturing and migrating out to sea (McDowall 1990). Their high position in the food chain, high tissue lipid content, and close association with the sediment maximise their dietary exposure potential for lipid-soluble and biomagnifiable contaminants with estrogenic activity. In addition, the shortfin eel has considerable cultural significance to Mäori and economic importance to commercial fisheries. This paper describes the development of an indirect, competitive, enzyme-linked immunosorbent assay (ELISA) that is specific to shortfin eel Vtg. This method was used to determine the plasma Vtg concentration of eels exposed to selected estrogenic compounds via the intraperitoneal (ip) route.

MATERIALS AND METHODS

Fish

Sexually immature "yellow" shortfin eels of mixed sex and age weighing approximately 100-400g were supplied by a commercial eel fisherman (Taumutu, Canterbury, New Zealand). All eels were captured in knotless nylon (Rachel netting) fyke nets from Te Waihora (Lake Ellesmere, Latitude -43.78, Longitude 172.46). Healthy eels of similar size were selected. To minimise transport stress eels were transported in ice-water to an indoor animal facility. Animals were held in a series of aerated black polyethylene tanks each with a total volume of 350 L. Water was drawn from an artesian supply into large header tanks from where it flowed into the eel holding tanks. Mechanical agitation combined with a period of water retention in the header tanks was required to dissipate the periodic high levels of dissolved gases that can be toxic to fish

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(Svobodová *et al.* 1993). The water flow-rate ranged from 100 to 128 L/h resulting in a tank refresh period of 3-3.5 h. Photoperiod was set to a 12-h light, 12-h dark cycle. The concentration of dissolved oxygen (DO) was maintained over 80% by aeration with air stones. Over the experimental period, water temperature ranged between 15 and 18°C. Eels were fed every two days with fresh ovine liver supplemented with frozen squid and live earthworms (*Eisenia fetida*, tiger worm; *Lumbricus rubellus*, red worm; The Wormery, Christchurch, New Zealand) until satiated.

Vitellogenin induction and blood sampling

E_2 (Sigma, USA) was made into a fine powder and mixed with corn oil (5 mg/mL) to produce a uniform suspension. Eels were anaesthetised using benzocaine (250 mg/mL) in ice-cold water prior to ip injection of E_2 (10 mg/kg) given once weekly for 3 weeks. Food was withdrawn 24 h prior to injections to facilitate E_2 absorption. Eight days after the last injection, eels were anaesthetised in benzocaine (250 mg/L) for 5 min. Blood was collected from the caudal vein immediately posterior to the anal vent using a heparinised syringe and needle, and was transferred to tubes containing the protease inhibitor aprotinin (20 TIU/mL blood). Whole blood was centrifuged at 317 x *g* (rotor 3042, Heraeus, Germany) for 15 minutes at 8°C and the plasma was collected, then stored at –20°C.

Purification of plasma vitellogenin

Vitellogenin was purified using an adaptation of the method of Silversand *et al.* (1993). Plasma from E_2- and control- injected eels was diluted (1:30) with ultra-pure (MQ) water and filter-sterilised through a 0.22-µm syringe filter (Millex-GS, Millipore, USA). Then 500 µL of the diluted plasma was injected into a loop connected to an anion-exchanger (Mono Q HR 5/5 column 50 x 5 mm I.D., Pharmacia, Sweden) attached to a fast protein liquid chromatography (FPLC) system with a LCC-501-plus controller unit, P-500 pumps, pre-filter, sample loop of 500 µL, UV-1 UV monitor with an HR flowcell, conductivity monitor and SuperFrac sample collector (Pharmacia, Sweden). All buffers used in this procedure contained 200 TIU/L aprotinin, and were filter-sterilised (Steritop 0.22-µm GP Express membrane Millipore, USA) and then degassed (200 torr) for 20 min. Before the plasma sample was injected, the column was equilibrated with buffer A (50 mM Tris base, 200 TIU/L aprotinin, pH 8) until a stable 280-nm baseline was reached. Unbound material was washed through the column with 2 mL of buffer A. Bound material was eluted by running buffer B (0.5 M NaCl, 50 mM Tris base, pH 8) through the column (1.0 mL/min). The concentration of buffer B was increased in three step-wise progressions. The first was a 2-mL linear NaCl gradient (0-55% buffer B composition), followed by a 12-mL linear NaCl gradient (55-80% buffer B composition) and finally a 2-mL linear NaCl gradient (80-100% buffer B). The protein concentration in the column elutant was measured at 280 nm. The flow rate through the column was 1.0 mL/min and 1.0-mL fractions were collected. The pooled fractions from each animal across multiple runs of E_2-dependent protein were transferred into dialysis tubing (MWCO 12-14 kDa, Spectrum, USA) and dialysed against 0.5 mM Tris base (pH 8.0) for 12 h at 4°C with stirring. Proteins in the dialysed fractions were concentrated by passive diffusion in polyethylene glycol 4000 flakes for 2-4 h at 4°C, and stored at –20°C. The protein concentration in the purified samples was determined by the method of Bradford (1976) using bovine serum albumin (BSA) standards.

Preparation of polyclonal antisera

Specific polyclonal antisera against the purified Vtg were raised in 10-week-old New Zealand white rabbits. Aliquots of the purified Vtg were diluted with 1.5 mL of phosphate-buffered saline and 1.5 mL of Freund's complete adjuvant and mixed into an emulsion. Three rabbits were given 3-4 subcutaneous injections with the immunogen (300-400 µg) at different points adjacent to the spine. Additional booster injections (1.5-2.0 mL) were administered 1 (280-380 µg) and 2 (240-320 µg) months later. The rabbits were ear bled 5-6 days after each booster injection. Titre checks (lowest concentration containing readily detectable levels of specific antibodies) on serum from each rabbit after the first and second booster injections revealed an antibody titre of 10,000. Positive displacement of the antisera with free Vtg was observed with all sera. Another titre check following the last booster injection revealed titres between 20,000 and 50,000. Rabbits were exsanguinated 30 days after the third injection; blood was collected and allowed to clot at 4°C overnight. Antisera were collected after centrifugation at 5,000x*g* at 4°C for 10 min and frozen in 10 mL aliquots.

