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# Relationship between Reproductive Success and Male Plasma Vitellogenin Concentrations in Cunner, *Tautogolabrus adspersus*

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**The gene for vitellogenin, an egg yolk protein precursor, is usually silent in male fish but can be induced by estrogen exposure. For this reason, vitellogenin production in male fish has become a widely used indicator of exposure to exogenous estrogens or estrogen mimics in the aquatic environment. The utility of this indicator to predict impacts on fish reproductive success is unclear because information on the relationship between male plasma vitellogenin and reproductive end points in male and female fish is limited. In the research reported in this article, we investigated whether the presence of male plasma vitellogenin is a reliable indicator of decreased reproductive success in mature fish. Adult and sexually mature male and female cunner (*Tautogolabrus adspersus*) were exposed to 17β-estradiol, ethynylestradiol, or estrone, three steroidal estrogens that elicit the vitellogenic response. Data were gathered and pooled on egg production, egg viability, egg fertility, sperm motility, and male plasma vitellogenin concentrations. All males, including two with plasma vitellogenin levels exceeding 300 mg/mL, produced motile sperm. Neither percent fertile eggs nor percent viable eggs produced by reproductively active fish demonstrated a significant correlation with male plasma vitellogenin concentrations. Male gonadosomatic index and average daily egg production by females showed significant, but weak, negative correlation with male plasma vitellogenin concentrations. Results suggest that male plasma vitellogenin expression is not a reliable indicator of male reproductive dysfunction in adult cunner exposed to estrogens for 2–8 weeks during their reproductive season, at least in relation to capacity to produce motile sperm or fertilize eggs. Male plasma vitellogenin expression may serve as an indicator of reduced female reproductive function caused by estrogen exposure. *Key words:* cunner, estrogens, fish, indicator, reproduction, vitellogenin. *Environ Health Perspect* 111:93–99 (2003). [Online 4 December 2002] doi:10.1289/ehp5531 available via *http://dx.doi.org/***

Vitellogenin is a large serum phospholipoglycoprotein normally produced in the liver of female oviparous vertebrates in response to circulating endogenous estrogen. It is a precursor of egg yolk proteins and, once produced in the liver, travels in the bloodstream to the ovary, where it is taken up and modified by developing eggs. Vitellogenin is normally undetectable in the plasma of immature and male oviparous animals because they lack circulating estrogen, although the vitellogenin gene in these animals can be induced by estrogen exposure (*1,2*). As a result, the presence of notable vitellogenin in the plasma of immature and male animals is considered evidence that they have been exposed to exogenous estrogens or estrogen mimics (*1–3*). The relevance of this indicator to actual impacts on fish populations is unclear because information on the relationship between male plasma vitellogenin and reproductive success in fish is limited. We investigated whether elevated male plasma vitellogenin is a reliable indicator of decreased reproductive success in a laboratory model. In this article, we report on the correlation between measures of reproductive output in laboratory studies with adult cunner (*Tautogolabrus adspersus*) and the widely used indicator of estrogen exposure, male plasma vitellogenin expression.

Vitellogenin in male fish has been used as an indicator of exposure to estrogenic contamination in a well-documented series of studies conducted in rivers of the United Kingdom (*2,4–8*). Using caged male trout, Sumpter and Jobling (*2*) conducted a nationwide survey to assess estrogenic effects in British rivers and reported that at all sites where fish survived, there was a pronounced increase in plasma vitellogenin concentrations. Later findings suggest that the steroidal estrogens present in sewage effluent could account for the vitellogenic response observed in these male fish (*7,8*). Evidence continues to mount that natural and synthetic steroidal estrogens such as 17β-estradiol, ethynylestradiol, and estrone are potent environmental inducers of the vitellogenic response in male fish, more so than some of the well-known environmental estrogen mimics such as alkylphenols or *o,p′*-DDT (dichlorodiphenyl trichloroethane) (*7–10*).

Several studies have linked high plasma vitellogenin concentrations in males of aquatic species with increased mortality and tissue histopathology (*11–14*), but information on the relationship between male plasma vitellogenin and reproductive success is limited. Such information is critical if male plasma vitellogenin is to be applied as an indicator not only of individual exposure but also of population impacts through effects on fish reproductive success. Sumpter and Jobling (*2*) speculated that although they could not "ascribe any deleterious consequences to the unnatural synthesis of vitellogenin" in male fish, it was "probable that these changes from the normal pattern will adversely affect reproduction." Several years later, Kime et al. (*15*) theorized that in females, increased plasma vitellogenin could have effects on egg numbers and size, gonadosomatic index (GSI), and spawning success. The same authors speculated that, in males, increased plasma vitellogenin might result in compromised testicular function, decreased sperm production and quality, and disruption of sperm maturation. Kramer et al. (*16*) correlated reproductive function in fathead minnows with plasma vitellogenin expression, measured as alkaline-labile phosphorus (ALP). Using fish exposed to waterborne 17β-estradiol, these researchers found that ALP concentrations in females or males were inversely related to egg production. Fertility of eggs or motility of sperm, which would serve as a measure of male physiologic function, was not determined in their study. Jobling et al. (*17*) found a significant correlation between elevated plasma vitellogenin

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concentrations and occurrence of intersexuality in wild populations of roach (*Rutilis rutilis*) from British rivers.

