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Regulation of Vitellogenin and Other Serum Proteins By Estrogen and Xenobiotic Estrogens In Salamanders

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Regulation of Vitellogenin and Other Serum Proteins By Estrogen and Xenobiotic Estrogens In Salamanders

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ABSTRACT

Environmental estrogens pose potential health risks to humans and wildlife by disrupting physiological and developmental processes; therefore, our laboratory has been designing *in vivo* bioassays on induction of the egg-yolk precursor protein vitellogenin. I have provided information on vitellogenin induction by estrogens in tiger salamanders (*Ambystoma tigrinum*), spotted salamanders (*Ambystoma maculatum*), hellbenders (*Cryptobranchus alleganiensis*), Morelet's crocodiles (*Crocodylus moreletii*), and developed vitellogenin immunoassays in these species. Salamanders were estrogen-injected; blood was collected at intervals. Estrogen exposed male salamanders of all species, produced substantial quantities of a 200-kDa protein, the approximate size of vertebrate vitellogenins. Sequencing of estrogen-induced proteins showed substantial homology to known vertebrate vitellogenins. Tiger salamander vitellogenin N-terminus antibodies were produced, and crossreacted with serum from estrogen-treated but not control tiger salamanders. Vitellogenin assays for these species could study annual reproductive cycles, test waters for the presence of estrogenic agents or assess exposure of wild populations to environmental estrogens.

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Introduction

In the early 1970's, on a small, secluded street in Pittsburgh, PA there was a group of residents whose properties were near an automobile junkyard. These same residents, their children, and even grandchildren, seemed to be predisposed to a variety of illnesses, ranging from serious thyroid conditions to miscarriages to even various forms of cancers. It was not until years later that this junkyard was discovered to be a dumpsite for toxic waste and chemicals, in particular polychlorinated biphenyls (PCBs). The chemicals were suspected to be giving the residents the predisposition to these illnesses, and are now classified with many other chemicals as endocrine disruptors.

According to the endocrine disruptor hypothesis, there are "certain chemicals (primarily man-made) that act as, or interfere with, human hormones (specifically estrogens) in the body, and thus cause a range of defects and diseases related to the endocrine system" (Lukachko, 1999). Some endocrine disruptors are extremely harmful to humans due to their persistence in the environment, high lipophilicity, long half-life, high bioaccumulation, and low rate of biodegradation. Once they enter into our environment, it is a very long, hard road to removal. In order to better understand how endocrine disruptors may interfere with our endocrine system, it is important to understand how hormones normally function.

Steroid Hormones

Hormones are substances secreted by an endocrine gland or cell into the blood, where they may exert regulatory activity, typically in cells other than those in which they were produced, which result in a response depending on the presence of a specific receptor (O'Malley and Strott, 1991; Carlstedt-Duke et al., 1995). Steroid hormones are a class of molecules that regulate gene functions associated with specific developmental and physiological processes. Steroid hormones are divided into six major classes; estrogens, progestins, androgens, glucocorticoids, mineralocorticoids, and vitamin D derivatives. These hormonal classes show a high degree of similarity in structure but have widely different functions (O'Malley and Strott, 1991; Baulieu, 1984). Estrogens are involved in regulating female reproduction, androgens are involved in regulating male reproduction, progestins aid in pregnancy, glucocorticoids regulate aspects of metabolism and stress responses, mineralocorticoids assist in salt metabolism, and vitamin D and its derivatives are important in bone metabolism (O'Malley and Strott, 1991; Bolander, 1994).

Steroid hormones are secreted by a variety of glands into the bloodstream, where they circulate bound mostly to plasma transport proteins. These hormones, being lipophilic, can diffuse across the plasma membrane and into cells. In the target cells, steroid hormones combine with their specific receptors, and the hormone/receptor complex then alters cell function (O'Malley and Strott, 1991; Korach, 1994; McMaster et al., 2001). Receptors for some steroids are present in most cells; for example, those for mineralocorticoids and glucocorticoids. However, receptors for other steroids are present in more limited cell types; for example,

receptors for estrogens are present mostly in female reproductive tissues, and receptors for androgens are present mostly in male reproductive tissues.

Regardless of the type of steroid hormone, all operate through a similar mechanism. In order for steroid hormones to regulate specific functions, they must bind to and activate receptors, which are gene-regulatory molecules. The receptors bind steroid hormones very tightly and are extremely specific; they are also present in low concentrations in cells. Upon binding of the hormones to their specific receptors, the receptors undergo a conformational change, which alters the receptor from an inactive to an active state. Before binding, the receptors are associated with chaperone proteins, more specifically known as heat shock proteins. Binding results in the dissociation of the chaperone protein and allows the hormone receptor complex to interact with the genome by binding to the DNA at specific sites within hormone-dependent genes (Carlstedt-Duke et al., 1995). Binding of the hormone/receptor complex to the regulatory elements of genes either activates or suppresses their transcription.

In early studies it was thought that unliganded steroid receptors resided in the cytosol, and after hormone binding, the hormone/receptor complex moved into the nucleus. It was later found that even in the absence of the hormone, some receptors reside in the nucleus (Carlstedt-Duke et al., 1995; Smith and O'Malley, 1999). The steroid hormone receptor is comprised of three distinct functional domains. The first is the amino-terminal domain, which regulates transcriptional activity. The second is the DNA-binding domain, which is responsible for dimerization, DNA-binding, and some transcriptional functions. The third is the carboxy-terminal domain, which contains the hormone-binding site and interacts with the chaperone proteins (Carlstedt-Duke et al., 1995). The receptor's hormone-binding domain recognizes the three-dimensional configuration of a particular hormone with high affinity, and has low affinity

for other hormones. In the absence of hormones, the receptor protein binds to a heat shock protein (usually hsp90). The role of the heat shock protein is to maintain the correct folding of the receptor and to mask the DNA-binding site of the receptor protein, blocking genome interactions in the absence of the appropriate steroid hormone (Carlstedt-Duke et al., 1995). Not only do the heat shock proteins assist in folding, they also protect the receptors from degradation via cellular proteases (O'Malley and Strott, 1991). After the hormone binds to the receptor, the hormone/receptor complex binds to the DNA as dimers. Because dimers bind more tightly and stably than a monomer, they have a greater effect on gene expression (O'Malley and Strott, 1991; Toft et al., 1967).