Polyacrylamide gel electrophoresis (PAGE)

The separation of proteins by PAGE was performed using a discontinuous buffer system and dissociating conditions as described by Laemmli (1970). Resolving (7.5% acrylamide, 0.1% SDS, 0.375 M Tris HCl pH 6.8) and stacking gels (3.8% acrylamide, 0.1% SDS, 0.182 M Tris HCl pH 6.8) were degassed (180 torr) for 30 min at room temperature (23-25°C) and polymerised by the addition of 0.1% TEMED, 0.1% ammonium persulphate and left to set for 60 min. SDS-reducing sample buffer (63 mM Tris HCl, 10% glycerol, 2% SDS, 0.1% bromophenol blue) containing 5% β-mercaptoethanol, was added to the protein samples, heated at 100°C for 4 min, and cooled immediately in ice. Molecular weight protein markers (High molecular weight, BioRad, USA) were diluted 1:20 with sample buffer and prepared as for the plasma samples. Gels were submersed in running buffer (25 mM Tris base, 192 mM Glycine, 1% SDS pH 8.3) prior to loading wells with sample (20 µL of protein and sample buffer). Proteins were separated electrophoretically (150 V and 60 mA; Mini-PROTEAN® II Electrophoresis Cell and Power Pac 2000; BioRad, USA) at 4°C for approximately 60 min, and visualised with coomassie blue solution (0.15% coomassie blue, 30% methanol, 10% acetic acid). Background stain was removed with destainer solution (30% methanol, 10% acetic acid). A permanent record of each gel was captured digitally (Gel Doc 2000 scanner, BioRad, USA). The relative molecular mass of the purified protein under reducing conditions was calculated (Quantity one-4.0.1 software, BioRad, USA).

Immunoelectrophoresis

Characterisation of the 1°Ab by immunoelectrophoresis was adapted from the procedures described by Lewis (1983). A 1% agarose gel (LE, Boehringer Mannheim, Germany) was prepared in 0.1 M Tris-HCl (pH 8.8) and poured onto glass plates to thickness of approximately 2-4 mm. Wells (1 mm diameter and 10 mm apart) were punched into the gel and protein samples mixed with sample buffer (0.1 M Tris-HCl, glycerol, 1% bromophenol blue). Gels were run at 150 V and 120 mA for 50 min in a horizontal submersible gel apparatus (Mini Sub™ DNA Cell, BioRad, USA). Following electrophoresis, troughs (2 mm wide) were cut in the gel between the sample wells parallel to the electrophoretic migration and the

undiluted 1°Ab was dispensed into the troughs and incubated overnight at room temperature in a humidity chamber. Gels were soaked in saline (0.9% NaCl) for 3 h and blotted for a further 3 h to remove the remaining non-precipitated protein. The precipitated proteins were visualised by staining with coomassie brilliant blue G-250 for 30 min, and destained until the desired band intensity was obtained. A permanent record of the gel was taken using a gel imaging system (Gel Doc 2000, BioRad, USA).

Western blot analysis

After PAGE, the proteins were immobilised onto activated polyvinylidene difluoride (PVDF) membrane (Immobolin-P, Millipore, USA) as described by Bittner *et al.* (1980). Electrophoretic transfer was performed overnight at 30 V (Mini Trans-Blot apparatus, BioRad, USA) and 8°C in transfer buffer (25 mM Tris base, 192 mM glycine, 1% SDS). After the transfer, membranes were developed according to the method of Towbin *et al.* (1979). The PVDF membrane was blocked for 3 h with TBS buffer (0.01 M Tris base, 0.15 M NaCl, pH 7.8) containing 2.5% BSA and then washed (TBS-T 0.01 M Tris base, 0.1% Tween 20) three times (15 min with shaking). The membrane was then probed with the 1°Ab (1:10,000 dilution in TBS-T containing 1% BSA) for 1 h, washed three times, and incubated with alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit, SIGMA, USA) diluted 1:3,000. Specific binding was visualised using the BioRad pre-mixed substrate reagents.

Optimisation of the ELISA for eel vitellogenin

Purified Vtg (0.4646 µg/µL) was diluted with sodium bicarbonate buffer (50 mM $NaHCO_3$, 0.5% gentamycin, pH 9.6) and 150 µL dispensed into each well (96-well polystyrene flat bottom Costar®, USA) and incubated at 37°C for 3 h. Well contents were discarded and washed five times with 200 µL TBS-T buffer (10 mM Tris base, 0.15 M NaCl, 0.1% Tween 20, 0.5% gentamycin pH 7.5). Initially, wells were saturated with 200 µL of TBS-T containing 0.5% ELISA-grade BSA (TBS-T BSA) and incubated at 37°C for 30 min. The wells were washed a further five times with TBS-T. Purified Vtg standards (2323 to 4.537 ng/mL) and plasma samples (1:500 dilution) were diluted using TBS-T-BSA and 50 µL added to the corresponding wells. Primary antibody was diluted 1:80,000 in TBS-T-BSA and 100 µL was added to all wells except the assay blank. The well contents were left overnight at room temperature to reach a state of equilibrium. The plate was then washed five times in TBS-T and incubated for 2 h at 37°C with goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Sigma, USA) diluted 1:100 in TBS-T BSA. The plate was then washed as previously described and incubated with O-phenylene diamine substrate (0.5 g/L 1,2-phenylene diamine, 0.015% hydrogen peroxide) and 32 mL ammonium acetate (50 mM) citric acid (50 mM) solution (pH 5.0). The reaction was stopped with 5 N H_2SO_4 and the absorbance read at 490 nm (340 ATTC microplate reader, SLT-Labinstruments, Austria). However, to negate high non-specific background, the TBS-T BSA buffer system was substituted with PBS-T 0.1% gelatin (25 mM sodium phosphate, 0.1% gelatin, 0.1% (v/v) Tween 20, 0.25 mM thiomersal) in all phases of the assay.

Quality control

Following the methodology of Keith *et al.* (1983) an arbitrary limit of detection (LOD) was set to three times the standard deviation above the mean of the assay zero. An ELISA data manipulation program (Titri v. 5.04; Gestur Vidarsson, Netherlands) was used to calculate the concentration of Vtg in assay zeroes using absorbances corresponding to individual assay zero wells (n=30). Sample values below the limit of detection were classified as "BLD". Samples above the limit of detection were multiplied by the dilution factor to express the Vtg concentration as ng/mL plasma.

Intra- and inter-assay coefficients of variation (CV) were calculated using internal references (frozen aliquots of diluted plasma from E_2-treated eels).

Intraperitoneal exposure experiment

E_2 and 4-nonylphenol (4-NP, Aldrich Chemical Company, USA) were selected as environmentally relevant Vtg-inducing agents. A low and high dose concentration of E_2 (0.05 and 5 mg/mL) and 4-NP (10 and 100 mg/mL) were suspended in corn oil. Immature "yellow" eels of mixed sex (100-400 g) were collected as described previously. Eels (n=8 unless specified otherwise) were injected ip with one of the test solutions, or corn oil vehicle, once weekly for three weeks. Individual animals were fin-tagged with coloured cotton to facilitate easy identification. Whole blood was collected from the caudal vein four days after the last dose. Plasma was isolated by centrifugation and stored at –20°C until analysis.

Statistical analysis

Prior to analysis, the data were tested to verify whether they satisfied the assumptions of the analysis of variance (ANOVA). Data were log transformed because of variance heterogeneity. A two-factor ANOVA was used to examine treatment and day of sampling effects (a consequence of the staggered sampling design). Geometric means are shown. Where ANOVA analysis revealed a significant treatment effect, one-sided or two-sided pairwise treatment comparisons (*t*-tests) were made and corrected using a Bonferroni adjustment (SYSTAT® 7.0 for Windows®, SPSS Inc., Illinois, USA).