In this article, we report on the correlation between measures of reproductive output in laboratory-exposed adult cunner of both sexes and male plasma vitellogenin expression. Cunner (*Tautogolabrus adspersus*) is a temperate reef fish normally found near areas with abundant submerged cover, such as rocks, stone walls, or pilings. Because of their habitat preference, they are likely to be found near shore in estuarine waters that may receive sewage treatment effluent. In Rhode Island (USA) waters, adult cunner spawn daily over approximately a 6-week period beginning in mid-spring and ending in early summer (*18*). Cunner exhibit a well-characterized courtship behavior that culminates in a sudden rush to the surface of the water with simultaneous release of gametes by male and female fish (*19–21*). Interestingly, this species was once abundant in the estuary of Narragansett Bay, Rhode Island, but numbers caught in survey trawls exhibited a dramatic decline starting in 1969 and have been totally absent from trawls since 1989 (*22*). The reasons for this decline are unclear. There are still isolated areas of the bay that have notable cunner populations, and we have collected fish from such an area to conduct laboratory exposure experiments using 17β-estradiol, ethynylestradiol, and estrone, all potent estrogens found in sewage treatment effluent (*7,8,23*). These three chemicals are steroidal estrogens that have been shown to elicit the vitellogenic response in male fish with aqueous exposures in the nanogram per liter range or lower (*7,9,24*). We compiled data from a series of experiments using these three different steroidal estrogens and exposures of both short and long duration, so that the correlations between male plasma vitellogenin concentrations and reproductive end points reported here are not limited to a single chemical or exposure regime. Because male plasma vitellogenin is routinely used as an indicator in fish collected from the field, where length and type of chemical exposure might vary greatly, pooling our data from various exposure scenarios is realistic. We focus not on differences in vitellogenin induction with chemical treatment or length of exposure, but on whether male plasma vitellogenin concentrations indicate an effect (reproductive impairment) regardless of exposure length or type.

In laboratory studies, we gathered data on egg production, egg viability, egg fertility, sperm motility (presence or absence), male GSI, and male plasma vitellogenin concentrations in cunner over a series of exposure experiments to 17β-estradiol, 17α-ethynylestradiol, and estrone. On a daily basis, we measured three parameters to determine reproductive output: total egg production per female, percentage of total eggs that were viable (i.e., developing normally) after 24 hr, and percentage of total eggs that were fertilized. Total egg production measures female reproductive function, because ripe female cunner spawn daily, even without a male present (data not shown). Percent developing eggs, which consists of fertilized eggs with developing embryos 1 day after eggs are spawned, indicates ability of both sperm and egg to produce a viable embryo. Percent fertilized eggs, on the other hand, includes both viable (developing) and nonviable (in arrested development) eggs and so reflects the ability of males to fertilize eggs and is independent of the ability of those fertilized eggs to develop into viable embryos.

## Materials and Methods

***Animals.*** Cunner were collected from Narragansett Bay off the Jamestown pier at the southern tip of Jamestown, Rhode Island (USA), during the summers of 1999 and 2000. The cunner were captured using standard wire-mesh Gee's minnow traps (Cuba Specialty Manufacturing Company, Fillmore, NY) modified to allow entry of larger fish and baited with fish scraps, mussels, and squid. Captured cunner were transported to the laboratory in coolers filled with ambient seawater and then held in large rectangular (4,400 L) or round (1,000 L) holding tanks that were aerated and received ambient, flow-through bay seawater. Fish were fed a diet of thawed krill, squid, and mussels *ad libitum* and were overwintered in the laboratory at ambient bay temperatures. Ripe fish were sexed either when they arrived in the laboratory or before the start of an experiment by gently stripping gametes manually. The length of male fish in this study ranged from 11.7 to 19.0 cm, with an average (± SD) of 14.3 ± 1.6 cm. Weight ranged from 22.6 to 98.4 g with an average of 44.2 ± 15.7 g.

***Experimental design and sampling.*** Before beginning exposures, two male and three female fish were transferred to each experimental tank. Each experimental tank was an 80-cm-tall, 114-L-capacity, high-density polyethylene barrel (47 cm diameter) with a clear Plexiglas cover. All tanks were aerated and received flow-through seawater at 1 L/min. To provide submerged cover for the fish, each tank also contained one 20-cm length of 10-cm-diameter PVC pipe. Fish were allowed to acclimate to experimental conditions for 3–5 days before an experiment was started. Further details of the experimental system can be found in Gutjahr-Gobell et al. (*18*). Two different scenarios were followed for exposure experiments (Figure 1). One consisted of a 7–8-week exposure period that began while fish were acclimated from overwintering to spring spawning conditions. The second consisted of a 2-week exposure period started once spawning was already well underway. In both cases, reproductive end points were measured under natural spawning conditions (i.e., 18°C water and a photoperiod of 15 hr light and 9 hr dark, with