Once the receptor has become activated, it alters cell function by binding to the DNA at specific sites called hormone-response elements (HREs), which are specific sequences of DNA located near hormone-dependent genes (Carlstedt-Duke et al., 1995; Nawaz et al., 1992). The hormone response elements are characterized by short inverted repeats separated by a fixed number of nucleotides, which varies depending on the steroid receptor types (Carlstedt-Duke et al., 1995; O'Malley and Strott, 1991; Nawaz et al., 1992; Ryseck et al., 1989). Binding of the hormone-receptor complex to HREs causes a disruption of the chromatin structure, supplying a naked region of the DNA. By modulating the chromatin structure, access is allowed to transcription factors (Carlstedt-Duke et al., 1995; O'Malley and Tsai, 1992; Ryseck et al., 1989). The receptor attracts transcription factors and RNA polymerase to transactivation domains on the receptor, initiating transcription. The production of mRNA via gene transcription requires the binding of transcription factors and RNA polymerase to the gene promoter to form a complex (Carlstedt-Duke et al., 1995; O'Malley and Tsai, 1992).

Endocrine Disruptors

Endocrine disruption is a topic that has received increased attention due to the actions and dedication of environmental activists beginning with Rachel Carson (and her book Silent Spring), and more recently followed by Theo Colburn (and her book Our Stolen Future). Considerable quantities of potentially endocrine disrupting chemicals are mass-produced and introduced into the environment each year. Endocrine disruptors have been defined as "exogenous agents that interfere with the production, release, transport, metabolism, binding action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" (Ginsburg, 1996). Alternatively, the Environmental Protection Agency (EPA) defines endocrine disrupting chemicals to be agents leading to toxic outcomes such as various types of cancer and a whole host of adverse reproductive effects (Kavlock, 1997). They range in chemical structure from pesticides, chlorinated compounds, plasticizers, to even the breakdown components of household detergents (Lukachko, 1999).

Endocrine disruptors exert their deleterious effects by interfering with the normal functioning of the endocrine system. The endocrine system is a complex communication system regulating normal growth, development, reproduction, behavior and physiological functions of humans and animals. There are a variety of sectors that work together to make up this system, including the adrenal cortex, ovaries, testes, pituitary, thyroid, and the parathyroid (Lukachko, 1999). These glands secrete hormones, which act as information carriers, to activate specific receptors in tissues and organs throughout the body. The most horrific effects of environmental contaminants are mediated by their ability to mimic these natural hormones in the body. The

most prominent and pervasive of these endocrine disruptors are those known to mimic the steroid hormone estrogen. While some synthetic compounds were manufactured to be estrogenic, most were not designed to have endocrine functions, and are inadvertently estrogenic (termed xenobiotic estrogens).

Exposure of humans to endocrine disruptors has been suggested to cause a number of health problems, including: increased risks of cancers (breast, prostrate, and testicular), reduced sperm counts, birth defects to the reproductive organs, problems in pregnancy, and effects on the thyroid gland (Lukachko, 1999; Danzo, 1998). Endocrine disruptors are insidious because they affect the endocrine system by mimicking natural hormones, and often the effects are not noticed until after a large time lapse. For many chemicals, the exposure may occur in one generation (e.g., a pregnant mother), and may not cause any harm at all to this generation; however, the compound may elicit its deleterious effects on the second generation (e.g., the progeny of the mother). Even though the effects from these chemicals on one generation may be very minimal, if they are experienced at critical stages in development, they can lead to permanent, devastating effects on a second-generation developing fetus.

Not only are the effects of endocrine disrupting chemicals potentially so grave on humans, they are also known to be devastating to wildlife populations. The current decline of amphibian populations may reflect a worldwide trend of environmental degradation. Endocrine disruption in wildlife populations is far from consistent; it ranges from crossed beaks in birds, to alligators with small penises, to the disappearance of entire populations. Chemicals contaminate the habitats of fish and wildlife; these chemicals can permeate through the skin of amphibians, accumulate in the animal's endocrine systems, and cause serious defects. These endocrine disrupting chemicals, due to their persistence in the environment, bioaccumulate in the fatty

tissues of organisms, and increase in concentration as they move up throughout the food web, harming species far from the original source of contamination. The most prominent effects upon wildlife populations are: thyroid system disorders, inability to breed, abnormal mating and parenting, interrupted sexual development, and reduced immune response (Colborn et al., 1993; Hayes et al., 2002; Clark et al., 1998; Raloff, 1994; Carey and Bryant, 1995). The most prominent studied populations are: sea gulls, harbor seals, bald eagles, oysters, snails, fish, whales, trout, panthers, alligators, turtles, other types of reptiles and birds (Shimasaki et al., 2003; Kavlock, 1997; Toppari et al., 1996). Recently considered potential sources of endocrine modulators which affect wildlife are: runoff from sewage treatment plants, pulp/paper mills, and other manufacturing factories (Luchako, 1999). An example is the increased amount of women using birth control. This places an increased amount of estrogen and their breakdown products through sewage treatment plants and into the waterways, leaving wildlife at a disadvantage because these waters serve as nurseries for offspring and breeding ponds for mature animals. These environments may be triggering endocrine related effects in fish and aquatic amphibian populations.