RESULTS

Purification of *Anguilla australis* vitellogenin

The concentration of protein in the column elutant was monitored spectrophotometrically at 280 nm. The measurement of an absorbance peak spanning fractions 5-9 (maximum at 0.28 M NaCl) in plasma from control and E_2-treated eels coincided with the rapid increase in buffer salt concentration (Figure 1). As the concentration of NaCl was increased, two further absorption peaks corresponding to fractions 10 and 11 (maximum at 0.32 M NaCl) and 12 (maximum at 0.34 M NaCl) were observed in plasma from the E_2-injected eels (Figure 1). Proteins in the FPLC fractions 9-12 from E_2-treated eel plasma were separated using SDS-PAGE revealing a major protein band at 197 kDa (Figure 2A). Two lighter protein bands at 123 and 116 kDa were also observed (Figure 2A). These protein band profiles were consistent between purified protein fractions from different eels (Figure 2A), and corresponded to an E_2-dependent protein band present in plasma from E_2-treated eels but absent in controls (Figure 2A).

Characterisation of antisera and immunoassays

Titre checks on serum from each rabbit prior to harvesting the plasma revealed antibody titres between 20,000 and 50,000. Positive displacement of the antisera with free Vtg was also observed with all batches of sera. Western blot analysis of E_2-treated eel and control plasma and the purified protein after SDS PAGE revealed that the antisera recognised the purified protein, while no cross-reactivity was observed against purified Vtg from rainbow trout (*Oncorhynchus mykiss*) or the African clawed toad (*Xenopus laevis*) (data not shown). However, the antisera also displayed extensive non-specific recognition of proteins in both the control and treated plasma (Figure 3). Non-specific binding was confirmed by preliminary ELISA when the epitope specificity of antisera for purified eel Vtg and plasma from E_2-treated eels was evaluated.

Assuming that the antisera-antigen binding is specific, then titration curves of serially diluted purified Vtg and plasma from induced eels will be parallel (Chard 1982). At dilutions of treated plasma greater than 1:8000 there was a lack of parallelism (Figure 4). Furthermore, a titration curve of control plasma indicated non-titratable antisera recognition of a component of the control plasma (Figure 4). This interference was also observed in cell medium collected from non-stimulated eel primary hepatocyte cultures (data not shown). In contrast, titration curves derived from the plasma of non-treated immature Chinook salmon (*Oncorhynchus tshawytscha*) and laboratory mice (C57BL/6) showed no antisera recognition (data not shown). Attempts to remove the non-specific recognition by varying buffer types and concentrations, using serum instead of plasma, and passing plasma through centrifugation molecular filters were unsuccessful (data not shown).

Further characterisation of the 1°Ab by immunoelectrophoresis revealed that there was a minor IgG species that recognised an epitope in both the control and E_2-treated plasma (Figure 5). To negate effects of this IgG species the 1°Ab was adsorbed with control plasma, a process that saturates all available binding sites for this species of IgG. In Western blots, the integrity of the 1°Ab recognition of the 196 kDa band was retained but the non-specific background was removed (Figure 2B). Furthermore, parallelism was observed between titrations of plasma from E_2-treated eels and purified Vtg, while titrations of control eel plasma exhibited no signs of non-specific binding (Figure 6).

Optimisation of ELISA Assay

Despite resolving the non-specific binding by adsorbing the 1°Ab with control plasma, this process had created a very high background (absorbance) in the assay zeroes (no Vtg well coating). Furthermore, the rate of background absorbance was directly proportional to the concentration of primary antibody used (data not shown). By substituting 0.1% gelatin for BSA during the blocking step of plate development, this background absorbance dropped to almost zero, with little sign of an increase with increasing concentrations of 1°Ab. Following a checkerboard design the optimal conditions of Vtg well coating concentration and 1°Ab dilution rate were found to be 4 ng/well and 1:80,000 respectively (Figure 7).

Precision tests and specificity of the assay

The limit of detection (LOD) was calculated to be 35.0 ng/well. Using the data from internal references, an inter-assay CV was calculated to be 19.8%. An intra-assay CV was derived from the mean CV values for every sample on one plate measured in triplicate and was calculated to be 12.2% (data not shown).

Intraperitoneal exposures

The mean eel plasma Vtg concentration was significantly greater ($P<0.001$) in the E_2 high treatment group (5 mg/kg) compared to controls (Figure 8). However, no significant difference was measured between the E_2 low-treatment group (0.05 mg/kg) and controls. Differences in the mean plasma Vtg concentration between the 4-NP high (100 mg/kg) and low (10 mg/kg) treatment groups and the control were not significant.

DISCUSSION

In the FPLC separation, three distinctive absorbance peaks were measured in plasma from E_2-treated eels. The first peak was associated with a pulse of plasma protein, most likely albumin, that has a low affinity for the column matrix and is eluted early due to the rapid increase in the buffer salt concentration (Wiley *et al.* 1979; Silversand *et al.* 1993; Palmer *et al.* 1998). The second peak was broad and short, which suggests that low levels of one or more proteins are eluted from the column at that point (Wilson and Walker 1994). In contrast, the narrower and higher third peak suggests that a large amount of a specific protein is eluted later from the column (Wilson and Walker 1994). Similar elution profiles have been reported during purification of Vtg from Atlantic halibut (*Hippoglossus hippoglossus*) (Norberg 1995), rainbow trout (*Oncorhynchus mykiss*), cod (*Gadus morhua*) and wolf fish (*Anarchichas lupus*) (Silversand *et al.* 1993). In these studies the last and highest absorbance peak was associated with the elution of Vtg.

Polyacrylamide gel electrophoresis of the fractions corresponding to the E_2-dependent absorbance peaks supports the FPLC findings. The presence of a high molecular weight and dominant protein band that is also accompanied by lesser amounts of smaller proteins has been shown in Atlantic halibut (Norberg 1995) and African clawed frog (Palmer *et al.* 1998) Vtg preparations. It was speculated that these are breakdown products of Vtg (Norberg 1995; Palmer *et al.* 1998) because Vtg is sensitive to proteolytic cleavage (Denslow *et al.* 1999). Characterisation of plasma from three different species of E_2-induced fish (Silversand *et al.* 1993) show that these bands increased in intensity when protein inhibitors were absent during the purification, suggesting they are breakdown products of Vtg. The molecular weight of the dominant protein purified from plasma from E_2 injected eels (197 kDa) is slightly larger than the molecular weights reported for other fish species (167 kDa for cod, 170 kDa for rainbow trout, 175 kDa for turbot, 176 kDa for wolfish) (Silversand *et al.* 1993). However, this value does concur with the estimated molecular weight of 200 kDa for Vtg in tilapia (*Oreochromis mossambicus*) (Kishida and Specker 1993) and the African clawed frog (Palmer *et al.* 1998).

Administration of this protein to rabbits via subcutaneous injections produced a marked immunogenic response (Peter Elder, pers comm. 2000) and supports the finding that Vtg is highly immunogenic to rabbits and mice (Denslow *et al.* 1999).