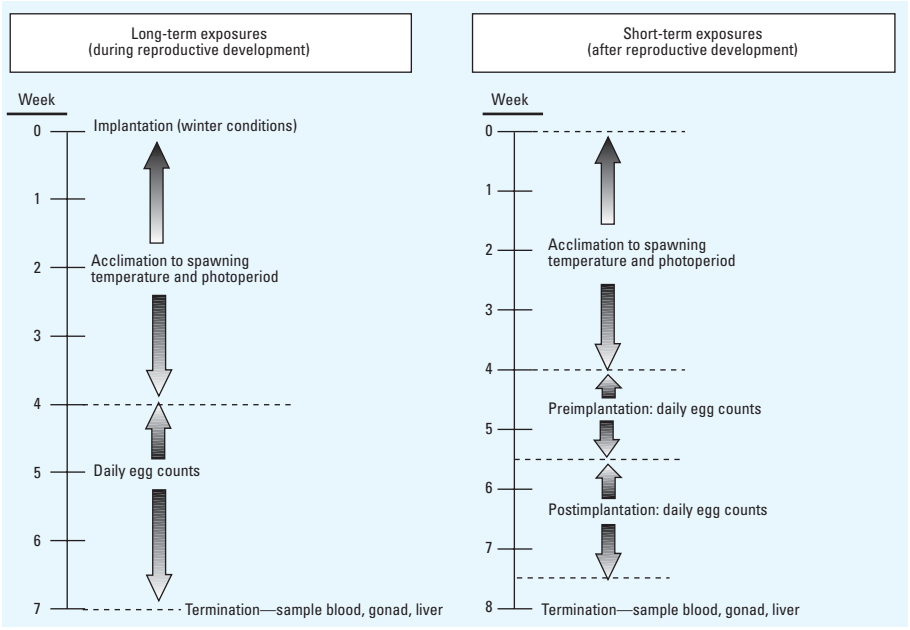


**Figure 1.** Summary of two different exposure regimes followed during the experiments from which our data on male plasma vitellogenin levels and various reproductive parameters were taken. In both scenarios, male plasma vitellogenin levels were correlated with egg production, percentage viability, or percentage fertility data taken the week before sampling for vitellogenin. Milt expression and sperm motility were measured at the same time as sampling for plasma vitellogenin.

computerized dimming of fluorescent lighting to simulate dawn and dusk). In some tanks under both scenarios, one of the two males in a tank was aggressive and killed the other male in the tank. Almost all of this male mortality preceded the start of the chemical exposures—in fact, only two males of 105 starting exposures died during the exposure period, and one of these was in a control treatment. To investigate the correlation between male plasma vitellogenin concentrations and male GSI, data from the 103 surviving male cunner were analyzed. These data were taken from tanks with either one or two males. To correlate individual male plasma vitellogenin concentrations to egg data, only egg data from tanks with a single male were used. This means that egg data and male vitellogenin concentrations were compared from a total of 49 tanks, each with a single male cunner.

During experiments, all fish were fed fresh or thawed mussels *ad libitum* every day. Tall experimental tanks were used to accommodate the vertical spawning run of cunner. Because cunner eggs are buoyant for a period of hours after spawning, each tank was fitted with a top drain to siphon surface water and spawned eggs. This siphon was directed into an egg collection cup for each tank. Egg collection cups consisted of two nested Nytex screens, a coarse upper screen (2 mm) to catch debris and a fine lower screen (0.4 mm) to collect eggs. The lower screen was always submerged in a pool of seawater to prevent eggs from drying out.

In the laboratory, cunner routinely spawned in the afternoon, and eggs were rinsed from collection cups the next morning for counting. Eggs from each collection cup were rinsed into 50–200 mL of seawater (depending on volume of eggs collected), and three 1-mL subsamples of a well-mixed suspension were counted using a dissecting microscope. For each subsample, the numbers of buoyant eggs, sunken eggs, and developing eggs were recorded. Only buoyant eggs were counted as viable, but eggs showing any development were considered fertile (whether buoyant or not). Total eggs spawned per female each day is the sum of buoyant eggs plus sunken eggs divided by the number of females in a tank. For correlation analysis, the plasma vitellogenin concentration of each male, determined from a plasma sample taken at experiment termination, was compared with egg data collected over the 7 days before experiment termination. To correlate individual male plasma vitellogenin concentrations to egg data for a tank, only data from tanks with a single male were used. Data from a total of 49 tanks, each with a single male cunner, were analyzed. These data were pooled from a series of experiments using one of three different steroidal estrogens (17β-estradiol, ethynylestradiol, or estrone) and exposures of both short and long duration.