A number of potential endocrine disruptors have been identified. Some important ones are 1) DES, 2) DDT, 3) PCBs, and 4) a number of contemporary contaminants. Diethylstilbestrol, or DES, is a pharmacological agent manufactured to have estrogenic activity. Although DES is structurally dissimilar to natural estrogens, it exhibits similar biological functions and hence, is an example of the worst-case scenario for endocrine disruption (McLachlan, 2001; Denslow et al., 2001). This drug was prescribed for the prevention of miscarriages in the 1950s through 1970s. The daughters of women who took this drug experienced increased numbers of defects (in the vagina, uterus, and Fallopian tubes),

reproductive cancers (known as clear-cell adenocarcinoma), sexual deformities and sterility. Consequently, DES has been described as the first transplacental carcinogen, a chemical that causes cancer in the daughter when administered to the mother (McLachlan and Arnold, 1996). Not only was this compound harmful to female offspring of treated mothers, but it also has resulted in increased frequency of undescended testicles, sperm abnormalities, prostrate disease, and testicular cancers in male offspring (McLachlan and Arnold, 1996; Palmer et al., 2001).

DDT and PCBs are endocrine disrupting chemicals no longer in wide use; nevertheless, they still have great importance due to their perseverance in the environment. The first recognized class of endocrine disruptors was the pesticide dichlorodiphenyltrichloroethane, better known as DDT. This compound was manufactured primarily as a malaria control agent, whose effectiveness allows it to still be used in some third world countries (Mbongwe et al., 2003). Being an artificial estrogen, DDT is most notably the best studied example of an exogenous hormone mimic (Mbongwe et al., 2003; Bustos, 1988). DDT was considered an ideal chemical because it was extremely persistent in the environment, had a high toxicity to invertebrates, and was thought to have a low toxicity to vertebrates. However, DDTs bioaccumulating effects resulted in elevated levels at the top of the food chain. Prior to being banned in the United States in 1972, DDT was found to induce poor survivorship, antibody response suppression, reduced reproductive success, reduced sperm counts, and eggshell thinning in a variety of birds, including seagulls, and particularly bald eagles (Gilbertson et al., 2003; Lukachko, 1999; Bitman and Cecil, 1970; Bustos et al., 1988; Raloff, 1994, McLachlan and Arnold, 1996). Since the 1980's, DDT has been identified as a wildlife feminizing chemical (McLachlan and Arnold, 1996) and also has shown to increase uterine weight in rats, prolong estrous cycles, and decrease ova implantation in mice (Bitman and Cecil, 1970). DDT inhibits

the enzyme Ca-ATPase, a calcium transporter from the blood to the oviduct, which induces eggshell thinning (Fairbrother et al., 1999). Although the mechanism of DDT action is controversial, the most widely accepted theory is that DDT binds to the cellular estrogen receptor, initiating the same sequence of events as the natural estrogen hormone does (Fairbrother et al., 1999). This stimulates the increase in uterine DNA synthesis, protein synthesis, and secretion (Stancel et al., 1995). The breakdown products of DDT may act as estrogens, or even compounds that block androgens. DDT has also been reported to disrupt the sodium/potassium exchange between neurons (Fairbrother et al., 1999).

Another class of endocrine disruptors used widely in the past are the chemicals called polychlorinated biphenyls, better known as PCBs. The best-known human example is in Anniston, Alabama, where PCBs can still be found in the air, water, soil, wildlife, and even in the residents themselves (Kroft, 2002). PCB exposure resulted in deformed fish, liver damage in exposed rats, and possibly causing cancer, heart disease, and diabetes in the town population. PCBs have also been shown to have effects on exposed bird populations by feminizing the male sex organs of exposed seagulls and causing a life-threatening bill deformity in the cormorant (Raloff, 1994; Fairbrother et al., 1999; McLachlan and Arnold, 1996). PCBs are similar to DDT in that they can have reproductive defects, induce uterotropic effects, stimulate prolonged estrous cycles, and impair fertility (Selcer et al., 2001; de Solla et al., 2002; Blais et al., 2003). Some of these compounds have been shown to become even more estrogenic as they are metabolized by the body (McLachlan and Arnold, 1996; Bergeron et al., 1994). Various PCBs are adept at binding to the aryl hydrocarbon receptor, estrogen receptors, thyroid hormone receptors, and several receptors in the liver. When binding to estrogen receptors, PCBs may either interfere with or initiate the same sequence of events as the natural estrogen hormone does (Fairbrother et

al., 1999; Korach, 1994). Some PCBs are proficient at antagonizing the natural estrogen actions, and also can cause the conversion of androgen to estrogen in some cells, impairing masculinity (Colborn, 1995; Bitman and Cecil, 1970; Bergeron et al., 1994).

While DES, DDT, and PCBs are of considerable historical importance, these substances are no longer in use in the U.S., and are now limited in their distribution. However, other chemicals have recently been recognized as frontrunners in endocrine disruption importance. Alkylphenols and their relatives, alkylphenol polyethoxylates (APEOs), are environmental contaminates that have proven estrogenic activity. These compounds are used in pesticides, detergents, paints, and even cosmetics (Danzo, 1998; Soto et al., 1991). Two of these compounds, nonylphenol and octylphenol, products of APEO degradation by microorganisms (Soto et al., 1991), are very stable in the environment and estrogenic in different model systems: for example, newts and frogs (Pfister et al., 2003; Mosconi et al., 2002). They have also been found to cause male reproductive deficits (Danzo, 1998). Two plasticizers, bisphenol-A and phthalate esters, are some of the most prevalent man-made chemical pollutants in the aquatic environment. These have both been shown to be estrogenic in vertebrates and invertebrates alike (Hutchinson, 2002; Danzo, 1998; Turner and Sharpe, 1997; Soto et al., 1995). A recent leader in the field; atrazine is the most commonly used herbicide in the United States, and probably in the world. Atrazine affects sexual differentiation, by inducing aromatase, which converts androgens into estrogens (Hayes et al., 2002; Dankwardt, 1998). In agricultural runoff alone, atrazine can be present at several parts per million, and can reach up to 40 parts per billion in precipitation (Hayes et al., 2002). Amphibians breeding in watersources such as streams, rivers, and lakes, can be exposed to very high concentrations of atrazine.