To establish that the prepared polyclonal antiserum is immunoreactive against Vtg, it is critical to determine this empirically. Western blot analysis showed that the antiserum displayed non-specific recognition of plasma proteins from both

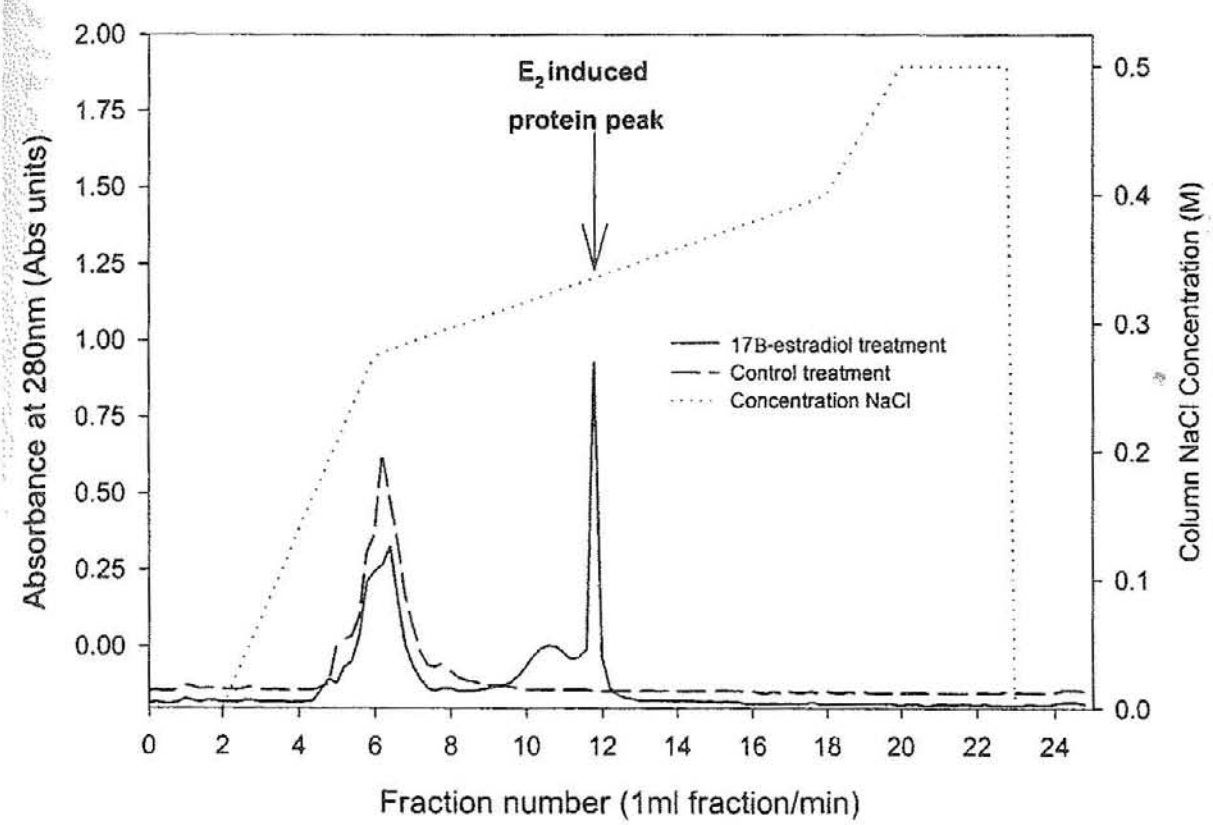

Figure 1. Absorption profile (280 nm) of diluted plasma from E_2-treated (——) and control (— — —) eels where 500 _L of diluted (1:30) plasma/per run was injected onto an anion-exchanger column connected to an FPLC system.

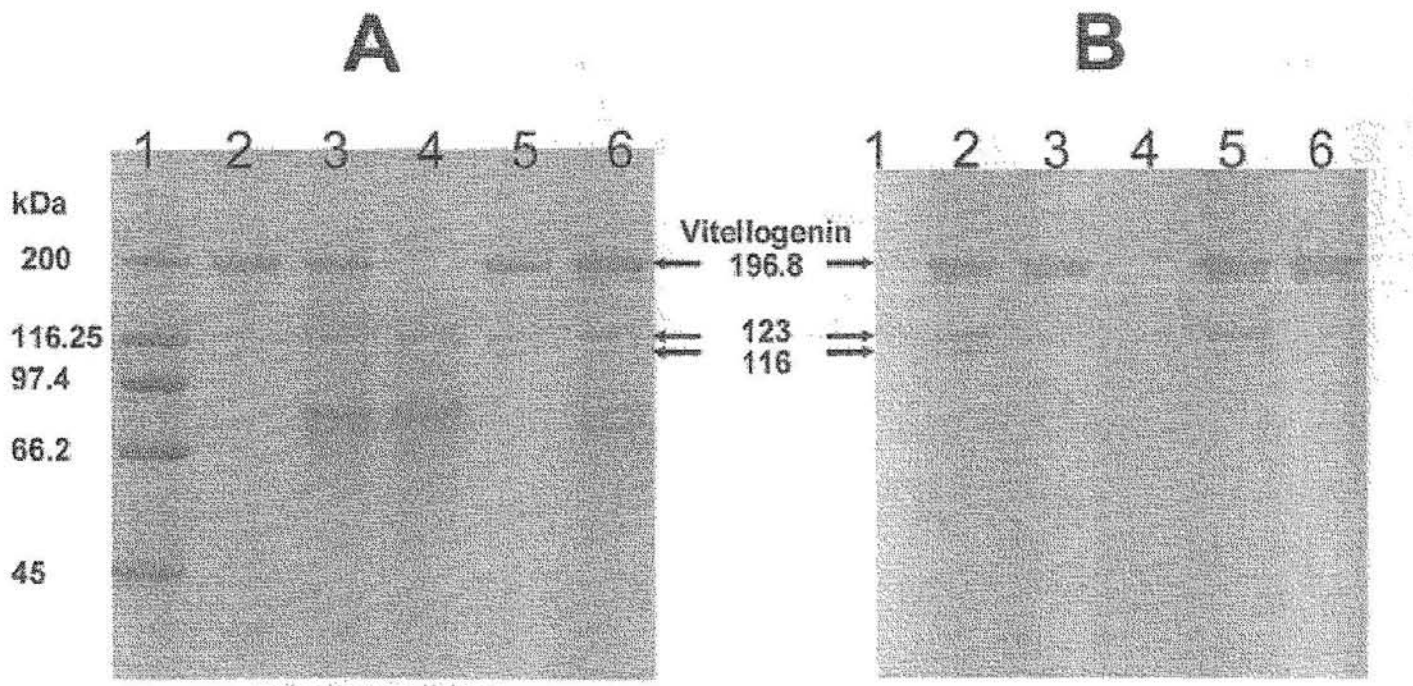

Figure 2. SDS-PAGE (A) and Western blot analysis (B) of purified Vtg and plasma collected from E_2-treated and control eels. Proteins were separated electrophoretically (150 V, 60 mA) in a 7.5% denaturing gel for 70 min at 4°C. Proteins were visualised with coomassie blue stain (A). After electrophoresis, the proteins were immobilised onto PVDF membrane and probed with adsorbed 1°Ab followed by the secondary antibody (goat anti-rabbit antiserum) conjugated to the alkaline phosphatase detection system. The membrane was developed for 7 min (B). Lanes 2 and 5 contain 1.2 µg of Vtg protein. Lanes 3 and 6 contain 8 µg of plasma protein from a control eel.