At the end of an experiment, each fish was anesthetized in seawater containing MS-222. A milt sample was obtained by gently applying pressure to the dried abdominal area of each male fish and collecting milt extruded from the urogenital pore with a micropipette. A drop of milt was immediately placed on a microscope slide, mixed with a drop of seawater, and observed under a microscope to check for sperm motility. Sperm in each milt sample was determined only to be motile or nonmotile, with no attempt to determine rate of movement or other parameters. Each fish was then bled using a heparinized syringe. Blood was drawn from a caudal vessel using a 1 mL tuberculin syringe with a 22-gauge needle rinsed with the anticlotting agent heparin (sodium solution, 1,000 U/mL; U.S. Biochemical Corp., Cleveland, OH). Blood samples were held on ice in a polypropylene microcentrifuge tube containing 25 µL heparin and 25 µL of the serine protease inhibitor aprotinin (5–10 trypsin-inhibitor unit/mL from bovine lung; Sigma A 6279; Sigma, St. Louis, MO). After all samples were collected, whole blood was centrifuged at 2,000 × *g* for 5 min and held on ice. Plasma was pipetted off of each sample and transferred into another microcentrifuge tube kept on ice. Aprotinin was added to the plasma samples (4 µL aprotinin/100 µL plasma), a subsample was removed for vitellogenin analysis, and all samples were stored at –80°C until assay.

After blood was sampled, fish were weighed, measured, and dissected. Freshly dissected livers and gonads were weighed and then preserved in Dietrich's fixative or 10% neutral buffered formalin, along with all other tissues saved for histopathologic evaluation. GSI was calculated as the ratio of gonad wet weight to whole fish wet weight minus liver weight, multiplied by 100. Liver weight was subtracted to eliminate any temporary effects accumulation of vitellogenin in liver tissue might have on body weight. To investigate the correlation between male plasma vitellogenin concentrations and male GSI, data from a total of 103 male cunner were analyzed. These data included GSI of males from both tanks with one male and tanks with two males.

***Chemical exposures.*** 17β-Estradiol [1,3,5(10)-estratrien-3,17β-diol; CAS no. 50-28-2] was obtained from Steraloids, Inc. (Wilton, NH, USA). 17α-Ethynylestradiol [17α-ethynyl-1,3,5(10)-estratrien-3,17β-diol; CAS no. 57-63-6] and estrone [1,3,5(10)-estratrien-3-ol-17-one; CAS no. 53-16-7] were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Cunner were exposed to 17β-estradiol, ethynylestradiol, or estrone using a subcutaneous slow-release implant system developed in our laboratory (*25*). To prepare the slow-release matrix for injection, Ethocel (a formulation of ethylcellulose that was a gift from Dow Chemical Company, Midland, MI, USA) was dissolved in methylene chloride and then mixed with a 5 mg/mL stock solution of estradiol, ethynylestradiol, or estrone dissolved in acetone. Coconut oil or castor oil, used as a carrier, was mixed in slowly to prepare a homogeneous solution. The mixture was placed in a light-tight vial and held under a vacuum while on a magnetic stirrer until all solvent was removed. An implant for control fish was prepared as above, but with no chemical. All of the estrogens used for exposures were implanted at three nominal concentrations, 0.05, 0.5, or 2.5 mg estrogen per kilogram of fish wet weight.

***Plasma vitellogenin.*** To obtain a purified sample of cunner vitellogenin, three males were injected twice, 5 days apart, with 17β-estradiol in coconut oil. For these injections, 17β-estradiol was dissolved in minimal amounts of acetone and then added to coconut oil to a final concentration of 2.5 mg 17β-estradiol per milliliter of solution. To induce vitellogenesis, male fish were injected intraperitoneally with 1 mL of this solution per kilogram of body weight. Control fish were injected in the same manner but with just acetone in coconut oil. Four days after the second injection, fish were bled and plasma was separated as described above.

Vitellogenin was purified from estrogenized plasma using POROS 20 HQ anion exchange chromatography and was fractionated with BIOCAD Sprint Perfusion system (Applied Biosystems, Foster City, CA) as described by Denslow et al. (*26*). Plasma was diluted with running buffer (20 mM Bis-Tris propane, 150 mM NaCl, pH 9.0) and loaded onto the column. Nonbinding proteins were eluted by washing with the running buffer. Bound vitellogenin was selectively released from the column using a linear gradient from 150 to 800 mM NaCl. The vitellogenin peak was identified by comparing the elution profiles of male and estrogen-induced male plasma. After pooling the vitellogenin fractions, the pH was adjusted to 7.0 and the following reagents were added: protease inhibitor ($10^3$ IU/mL aprotinin), preservative (0.02% sodium azide), and cryoprotectant (50% glycerol). Total protein of the sample was determined by Bradford assay. Purity of the vitellogenin is typically > 95% as verified by sodium dodecyl sulfate–polyacrylamide electrophoresis.