The relationship between wildlife and human abnormalities, diseases, and dysfunctions of the endocrine system and exposure to environmental contaminants is not fully understood and the subject of intense debate. Thus, there is a pressing need for assays capable of detecting endocrine disruptors and assessing their effects. There are three areas of utility for endocrine disruptor assays. 1) Testing compounds for endocrine disrupting effects; 2) Testing animal populations for exposure to such agents, and 3) Testing waters that are suspected to contain environmental contaminants.

To address these needs, Congress passed the Safe Drinking Water Act and the Food Quality Protection Act, which mandated that the EPA develop testing to characterize and detect the endocrine activity of pesticides, commercial chemicals, and environmental contaminants. These acts stated that EPA must develop an endocrine disruptor screening program, and then screen for endocrine disruptors in drinking water sources. The committee called EDSTAC (Endocrine Disruption Screening and Testing Advisory Committee) designed and implemented the program which focuses on providing methods and procedures to detect and characterize the endocrine activity of pesticides, commercial, and environmental contaminants (EDSTAC, 1998). This program was enabled to provide the most scientific data available for 87,000 plus chemicals in commerce (EDSTAC, 1998). Those endocrine disruptors will be identified and regulatory action will be enforced. Currently, the EPA is in the process of initial screening (via Tier I screening) and testing (via Tier II testing) of chemical substances that may have an effect similar to that produced by naturally occurring hormones in the body.

Many times what is troubling when evaluating endocrine disruptors is that the chemical structure of the synthetic compound may not resemble that of the natural hormones they mimic, therefore making it hard to predict which compounds will be endocrine disruptors. Even though

many synthetic chemicals were not manufactured with the intent of inducing hormonal activity, in some way they obtain the ability to alter the endocrine system (McLachlan and Arnold, 1996; Bitman and Cecil, 1970). Since the compounds may be structurally different than hormones, a chemical assay often cannot be used. Thus, biological assays must be used in order to assess the endocrine disrupting potential of compounds.

Endocrine disruption assays can be *in vitro* or *in vivo*. Each of these approaches has advantages and disadvantages. Some advantages of *in vitro* methods are cost, rapid detection, small amounts of material required, technique automation, complex mixtures can be tested, and there is a high specificity of response (Gray et al., 1997). The disadvantage of *in vitro* assays are they can provide both false positive and false negative results by not taking into account the physiology of the organism. The false positive results occur when a chemical is rapidly metabolized, is not absorbed or distributed to the target tissue, or is toxic in a different form *in vivo* (Gray, 1998; Gray et al., 1997). False negatives occur *in vitro* due to the inability to metabolically activate certain toxicants. The *in vitro* estrogenicity assays currently being used include the estrogen/androgen receptor reporter gene assays (Gray, 1998; Gray et al., 1997; EDSTAC, 1998), vitellogenin induction in trout hepatocytes (Pelissero et al., 1993), and growth of estrogen-dependent breast cancer cells (Soto et al., 1992; Arnold et al., 1996). *In vivo* advantages include accountability for absorption, distribution, metabolism, and excretion of the estrogenic chemical, the endpoints can be used in risk assessment, applicable over a wide-range of species, evaluation of the endocrine system as a unit, and it provides perspective to other toxicity endpoints (Gray, 1998; Gray et al., 1997). The disadvantages of the *in vivo* assays are they are very costly and it takes a long time to complete the assay. The *in vivo* estrogenicity assays that the EPA is currently proposing for screening are the rodent uterotrophic assay, the

rodent pubertal male/female assay, the fish reproduction assay, and the rodent reproduction assay (Turner and Sharpe, 1997; Soto et al., 1992; Soto et al., 1995). Another test created to assess estrogenicity is based on induction of vitellogenin in nonmammalian vertebrates (Palmer et al., 1998; Heppell et al., 1995; Sumpter and Jobling, 1995).

Testing animal populations and watersheds for the exposure and presence of endocrine disrupting chemicals is difficult because there is a need for background information for each species used, and a need for assays for each animal to test for estrogenicity. There are several basic field studies that can be used for testing. The first design is to place a prescreened animal into a test site, remove the animal after a specific time interval, and then check for endocrine disruption (Sumpter and Jobling, 1995; Purdom et al., 1994; Folmar et al., 2001; Folmar et al., 1996). The second design is a lab test involving removal of water from a suspected area, bringing it to the lab and testing it on an animal model (Sumpter and Jobling, 1995; Purdom et al., 1994; Folmar et al., 2001; Folmar et al., 1996).

The aforementioned vitellogenin assays have many advantages, such as cost, the use of aquatic organisms, and the ability to test waters for the occurrence of estrogenic contaminants (Bringolf and Summerfelt, 2003; Purdom et al., 1994; Folmar et al., 2001; Folmar et al., 1996; Selcer and Palmer, 1995). There is a lack of whole animal *in vivo* assays, and there is a lack of amphibian information, specifically salamander information. The lack of whole animal assays is particular troubling, especially since the majority of the assays performed thus far have only focused on fish populations. There is a need for biological assays that are capable of detecting physiologically relevant concentrations of xenobiotic estrogens in the remainder of the aquatic community. Even though there have been few studies on aquatic reptiles and amphibians, their numbers continue to decline. Salamanders, one type of amphibians, are rapidly declining, and

even though endocrine disruption is suspected, there have yet to be any studies to support this hypothesis. In this project, I have studied the regulation of vitellogenin and other serum proteins by estrogens in the tiger salamander, *Ambystoma tigrinum*.

Vitellogenin as a Biomarker

Vitellogenin is a large serum phospholipoglycoprotein that is a precursor molecule to the egg-yolk proteins of oviparous vertebrates (Ho, et al., 1982; Ho, 1987; Ho, et al., 1981). In a developing embryo, egg-yolk proteins are the source of metabolic energy. In mature female non-mammalian vertebrates, the liver, in response to circulating estrogens, synthesizes and secretes vitellogenin into the bloodstream (Ho, et al., 1982; Ho, 1987; Ho, et al., 1981). The vitellogenin is taken up from the bloodstream by the developing oocytes, and then cleaved into lipovitellins and phosvitins, the egg-yolk proteins (Selcer and Palmer, 2001). Vitellogenin is not normally present in the plasma of males and immature females; however, it is inducible by estrogen treatment. Estrogen promotes vitellogenesis in members of all non-mammalian classes (Ho et al., 1982; Ho, 1987; Smith and O'Malley, 1999). Although other factors play a role in modulating a vitellogenic response (Ho et al., 1982; Ho, 1987; Wangh, 1982; Wangh and Schneider, 1982), estrogen is the primary inducer of vitellogenin (Tata and Smith, 1979).