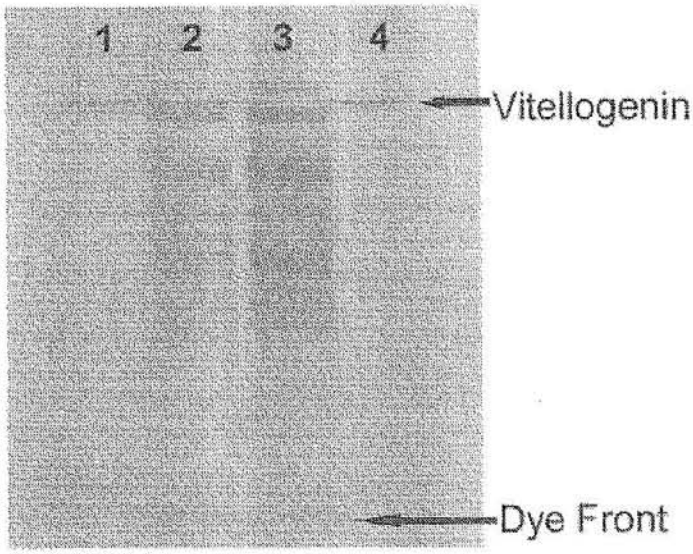

Figure 3. Western blot analysis of purified Vtg, and plasma from E_2 treated and control eels. Proteins were separated electrophoretically (150 V, 60 mA) in a 7.5% denaturing gel for 76 min at 4°C. The proteins were then transferred overnight to activated PVDF membrane. Immobilised proteins were probed with unadsorbed primary antibody (1:10,000 dilution) followed by the secondary antibody conjugated to the alkaline phosphatase detection system. The membrane was developed for 7 min. Lanes 1 and 4 contain 1 µg of purified Vtg. Lanes 2 and 3 contain 7 and 8 µg of plasma protein from E_2-treated and control eels, respectively.

control and E_2-treated plasma. Parallelism experiments confirmed these observations and showed that when plasma Vtg levels were below a critical threshold, the plasma titration curve would reach a plateau and would remain unaffected by further dilution except at very large dilutions. Similar to the Western blots, the parallelism experiments showed that the antiserum was also recognising a component present in both E_2-treated and control serum. This antibody/plasma factor interaction was also non-titratable except at very large dilutions. Changing the ELISA assay buffer pH, molarity, assay buffer type, and passing plasma through a molecular filter did not eliminate the non-specific recognition. However, results from passing samples through a molecular filter found non-specific binding in both the eluate and retentate, suggesting that the immunoreactive factor was less than 30 kDa and may also bind to factors found in the retentate. In addition, the presence of non-specific binding when supernatant from cultures of shortfin eel primary hepatocyte was used, suggests that the interfering factor is secreted by hepatocytes.

Further characterisation of the primary antiserum by immunoelectrophoresis revealed a major precipitation arc in both E_2 and control lanes indicating that the two species of IgG were recognising epitopes in the E_2-treated plasma and were thus responsible for the non-specific recognition. The spatial difference between the major precipitation arcs in the lane containing purified

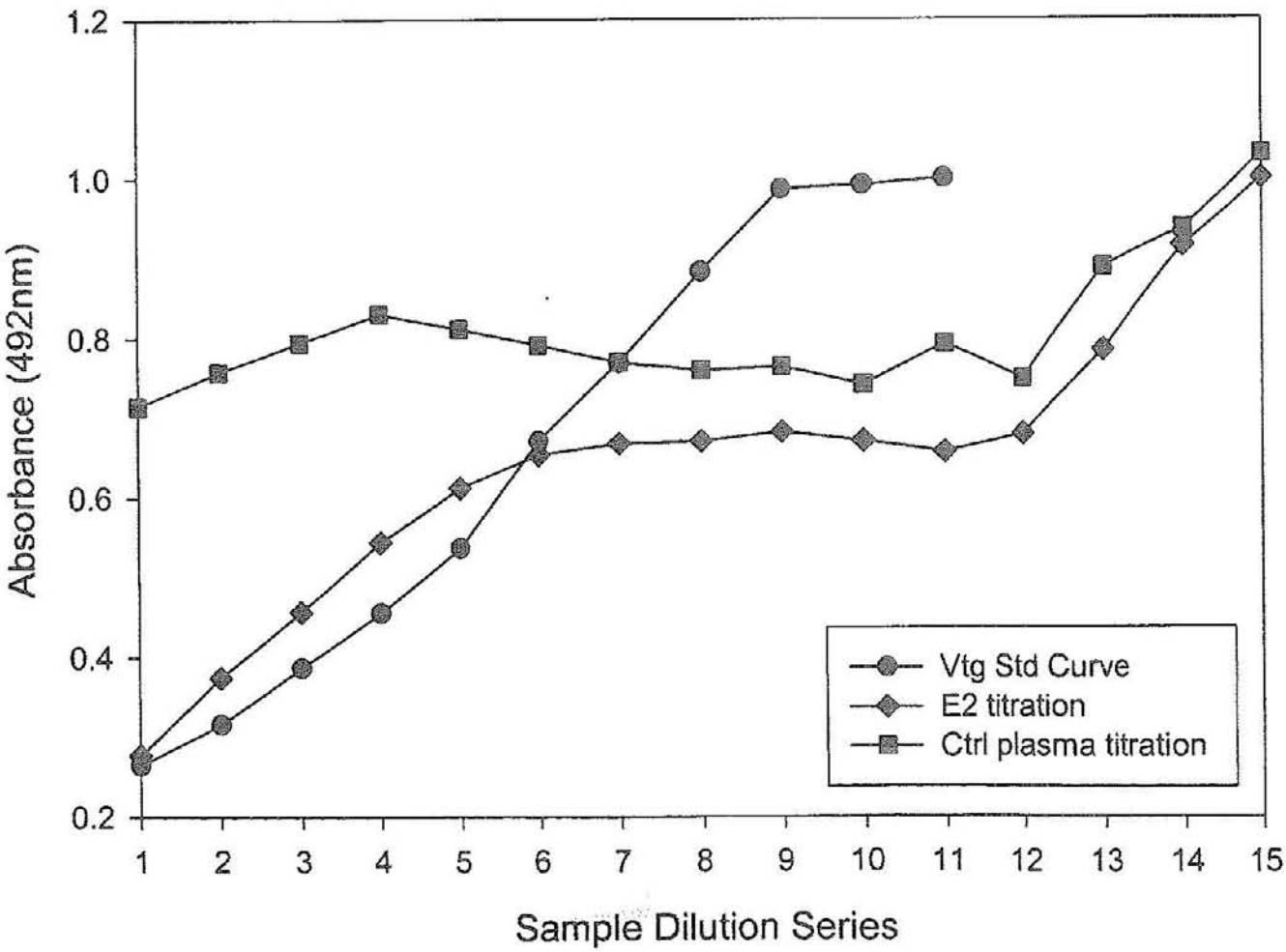

Figure 4. A preliminary ELISA to investigate the parallelism between titrations of purified eel Vtg and plasma from E_2-treated eels. Control (Ctrl) and E_2-treated plasma were serially diluted (beginning at 1:500 times). Purified Vtg was also serially diluted to produce a dilution curve ranging from 116.15 to 0.23 ng/well.