Vitellogenin in plasma samples from cunner in exposure experiments with either 17β-estradiol, estrone, or ethynylestradiol was quantified by direct enzyme-linked

immunosorbent assay conducted in the Biotechnology Program Laboratory at the University of Florida. The monoclonal antibody used, SWD 2D10, was made against saltwater swordfish egg yolk protein but was shown to cross-react with high sensitivity with cunner vitellogenin by Western blot of plasma collected from estradiol-induced males. With this antibody, we can detect 5 ng/mL purified cunner vitellogenin. However, as with other vitellogenin assays, we get significant interference from other plasma proteins, and to avoid this interference we must dilute the sample at least 1:100. This dilution decreases the sensitivity of the assay to 500 ng/mL in plasma. A standard curve using the homologous purified vitellogenin (cunner) was constructed ranging from 5 ng to 1.0 µg vitellogenin/mL of 1% bovine serum albumin in TBST (1% bovine serum albumin in 10 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.6). Control male plasma at the same dilution as in the unknown sample was added to each standard curve to correct for interferences. Standards and unknowns were incubated overnight in a moist chamber at 4°C, washed four times with PBST (10 mM sodium phosphate, 150 mM sodium chloride, 0.02% sodium azide, pH 7.2 containing $10^3$ IU/mL aprotinin, plus 0.05% Tween) buffer, and blocked with 1% bovine serum albumin in TBST for 2 hr at room temperature. After blocking, monoclonal antibody SWD 2D10 at a concentration of 2 µg/mL was added to each well and plates were incubated at 4°C overnight. The next day, samples were washed, incubated for 2 hr at room temperature with a biotinylated secondary goat anti-mouse whole immunoglobulin antibody, washed again, and probed with a strepavidin-labeled alkaline phosphatase for 2 hr at room temperature. After a final wash, the antibody–antigen complex was measured colorimetrically by adding 100 µL of 1 mg/mL *p*-nitrophenyl phosphate substrate, made in 30 mM carbonate and 2 mM magnesium chloride (pH 9.6), to the wells. The concentration of vitellogenin was calculated by interpolating the color absorbance at 405 nm from the standard curve. The standards, known positive controls, and unknowns were run in triplicate, and the coefficient of variation was maintained at less than 10%. The standard curve had a regression coefficient of $r^2 = 0.95$. The sensitivity of this assay is 0.0005 mg/mL (or 0.5 µg/mL) plasma sample.

***Statistics.*** Pearson product moment correlations (*27*) were calculated for relationships between plasma vitellogenin for each male fish and *a*) percentage of viable, developing eggs produced per tank, *b*) percentage of fertile eggs produced per tank, *c*) mean egg production per female per tank, *d*) mean egg production per gram female per tank, or *e*) GSI of male fish. Significant correlation was set at $p \leq 0.05$. Because data were pooled from fish exposed to three different steroidal estrogens (17β-estradiol, ethynylestradiol, or estrone) for both short and long duration, Pearson product moment correlations (*27*) were also calculated for each experiment independently to ensure that effects observed in the pooled data set were not driven by any single chemical or exposure (data not shown).

## Results

In our experiments, concentrations of vitellogenin in control males ranged from < 0.0005 to 1.521 mg/mL plasma (Table 1). For all exposures, the highest male plasma vitellogenin concentrations were observed in fish treated with the highest nominal concentration (2.5 mg/kg) of estrogen (Table 1). In both short- and long-term experiments, ethynylestradiol generally induced higher male plasma vitellogenin concentrations than did treatments with the same nominal concentration of either estradiol or estrone (Table 1). Neither the percent fertile eggs ($p = 0.30$, $r = -0.17$; Figure 2) nor the percent viable eggs ($p = 0.66$, $r = -0.07$; Figure 3) produced by reproductively active fish demonstrated a significant correlation with male plasma vitellogenin concentrations. Male GSI ($p = 0.0016$, $r = -0.27$; Figure 4) exhibited a weak but significant negative correlation with male plasma vitellogenin concentrations. All males, even two with plasma vitellogenin concentrations exceeding 300 mg/mL, still produced motile sperm. Total egg production showed a weak but significant correlation with male plasma vitellogenin concentrations, whether measured in terms of per female per day ($p = 0.04$, $r = -0.33$; Figure 5) or per gram female per day ($p = 0.009$, $r = -0.41$; Figure 6). When Pearson product moment correlations (*27*) were calculated for each experiment independently, there were no significant negative correlations between male vitellogenin concentrations and any reproductive end point.

## Discussion

As shown in Table 1, estradiol, ethynylestradiol, and estrone exposures all induced some level of vitellogenin production in male cunner. Male cunner collected from Narragansett Bay usually have no measurable plasma vitellogenin, although we have occasionally captured males with concentrations between 0.001 and 0.5 mg/mL plasma (data not shown). In actively reproducing female cunner collected from Narragansett Bay, plasma vitellogenin concentrations usually range between 10 and 50 mg/mL, although we have seen concentrations as high as 76 mg/mL plasma. Regarding other fish species, in a survey of flounder (*Platichthys flesus*) in U.K. estuarine and marine environments, Allen et al. (*28*) found males from contaminated areas with vitellogenin concentrations on the order of 1 mg/mL plasma. Jobling et al. (*17*) found male plasma vitellogenin concentrations between 0.001 and 0.01 mg/mL in wild roach (*Rutilus rutilus*) populations from rivers throughout the United Kingdom. Folmar et al. (*3*) reported vitellogenin values as high as 10 mg/mL in male carp (*Cyprinus carpio*) collected from the effluent canal of the St. Paul, Minnesota (USA), metropolitan sewage treatment plant. Vitellogenin concentrations in male cunner exposed to estrogens in our laboratory experiments ranged from < 0.0005 mg/mL (nondetectable) to 609 mg/mL. Given levels previously reported in the literature for other