Induction of vitellogenin may be a way to show evidence of exposure to endogenous or exogenous estrogens (Heppell et al., 1995; Denslow et al., 1995). Vitellogenin is normally female specific, but is inducible in male animals by estrogen and estrogenic compounds (Palmer et al., 1998; Selcer et al., 2001). This presence of vitellogenin in the male animal can be used as an indicator of exposure to xenobiotic estrogens. Since the structures of environmental estrogens

are dissimilar, this provides an assay that is capable of measuring estrogenic exposure for a number of various compounds to attempt in identifying environmental pollutants. Thus, vitellogenin can serve as a biomarker for environmental estrogens.

Estrogen Regulation of Other Serum Proteins

Along with induction of vitellogenin, estrogen action may cause other proteins to change, which can also serve as biomarkers. Selcer and Palmer, 1995 and Shapiro, et al., 1989 demonstrated in frogs and turtles that estrogen treatment results in the down regulation of major serum proteins. In the turtle, albumin, with a molecular weight of 66kDa, and a second unclassified protein, with a molecular weight of 170kDa, were both down regulated (Selcer and Palmer, 1995). Estrogen treatment decreased both proteins by about 50% as compared to the controls. This finding is significant because it helps to establish the prevalence and importance of the estrogen down regulation mechanism (Selcer and Palmer, 1995).

Estrogen is important in female vertebrates for the maturation and preparation of the reproductive tract, development of secondary sexual characteristics, and stimulation of vitellogenesis (Selcer and Leavitt, 1991). The predominant mechanism of estrogen action is through the control of cellular protein synthesis, and it is known to up regulate and down regulate a number of serum proteins. Two common examples of enhanced protein synthesis via estrogen are ovalbumin production by the avian oviduct (O'Malley et al., 1969) and vitellogenin production by the amphibian liver (Hayward et al., 1982). In contrast, estrogen represses the estrogen receptor in the MCF-7 human breast cancer cell line (Read et al., 1989) and results in

the disappearance of a number of serum proteins, the most widely studied of which is albumin (Follett and Redshaw, 1974; Pastori et al., 1991; Jackson and Shapiro, 1986).

Estrogen down regulation of specific proteins may represent an important and understated aspect in hormonal regulation of cell function and differentiation. The clarity of how widespread this regulation is among tissues and species remains unknown because it has only been described in a limited number of species. In order to establish the importance and prevalence of the estrogen regulation mechanisms, information on estrogen regulation of serum proteins from species representing different vertebrates groups is needed.

Estrogen has been shown to regulate a few types of proteins, in a wide variety of species. In *Xenopus laevis*, estrogen treatment of males has been shown to induce a key regulatory enzyme in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Chen and Shapiro, 1990). Its induction by estrogen was shown primarily from mRNA in the livers of males. A few years later, vigilin, a high-density lipoprotein-binding-protein was found in vertebrate cells (Dodson and Shapiro, 1997). Vigilin, thought to play an important role in RNA metabolism, is also an estrogen-inducible protein. Another protein was found in teleosts. When treated with estrogen, plasma calcitonin secretion levels also increase (Suzuki et al., 2003). Those proteins, however, play a small role compared to the most widely known estrogen regulated protein, vitellogenin.

In amphibians, estrogen treatment induces synthesis of vitellogenin in both sexes. Studies have found that along with this induction of vitellogenin, estrogen concomitantly decreases the quantity of total serum albumin being produced by the liver. Most of these studies have been performed on *Xenopus laevis*. Estrogen exposure to male *Xenopus* down regulates absolute albumin mRNA levels by more than 95% during the first 4 days following treatment,

after which albumin mRNA returns to normal levels over the following 12 days (May et al., 1982; Riegel et al., 1986; Schoenberg et al., 1989). Estrogen has also been studied in *Xenopus* with dexamethasone, a steroid required for preservation of albumin mRNA levels in culture. In only dexamethasone treatment (the absence of estrogen), albumin mRNA declined rapidly. Estrogen, however can override the effects of dexamethasone treatment (albumin preservation), and elicit a decline of over 80% in albumin mRNA levels (Jackson and Shapiro, 1986). Although suspected endocrine disruptors act along an estrogen-pathway, they do not seem to elicit the same albumin-down regulated estrogen phenomena. Though there are a lack of studies on amphibians exposed to endocrine disruptors, one study focuses on human and rat models and found that exposure to polychlorinated biphenyls (PCBs) may in fact lead to an increase in serum albumin levels due to increased protein synthesis (Borlak et al., 2002). More studies are necessary to indicate the trend of potential endocrine disruptor induction of albumin, but this may also prove to be a valuable biomarker in which to evaluate endocrine disruptors. Given that estrogen down regulation has been demonstrated in frogs and turtles, it may also be useful as a biomarker for estrogenic action in other species.