Vtg and E_2-treated plasma is likely to be an artifact of the Vtg purification process that may have altered the electromobility of the Vtg protein (Wilson and Walker 1994; John Lewis, pers comm. 2000).

Adsorbing the interfering species of IgG with excess epitope is a common practice during ELISA development (John Lewis, pers comm. 2000). Incubating the primary antiserum with control eel plasma creates saturating conditions that eliminate the non-specific immunoreactivity due to this IgG species. Western blots and ELISA parallelism experiments carried out with adsorbed primary antibody confirmed that the non-specific background had disappeared. Furthermore, the results confirmed that the specificity of the antiserum-Vtg response has been conserved.

Unfortunately, manipulation of the primary antiserum to eliminate non-specific binding created a high background value during ELISA analysis. Furthermore, the level of background was proportional to the concentration of primary antiserum used in the assay, suggesting that BSA was promoting binding of the adsorbed primary antiserum. Substituting BSA with 0.1% gelatin as a constituent of the ELISA buffers reduced the background to an acceptable level.

The use of E_2 as a model inducer of vitellogenesis has been reported for a range of fish species (Hara *et al.* 1993; Anderson *et al.* 1996; Arukwe *et al.* 1999a,b; Cooke and Hinton 1998), including the European (*Anguilla anguilla*) and Japanese (*Anguilla japonica*) eels (Komatsu *et al.* 1996; Luizi *et al.* 1997; Peyon *et al.* 1997; Komatsu and Hayashi 1998). As expected, shortfin eels that were exposed to multiple ip doses of E_2 (5 mg/kg) produced a 22-fold increase in plasma Vtg concentration compared to controls. However, Vtg concentration in eels exposed to E_2 at a lower dose (0.05mg/kg) were not significantly greater than control eels, even though this dose is slightly lower than those used to elicit a vitellogenic response in Atlantic halibut and rainbow trout (Norberg 1995; Anderson *et al.* 1996). Compared to other fish species, the genus *Anguilla* appears to be less responsive to E_2 exposure, a finding that is also reported for this study in shortfin eels. For example, studies that used salmon and trout species required only a single ip injection (1-5 mg/kg) to elicit a 40 to 90-fold increase in plasma Vtg (Anderson *et al.* 1996; Arukwe *et al.* 1999a,b). In contrast, studies of *Anguilla* species injected with multiple doses of similar concentrations of E_2, including this study, resulted in smaller increases in plasma Vtg concentrations (Burzawa-Gérard and Dumas-Vidal 1991; Burzawa-Gérard and Delavallée-Fortier 1992; Peyon *et al.* 1997). This would suggest that the lack of Vtg induction seen in the low-E_2 dose group (0.05 mg/kg) is probably due to the level of circulatory E_2 being insufficient to exceed the E_2-dependent Vtg induction threshold, a threshold that appears to be greater in eels compared with other fish species. Alternatively, the prolonged and unique sexually immature state maintained by species of *Anguilla* while occupying the freshwater environment may be due to the down-regulation of factors that potentiate the effects of E_2 (Peyon *et al.* 1996; Peyon *et al.* 1998).