**Table 1.** Male plasma vitellogenin values by experiment.

| Chemical | Experiment | Nominal concentration (mg/kg) | Plasma vitellogenin (mg/mL) Mean | Range |
|---|---|---|---|---|
| Estradiol | Short term | Control | 0.008 | ND–0.018 |
| | | 0.05 | 0.074 | 0.037–0.294 |
| | | 0.50 | 1.420 | 0.068–3.244 |
| | | 2.50 | 12.322 | 4.465–37.984 |
| Ethynylestradiol | Short term | Control | 0.507 | ND–1.521 |
| | | 0.05 | 53.371 | 13.324–88.351 |
| | | 0.50 | 27.555 | 14.371–48.048 |
| | | 2.50 | 479.57 | 389.02–609.24 |
| Estrone | Short term | Control | ND | ND |
| | | 0.05 | 0.067 | ND–0.236 |
| | | 0.50 | 4.384 | 0.614–13.563 |
| | | 2.50 | 53.436 | 9.010–152.59 |
| Estradiol | Long term | Control | ND | ND |
| | | 0.05 | 0.200 | ND–1.408 |
| | | 0.50 | ND | ND |
| | | 2.50 | 1.780 | 0.000–5.576 |
| Ethynylestradiol | Long term | Control | ND | ND |
| | | 0.05 | 0.560 | 0.195–0.856 |
| | | 0.50 | 8.930 | 6.290–14.265 |
| | | 2.50 | 9.517 | 5.169–13.831 |

ND, nondetectable (detection limit = 0.0005 mg/mL).

species of fish collected in nature, male plasma vitellogenin concentrations exceeding 10 mg/mL could be considered extraordinarily high. Nearly one-quarter of the male cunner used in our analysis had vitellogenin concentrations exceeding 10 mg/mL.

In our experiments, ethynylestradiol generally induced higher male plasma vitellogenin concentrations than did treatment of similar duration with the same nominal concentration of either estradiol or estrone (Table 1). This may be caused by enterohepatic recirculation of this particular estrogen in cunner, similar to that recently documented in trout (*29*). In injected trout, Schultz et al. (*29*) found that ethynylestradiol was extensively conjugated and secreted into the bile of treated fish. When the gall bladder emptied, the stored ethynylestradiol was released into the gut, where most was deconjugated and reabsorbed, in effect redosing the trout with ethynylestradiol.

A few other studies have investigated the effects of steroidal estrogens on various aspects of reproductive success in fish. For instance, several studies have investigated whether changes in male mating behavior, induced by exposure to exogenous estrogenic chemicals, might decrease reproductive success. Shioda and Wakabayashi (*30*) found that when male medaka, exposed for 2 weeks to 17β-estradiol in water, were placed with unexposed reproductive females, a decrease in both the total number of eggs and the number of fertilized eggs occurred. Plasma vitellogenin was not measured in the exposed males. Bjerselius et al. (*31*) investigated how the mating behavior of male goldfish was affected by a 24–28-day exposure to estradiol through either food or water. During a 15-min observation period, males from all estradiol exposures, except the lowest food concentration, exhibited reduced courtship activity and failed to spawn. Plasma vitellogenin was not measured in these males. Kramer et al. (*16*) found that relatively high concentrations of plasma ALP (an indirect method for measuring vitellogenin) in males were not correlated with an inhibition of male courtship behavior, which is responsible for inducing female egg laying in fathead minnows. Although we did not measure spawning behavior in exposed cunner, our data on fertility of spawned eggs indicate no significant correlation between ability of male cunner to fertilize eggs (expressed as percent fertile eggs) and male plasma vitellogenin levels.