Model Organisms

In the following studies, the primary test animal was the eastern tiger salamander, *Ambystoma tigrinum*. This particular salamander is especially prevalent in the Midwestern portion of the United States and on its eastern coast. It is normally between seven and eight and a quarter inches long with light olive, or yellowish-brown spots on a dark background (Conant and Collins, 1999). Its spots are highly irregular in distribution and shape, and these are well

extended down the sides of the abdomen. Males have a slightly longer tail than females. This salamander is the most widely distributed salamander species in North America, and is one of the few capable of tolerating the dry conditions of the many regions in which it lives. They cope with these conditions by living below the ground in hibernation sites. The tiger salamander likes to congregate in deep water, and is a winter early spring breeder. The larvae of tiger salamanders are hatched and live in the water, until maturation, when they develop lungs and live on land. Because this animal lives in the wild, it is difficult to assess the effects endocrine disrupting chemicals have on their population. This is why it is critical to study species like the salamander. The species is consistently declining, and the reason is unknown. There is speculation of endocrine disrupting chemicals, but this has yet to be proven (Blaustein and Wake, 1990; Raloff, 1994; Wake, 1991; Carey and Bryant, 1995). Tiger salamander vitellogenin has never been fully characterized, and was part of the work presented here. Since the vitellogenin was characterized and analyzed, the salamander can now be used as a model animal to assess the effects of estrogenic chemicals.

Due to unavailability of tiger salamanders for a final experiment, juvenile spotted salamanders, *Ambystoma maculatum*, were used in their place. These salamanders are very similar to tiger salamanders in their geographic distribution, abundance, and appearance. They are prevalent along the eastern coastal area and are one of the largest members of the mole salamander family.

Two other species were also used extensively in this project, Hellbenders, *(Cryptobranchus alleganiensis)*, and Crocodiles, *(Crocodylus moreletii)*. Serum samples from both animals were sent to us for analysis. Hellbenders are a type of salamander species also living in the eastern portion of the United States. Most states where the hellbender is found have

classified it as an endangered species. The reason for its disappearance remains unknown, making it a perfect case study for endocrine disruption analysis. *Crocodylus moreletii* are most prominent in Central America and are known as a small type of crocodile. These crocodiles were almost hunted to extinction, and while hunting is now illegal, they are still on the brink of extinction. Endocrine disruption is also speculated in this species of crocodiles, but this has yet to be proven.

Hypothesis

Because vitellogenin is presumed to be present in female oviparous vertebrates during the egg-laying season, the prediction was that female tiger salamanders would also contain vitellogenin and other estrogen-induced serum proteins during this time. I also predicted that males would lack vitellogenin but, after estrogen injections, would also produce the vitellogenin and other serum proteins. This abnormality could then serve as an assay to determine exposure to estrogenic compounds. It was also proposed that environmental estrogens would induce vitellogenin and other serum protein production in males and increase its production in females.

There are three specific aims that were addressed in this proposal:

1. To characterize the changes in serum proteins associated with estrogen exposure. This was achieved by treating immature animals and adult males and females with estrogen. Serum proteins were evaluated for differences between control and estrogen-treated individuals.

2. To develop immunoassays for detecting changes in vitellogenin and perhaps other proteins for the future use in studies of endocrine disruption. This entailed developing an antibody specific for the protein of interest, and developing specific immunoassays.

3. To employ the above-mentioned assays to evaluate responses to specific endocrine-disrupting chemicals.

Materials and Methods

Chemicals

17β-estradiol and ethinyl-estradiol were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Aroclor 1221 and Atrazine were purchased from Chem Service (West Chester, PA). These chemicals were certified standard grade.

Animals

Immature and adult male and female *Ambystoma tigrinum* were purchased from the Charles D. Sullivan Company, Inc. (Nashville, TN) and from the William A. Lemberger Company (Oshkosh, WI). The terrestrial salamanders were housed at room temperature in plastic aquaria with autoclaved foam pieces and water purified via reverse osmosis. They were fed Salamander Bites (HBH Enterprises, Inc., Utah) *ad libitum*. Some salamanders were subjected to estrogen treatment, others received vehicle alone, and some were subjected to the xenobiotic (endocrine disrupting chemical) treatments. Salamanders were anesthetized (ketamine 80mg/kg), and decapitated at the termination of the experiment, and blood was collected. Blood was allowed to clot for four-hours (h) at room temperature, and serum was prepared by centrifugation at 2,500 x *g* for 10 minutes, twice. Serum was stored at –20° Celsius (C).

Various amphibian samples were sent to us to be screened for vitellogenin. Male hellbenders samples were sent by J.C. Elrod and Y.W. Huang of the University of Missouri-Rolla, Rolla, MO. Male and female wild-caught crocodile sera from Belize was taken by T.R.

Rainwater and S.T. McMurry of Texas Tech University, Lubbock, TX. J. Bettaso and B. Palmer sent wild caught *Rana aurora* samples from coastal northern California from Humboldt and Del Norte Counties.

Experimental design

The experimental design was adapted from Palmer et al., (1998). Estrogens or xenobiotic estrogens were administered by intraperitoneal injection (i.p.), in order to evaluate estrogenic responses. Salamanders were injected with 17β-estradiol or ethinyl-estradiol dissolved in propylene glycol. The injection schedules are listed in the description of each experiment.

During the course of each experiment, all animals were housed in the Duquesne University Animal Care Facility using approved IACUC Animal Care Protocols submitted to the Dean.

Experiment 1: 17β-estradiol Injection of Male **Ambystoma tigrinum**

Twenty male aquatic salamanders were injected with a solution of 0.5 mg/ml 17β-estradiol stock solution or a vehicle solution of peanut oil (5-no injection, 5-vehicle injection, 10-estrogen injection, see Table 1). Five salamanders were housed together in the cold room (4°C) in each of four plastic Tupperware boxes in aged tap water purified via reverse osmosis due to retailer suggestions. The 0.5 mg/ml stock solution was prepared by dissolving 0.200 grams of 17β-estradiol into 20 ml of a 100% ethanol solution. The mixture was stirred well until all of the 17β-estradiol was dissolved. Forty ml of peanut oil (used as a vehicle to deliver the 17β-estradiol) was stirred in to create an emulsion. The heat was turned on slowly to boil away all of the ethanol creating a 0.5 mg/ml stock solution. The vehicle treatment contained only peanut oil.

There was 200 µl of solution delivered to each salamander during injection. The tanks were cleaned and water was changed every three days. Due to the low metabolic rate by living in the cold, the salamanders were not fed during the course of the experiment. The injection schedule was as follows is shown in Table 1.