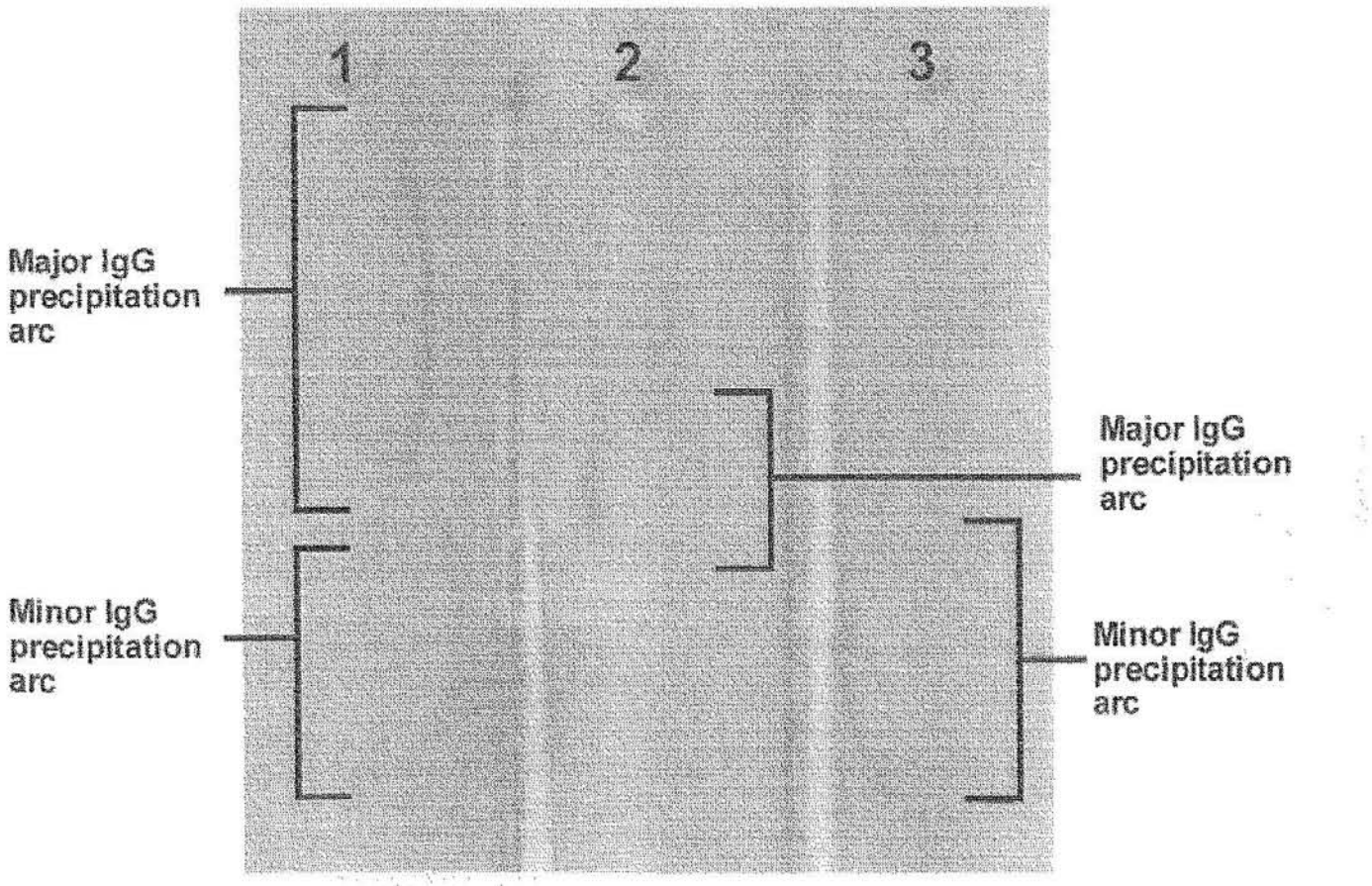

Figure 5. *Immunoelectrophoresis gel stained with coomassie blue stain. Lane 1 contains 1.2 µg of plasma protein from an E_2-treated eel. Lane 2 contains 1.2 µg of purified eel Vtg. Lane 3 contains 7.08 µg of plasma protein from a control eel. Proteins were separated electrophoretically on a 1% agarose gel run at 150 V (150 mA) and room temperature for 50 min. Primary antiserum was added to troughs adjacent to each lane and incubated overnight. Immunoprecipitated proteins were visualised with coomassie blue stain.*

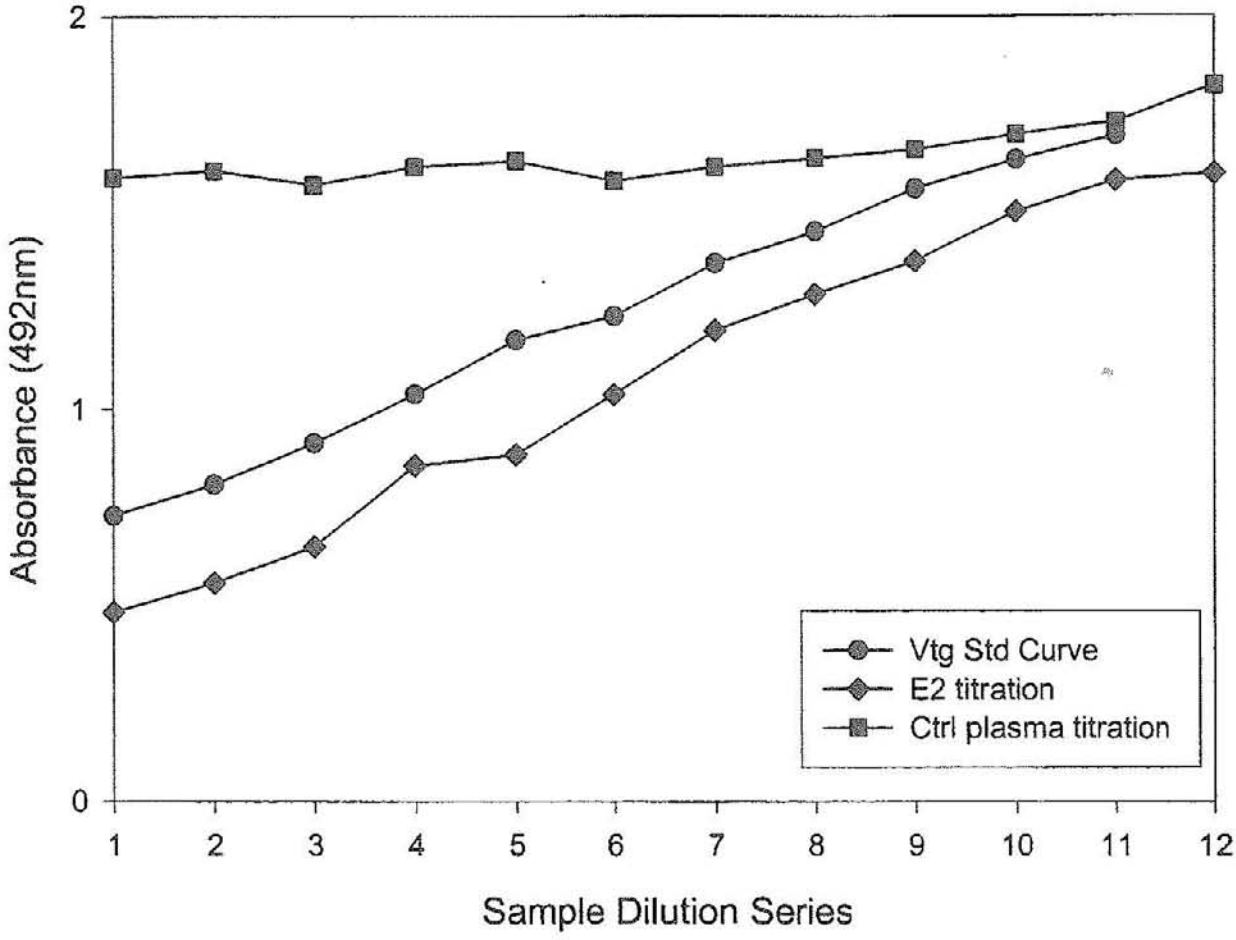

Figure 6. The binding specificity of adsorbed primary antiserum that has been diluted 160,000-fold (160K). Results are of an ELISA that investigated the parallelism between titrations of purified eel Vtg and plasma from E2-treated eels. Control and E2 plasma were diluted 1:500 (dilution 1). Each point after that is a 1:2 dilution of the previous sample. Similarly, Vtg stock (0.46 µg/L) was serially diluted to produce concentrations ranging from 116.15 to 0.23 ng/well.