Although the relationship between male GSI and reproductive success in fish is unclear, male GSI showed a weak but significant negative correlation with male plasma vitellogenin concentrations in our experiments. Other researchers have published similar findings. Panter et al. (*24*) reported that after a 21-day exposure to either estradiol or estrone in water, developing male fathead minnows exhibited a concentration-related elevation of plasma vitellogenin with a concomitant inhibition of testicular growth. Bjerselius et al. (*31*) observed a 2–3-fold reduction in GSI values compared with controls in male goldfish exposed for 24–28 days to estradiol via food or water. In addition, significantly fewer male goldfish exposed to 100 µg/g estradiol in their food had expressible milt compared with a control group, but plasma vitellogenin was not measured in these males. Jobling et al. (*32*) demonstrated that the effects of waterborne estrogenic alkylphenols or ethynylestradiol on rainbow trout GSI were dependent on the stage of reproductive development of the fish. In their study, GSI in developing male rainbow trout was significantly ($p = 0.0001$) and inversely correlated with increased plasma vitellogenin concentration, but these inhibitory effects on testicular growth were absent in sexually mature or regressing fish. The male fish in our experiments were actively reproducing and thus sexually mature, but we still observed a significant negative correlation between GSI and plasma vitellogenin concentration in male cunner. However, when data from the 10 males with the highest plasma vitellogenin concentrations (those exceeding 50 mg/mL) are removed from consideration, male GSI no longer exhibits a significant correlation ($p = 0.07$) with vitellogenin concentration, which suggests that male vitellogenin concentrations in reproductively active cunner have to exceed a threshold of approximately 50 mg/mL plasma before they are indicative of reduced male GSI. In addition, our data indicate that a low GSI may not necessarily affect male reproductive success. All reproductively active male cunner in our experiments, even

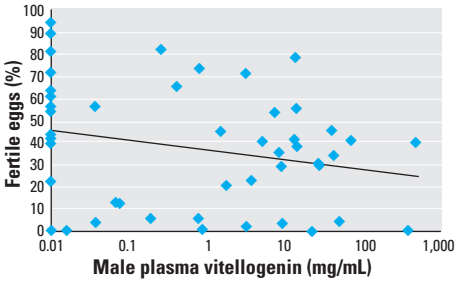


**Figure 2.** Correlation between percent fertile eggs and male plasma vitellogenin concentrations. Percent fertile eggs includes both buoyant, viable, developing eggs and sunken, nonviable eggs in various stages of arrested development. There was no significant correlation between percent fertile eggs and male plasma vitellogenin concentrations ($p = 0.300$, $R^2 = 0.029$, $r = -0.170$); $y = 1.919\text{Ln}(x) + 36.665$.

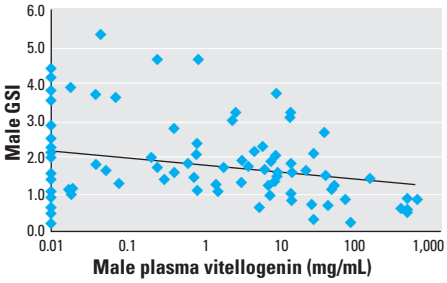


**Figure 4.** Correlation between male GSI, calculated with liver subtracted, and male plasma vitellogenin concentrations. There was a weak but significant negative correlation between male GSI and male plasma vitellogenin concentrations ($p = 0.0016$, $R^2$ 0.074, $r = -0.273$); $y = 0.0824\text{Ln}(x) + 1.81$.

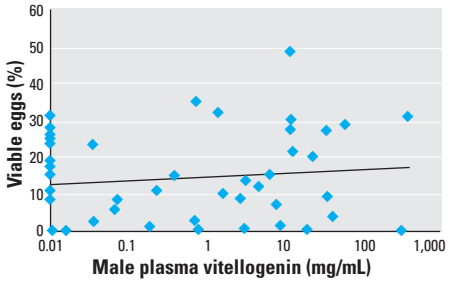


**Figure 3.** Correlation between percent viable eggs and male plasma vitellogenin concentrations. All viable eggs were buoyant, and almost all had clearly discernable developing embryos. There was no significant correlation between percent viable eggs and male plasma vitellogenin concentrations ($p = 0.660$, $R^2 = 0.005$, $r = -0.072$); $y = 0.3426\text{Ln}(x) + 14.435$.

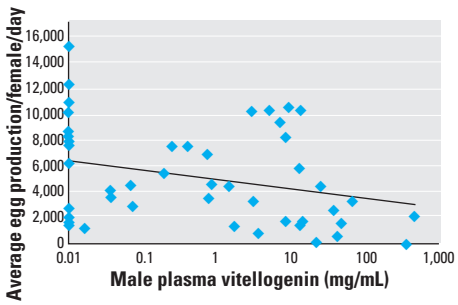


**Figure 5.** Correlation between total egg production per female per day and male plasma vitellogenin concentrations. Total egg production per female per day showed a weak but significant inverse correlation with male plasma vitellogenin concentrations ($p = 0.040$, $R^2$ 0.111, $r = -0.333$); $y = -301.5\text{Ln}(x) + 4885.4$.