Table 1: Injection Schedule for Experiment 1

Injection Number	***Day Number***	***Termination***
	Day 1	Arrival Date
1	Day 3	Friday May 24th: 5 initial controls which received NO injections
2	Day 7	
3	Day 11	
4	Day 15	
5	Day 19	
6	Day 23	
7	Day 27	Thursday June 20th: All remaining salamanders, 5 vehicle treated, 10 estrogen treated

Experiment 2: Ethinyl-estradiol Injection of Male and Female **Ambystoma tigrinum**

Nine female and twenty male terrestrial salamanders were injected with various doses of ethinyl-estradiol stock solution or vehicle solution of propylene glycol (see table 2). Four or five salamanders were housed together at room temperature in each of eight plastic Tupperware boxes with minimal aged tap water purified using reverse osmosis. The stock solutions were prepared by dissolving 60 mg of ethinyl-estradiol into 6 ml of a 100% acetone solution. The mixture was stirred well until all of the ethinyl-estradiol was dissolved. This solution was then serially diluted by taking out 600 ml, placing this into the medium dose, taking 600 ml of the new medium dose and placing that into the low dose. Of the three-doses, 4.5 ml was taken out from each, and put into three separate 45 ml solutions of propylene glycol (used as a vehicle to

deliver the ethinyl-estradiol) to create an emulsion. The vehicle solution was made by adding 4.5 ml acetone to 45 ml of propylene glycol. For two days, these four solutions were dried with a nitrogen-gas stream for 30 minutes, and allowed to evaporate in the hood overnight for two nights to remove the acetone. The final stock solutions were 0 mg/ml, 0.01 mg/ml, 0.1 mg/ml, and 1 mg/ml. There was 0.25 mg of solution delivered to each salamander during injection. The tanks were cleaned and water was changed every three days. The salamanders were fed salamander bites the day before their tanks were cleaned each time. The injection groups are shown in Table 2.

Table 2: Injection Regimen for Experiment 2

Sex	***Number in Group***	***Injection***	***Estrogen or Control***	***Dosage***
Female	5	Once	Estrogen	High (1mg/ml)
Female	4	Once	Control	Vehicle (0mg/ml)
Male	5	Once	Estrogen	High (1mg/ml)
Male	4	Once	Control	Vehicle (0mg/ml)
Male	5	Every 3rd-day	Estrogen	High (1mg/ml)
Male	5	Every 3rd-day	Estrogen	Medium (0.1mg/ml)
Male	4	Every 3rd-day	Estrogen	Low (0.01mg/ml)
Male	4	Every 3rd-day	Control	Vehicle (0mg/ml)

The injection schedule is shown in Table 3.

Table 3: Injection Schedule for Experiment 2

Injection Number	***Day Number***	***Termination***
	Day 1	Arrival Date
1	Day 10	
2	Day 14	Once injected salamanders were all terminated
3	Day 18	
4	Day 22	
5	Day 26	
6	Day 30	
7	Day 34	
8	Day 38	
	Day 41	All remaining salamanders

Experiment 3: Ethinyl-estradiol Injection of Juveniles **Ambystoma tigrinum**

Twenty juvenile terrestrial salamanders (not sexed) were injected with a high dose (1 mg/ml) ethinyl-estradiol stock solution or vehicle (0 mg/ml) solution of propylene glycol (4-vehicle injection and 16-estrogen injection). Four salamanders were housed together at room temperature in each of five plastic Tupperware boxes with minimal aged tap water purified using reverse osmosis. The stock solutions were used from experiment 2 (0 mg/ml, and 1 mg/ml). There was 0.20 mg of solution delivered to each salamander during injection. The tanks were cleaned and water was changed every three days. The salamanders were fed salamander bites the day before their tanks were cleaned each time. Each salamander received three weekly injections (each Wednesday). The injection schedule is shown in Table 4.

Table 4: Injection Schedule for Experiment 3

Injection Number	***Day Number***	***Termination***
	Day 1	Arrival Date
1	Day 2	
2	Day 9	
3	Day 16	
	Day 23	Terminated all remaining salamanders

Experiment 4: Ethinyl-estradiol Injection and Endocrine Disrupting Chemicals of Juvenile **Ambystoma maculatum**

Twenty-two juvenile terrestrial salamanders (not sexed) were injected with high dose (1 mg/ml) ethinyl-estradiol stock solution or vehicle (0 mg/ml) solution of propylene glycol (5-vehicle injection and 17-estrogen injection). Twenty-four juvenile terrestrial salamanders (not sexed) were injected with Aroclor 1221 or Atrazine (6-high dose Aroclor 1221, 6-low dose Aroclor 1221, 6-high dose Atrazine, 6-low dose Atrazine). Five or six salamanders were housed

together at room temperature in each of eight plastic Tupperware boxes with minimal aged tap water purified using reverse osmosis. All stock solutions were made fresh. The ethinyl-estradiol stock solutions were made by adding 1 ml 100% acetone to 50 ml of propylene glycol (used as a vehicle to deliver the ethinyl-estradiol) along with 50 mg ethinyl-estradiol. The mixture was stirred well until all of the ethinyl-estradiol was dissolved. The vehicle solution was made by adding 1 ml acetone to 50 ml of propylene glycol. The Aroclor 1221 high dose solution was made by adding 1ml acetone to 50 ml of propylene glycol along with 250 mg Aroclor 1221. The Aroclor 1221 low dose solution was made by adding 1 ml acetone to 50 ml of propylene glycol along with 5 mg Aroclor 1221. The Atrazine high dose solution was made by adding 6ml acetone (due to the solubility of atrazine) to 50 ml of propylene glycol along with 250 mg Atrazine. The Atrazine low dose solution was made by adding 1ml acetone to 50 ml of propylene glycol along with 5 mg Atrazine. These solutions were stirred with heat for two days, and allowed to evaporate in the hood overnight to evaporate away the acetone. The final stock solutions of ethinyl-estradiol were 0 mg/ml and 1 mg/ml. The tanks were cleaned and water was changed every three days. The salamanders were fed salamander chow the day before their tanks were cleaned each time. Each salamander received weekly injections (either Tuesday or Wednesday) of 0.1 cc per injection. The injection groups are shown in Table 5.