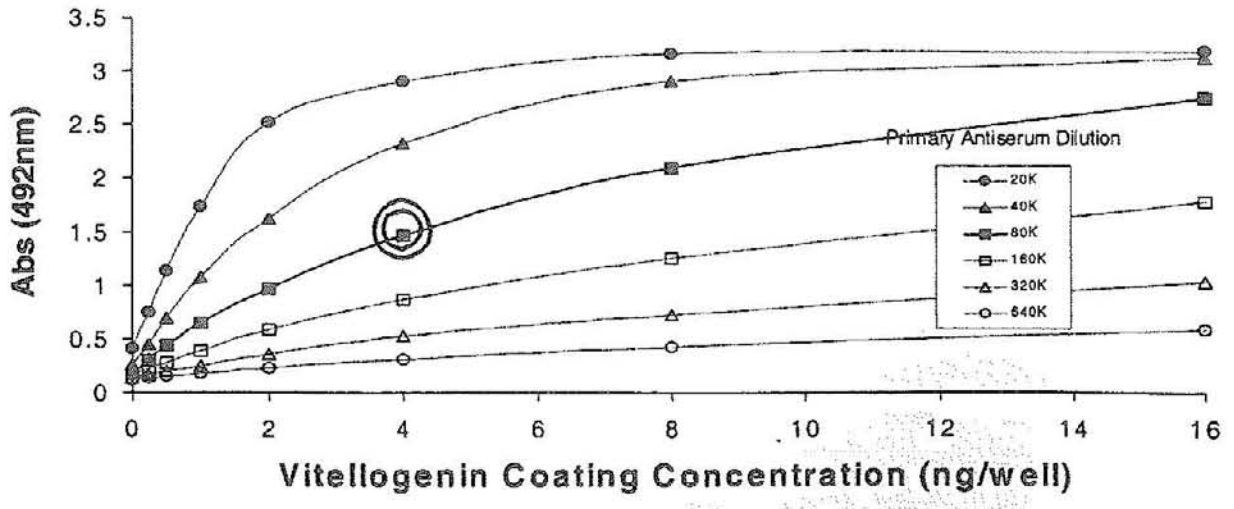

Figure 7. The optimisation of Vtg ELISA conditions. A checkerboard plate design was used to optimise the Vtg well coating concentration and the adsorbed primary antibody dilution factor. Serial dilution of the adsorbed 1°Ab ranged from 20,000-fold to 640,00-fold (ò 20K, p 40K, ¢ 80K, £ 160K, r 320K, ô 640K). A double ring highlights the optimum conditions.

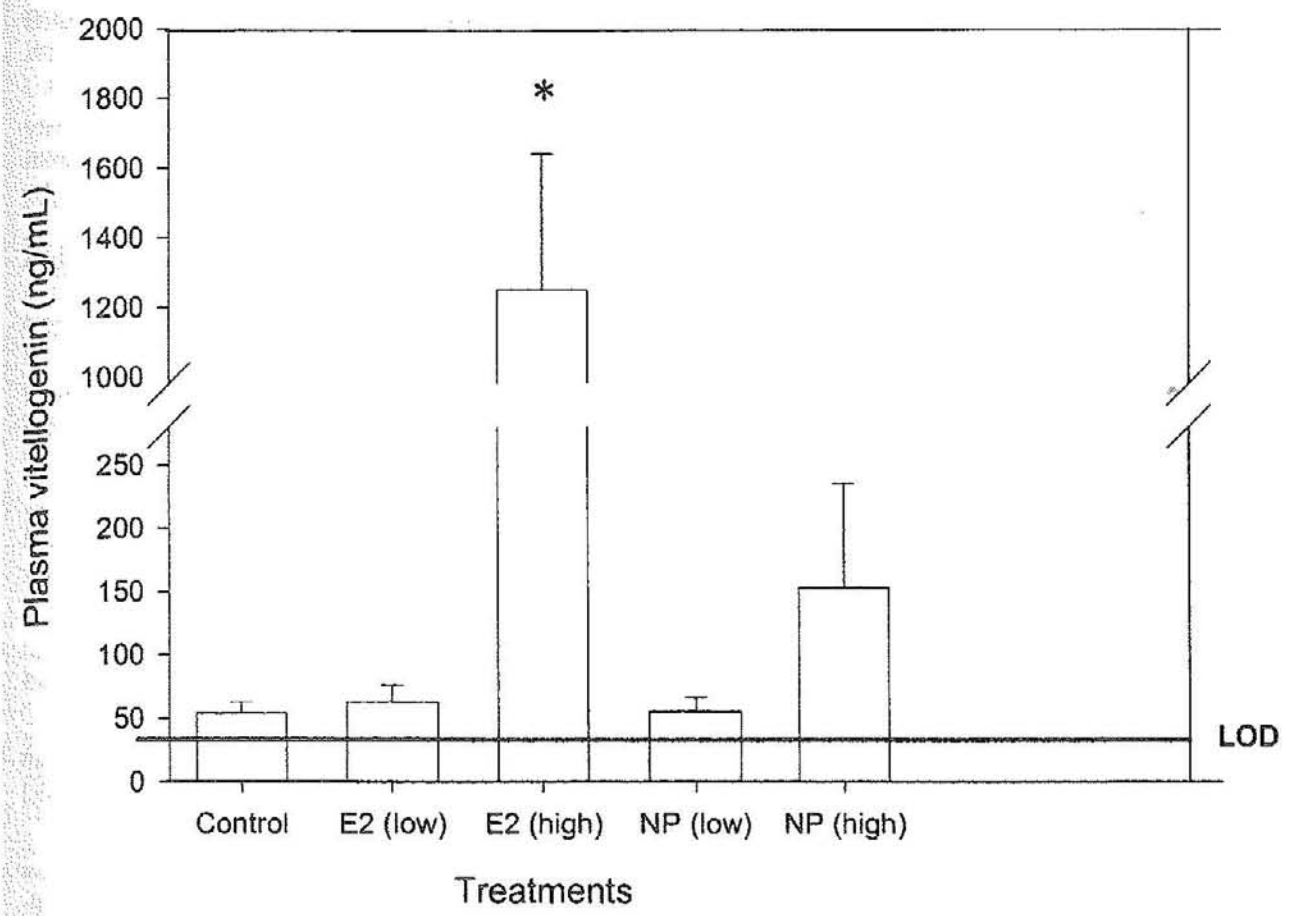

*Figure 8. The concentration of Vtg in plasma from immature eels 4 days following multiple ip dosing of E_2 (low – 0.05 mg/kg and high – 5.0 mg/kg) and 4-NP (low – 10 mg/kg and high – 100 mg/kg). Geometric treatment group means (+SD) are shown. Sample size is n=8, except for E_2 (H) where n=7. * denotes a significant difference from the control (P<0.0001). LOD denotes the limit of detection.*

Treatment with multiple doses of 4-NP (100 mg/kg) to shortfin eels produced a small (threefold) but non-significant increase in plasma Vtg. Exposure to a low dose (10 mg/kg) of 4-NP was insufficient to produce any difference to control fish. These results support previous findings that eel Vtg induction is more resistant to a challenge by E_2 and other compounds that act through the E_2 receptor than other fish species. For example, Atlantic salmon (*Salmo salar*) exposed to a single ip injection of 4-NP at 25 and 125 mg/kg induced 1.7- and 32-fold increases, respectively, in plasma Vtg (Arukwe *et al.* 1998; Arukwe *et al.* 1999a,b). Flounder (*Platichthys flesus*) exposed to 4-NP (10 to 200 mg/kg) four times a week for 2 weeks, showed a 10- to 80-fold increase in plasma Vtg (Bjerregaard *et al.* 1998).

Using standard chromatographic separation and protein analysis techniques, the production of stocks of IgG rabbit anti-eel Vtg 1°Ab were achieved. Further characterisation revealed non-specific IgG interference and high background levels in the ELISA assay, and these were eliminated. The production of a sensitive ELISA assay to measure shortfin eel plasma Vtg levels provided evidence that supported findings from studies with other species of eels that also showed that eels are less responsive to chemicals acting via the E_2 receptor to induce plasma Vtg concentrations. Combining this *in vivo* approach with *in vitro* primary eel hepatocyte culture assays will further enhance the value of this ELISA assay in environmental testing for estrogenic compounds.

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