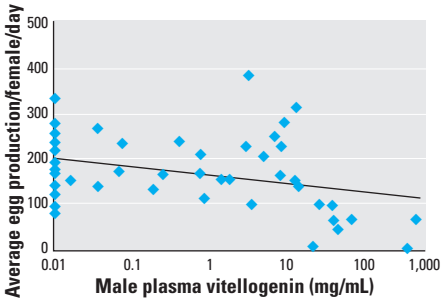


**Figure 6.** Correlation between total egg production per gram female per day and male plasma vitellogenin concentrations. Total egg production per female, normalized to female weight, showed a significant inverse correlation with male plasma vitellogenin concentrations ($p = 0.009$, $R^2 = 0.170$, $r = -0.412$); $y = -8.0118\text{Ln}(x) + 161.7$.

those with low GSI, still produced motile sperm. Gimeno et al. (*33*) observed that sexually mature male carp exposed to a nominal concentration of 1 µg/L 17β-estradiol in water for 1, 2, or 3 months exhibited significantly decreased GSIs and failed to produce any milt, although males similarly exposed to 0.1 µg/L 17β-estradiol showed no significant effect on either GSI or milt production. Vitellogenin concentrations measured at 2 and 3 months in fish from either estradiol treatment were four to six orders of magnitude higher than control concentrations. These results suggest that because vitellogenin was greatly elevated in male carp from both estradiol concentrations but effects on GSI and milt production were noted only at the higher estradiol concentration, elevated male plasma vitellogenin does not necessarily indicate male reproductive dysfunction. In our experiments, two male cunner with highly elevated plasma vitellogenin (exceeding 300 mg/mL) and low GSI continued to produce milt and motile sperm, indicating that neither elevated male vitellogenin nor lowered GSI alone is a reliable indicator of male reproductive dysfunction.

Can elevated male plasma vitellogenin be used as an indicator of reproductive impairment in females exposed to the same conditions? Data relating male plasma vitellogenin concentration to female egg production are limited. From studies with medaka exposed during development to the estrogenic chemical DDT, Cheek et al. (*34*) concluded that vitellogenin production in adult males could be interpreted as a signal that embryos produced in that environment may suffer reproductive effects such as gonadal feminization and reduced fertility as adults. Kramer et al. (*16*) correlated reproductive function in fathead minnows with plasma vitellogenin expression, measured as ALP. Using fish exposed to waterborne 17β-estradiol, these researchers found that ALP concentration in females or males was inversely related to egg production, which is the same type of relationship we observed. Lange et al. (*35*), conducting a long-term study with fathead minnows exposed to waterborne ethynylestradiol, noted that males exposed to 0.2 or 1 ng/L ethynylestradiol for up to 301 days posthatching showed no statistically significant reductions in total eggs laid, mean number of eggs laid per female, or mean number of eggs laid per female per breeding day, but these males also exhibited no elevation in plasma vitellogenin. In the same study, all fish exposed to higher ethynylestradiol concentrations (4 or 16 ng/L) developed as phenotypic females. Our pooled data show a weak but significant negative correlation between male plasma vitellogenin levels and average egg production per female per day ($p = 0.04$, $r = –0.33$). When egg production is normalized to account for differences in the size of females (average egg production per gram female per day), the significant negative correlation ($p = 0.009$, $r = –0.41$) is a bit stronger. Although the reasons for this correlation between male plasma vitellogenin concentrations and female egg production may not be obvious, the work of Reis-Henriques et al. (*36*) suggests an explanation, assuming that elevated vitellogenin levels in males are mirrored by similar increases in females. These researchers found that elevated plasma vitellogenin in females is associated with a decrease in estradiol production, which in turn affects egg production (*36*). Our preliminary histologic evaluation of female gonad tissue indicates that the ovaries of fish exposed to the highest levels of estrogens had fewer oocytes in later developmental stages, supporting the theory that physiologic changes are behind the decreases in egg production. Close examination of the data indicates that the nine males with the highest plasma vitellogenin concentrations are driving the significant correlations with egg production, suggesting to us that a threshold concentration for male plasma vitellogenin may need to be exceeded to predict significant biologic effects in females. If data from the nine males with vitellogenin concentrations in excess of 15 mg/mL plasma are removed from consideration, egg production no longer exhibits a significant correlation ($p = 0.49$, per female; $p = 0.26$, per gram female). This suggests that male vitellogenin concentrations in reproductively active cunner need to exceed some threshold level (15 mg/mL plasma in our experiments) before they are indicative of deleterious reproductive effects in females.

The data presented in this article indicate that induction of vitellogenin in adult male fish cannot be used as a dependable biomarker for reproductive dysfunction in all situations; we specifically present one such case: adult male cunner exposed to estrogens through implantation for a relatively short period. Our results show that the widely used indicator of estrogen exposure, male plasma vitellogenin expression, is not a dependable indicator of male reproductive dysfunction in adult cunner exposed to estrogens for 2–8 weeks during their reproductive season, at least in relation to capacity to produce milt and motile sperm or to fertilize eggs. In our experiments, high concentrations of plasma vitellogenin (~50 mg/mL) in male cunner were indicative of lowered GSI, but a reduced male GSI did not predict a reduced ability to fertilize eggs. However, in simultaneously exposed cunner of both sexes, male plasma vitellogenin concentrations exceeding a threshold level (~15 mg/mL plasma) was a reliable indicator of reduced female reproductive function due to estrogen exposure. These results do not exclude the possibility that there may be instances when induction of male vitellogenin may be used, in conjunction with other data such as evidence of intersexuality, to indicate that reproductive impairment is occurring in a fish population.

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