Table 5: Injection Regimen for Experiment 4

Number in Group	***Injection***	***Estrogen or Control***	***Dosage***
5	None	Initial Controls	None
5	Weekly	Control	Vehicle (0mg/ml)
5	Weekly	Estrogen	High (1mg/ml)
6	Weekly	Estrogen	High (1mg/ml)
6	Weekly	Estrogen	High (1mg/ml)
6	Weekly	Aroclor 1221	Low (0.5mg/ml)
6	Weekly	Aroclor 1221	High (5mg/ml)
6	Weekly	Atrazine	Low (0.5mg/ml)
6	Weekly	Atrazine	High (5mg/ml)

The injection schedule is listed in Table 6 (estrogen and vehicle were injected on Tuesdays while Atrazine and Aroclor 1221 were injected on Wednesdays).

Table 6: Injection Schedule for Experiment 4

Injection Number	***Day Number***	***Termination***
	Day 1	Arrival Date
	Day 5	5 Initial controls which received NO injections
1	Day 9 Day 10	
2	Day 16 Day 17	
3	Day 23 Day 24	
	Day 26	Terminated all remaining salamanders

*Hellbender Experiment (***Cryptobranchus alleganiensis*)**

Blood was taken from two male hellbenders by J.C. Elrod and Y.W. Huang of the University of Missouri-Rolla, Rolla, MO prior to estrogen-injection to establish control values for each animal. They were then injected intraperitonally (with a concentration of 2.5mg/kg body weight of 17-β estradiol dissolved in ethanol), and blood was taken 7 days after this first injection for a first reading. The hellbenders were then injected with the same dose of estrogen again, and 7 days after this injection, blood was taken for a second reading. After blood was collected it was placed into heparinized vaccutainers and spun down by centrifugation. Plasma was then collected, put into cryovials, and placed on liquid nitrogen (–170° C) for storage.

*Crocodile Experiment (***Crocodylus moreletii***)*

Male and female crocodile sera was taken by T.R. Rainwater and S.T. McMurry of Texas Tech University, Lubbock, TX, and were used to test for the presence of vitellogenin in wild-caught crocodiles from Belize.

Rana aurora *Experiment*

A number of wild caught *Rana aurora* samples (13 females, 3 non-distinguishable of sex, 81 sub-adults (juveniles), and 58 males) from coastal northern California from Humboldt and Del Norte Counties were taken by J. Bettaso and B. Palmer to be screened for vitellogenin.

Purification of Ambystoma vitellogenin

Ambystoma tigrinum serum was fractionated using a diethylaminoethyl (DEAE) chromatography procedure that was used previously and found effective for purification of turtle, and frog vitellogenins (Selcer and Palmer, 1995; Palmer et al., 1998). The procedure was run on a BioRad Econo Chromatography system (BioRad, Melville, NY, USA) using a DEAE-agarose column (1.0 X 15 cm). The equilibration buffer was 25 mM tris (hydroxymethyl) aminomethane (Tris)-HCl, with 1 mM monothyioglycerol, pH 7.5. The gradient buffer was 500 mM NaCl in equilibration buffer. Pooled sera from 17β- or ethinyl-estradiol-treated individuals totaling 100 µl was diluted 1:10 v/v in equilibration buffer and loaded onto the column. The column was washed with 30 ml equilibration buffer to remove proteins that did not adhere to the DEAE. Proteins were eluted using a linear gradient of NaCl (0 to 500 mM NaCl). A total volume of 90 ml was used. Protein was monitored by ultraviolet absorbance at 280 nm throughout the

chromatography run. Fractions of 3 ml were collected throughout the procedure. Protein levels were determined separately for each fraction by a Coomassie protein assay. Fractions containing significant amounts of protein were evaluated for the presence of vitellogenin by denaturing gel electrophoresis. DEAE fractions found to contain vitellogenin, a 200kDa protein, were then pooled and dialyzed against DEAE equilibration buffer overnight using Spectra/Por 1 dialysis membrane (Spectrum, Houston, TX, USA). After dialysis, the fractions were run again on the DEAE column under the same conditions as used for the serum. The resulting vitellogenin-containing fractions were pooled, dialyzed against water, and lyophilized under a vacuum. The purified vitellogenin was stored in the –20°C until further use.

Protein Gel for Sequencing

The gel was run according to the gel electrophoresis and the immunoblotting protocol, with adaptations as noted. The samples used for the gel were the serum salamander estrogen-treated samples. Directly after the gel was run, it was put into the western apparatus for acclimation and the western was run using Immobilon-P^{SQ} PVDF transfer membrane (Millipore, Bedford, MA). After the western ended, the membrane was placed in nanopure water for 5 – 10 minutes changing the water three times. The membrane was then placed in a 20% methanol solution for 5 – 10 minutes, followed by a 50% methanol solution for 5 – 10 minutes. Lastly, the membrane was placed in a 100% methanol solution for only a few seconds. Stain (0.025% Coomassie Blue R-250 in 40% methanol) was then applied for approximately 45 seconds (at a maximum). The membrane was then destained (50% methanol) three times (approximately 5 minutes each time). The bands were cut out while they were still wet and the membrane was

dried for approximately 30 minutes. The bands and membrane were put in the –20°C freezer and sent to ProSeq, Inc. (Boxford, MA) for sequencing.

Polyclonal antibody production

The N-terminal amino acid sequence of salamander, hellbender, and crocodile vitellogenin was determined and sent to Invitrogen (Carlsbad, CA) for antibody production. Polyclonal antibodies against purified *Ambystoma tigrinum* vitellogenin were generated in rabbits. Rabbits were immunized initially with DEAE-purified vitellogenin (500 μg) in Freund's complete adjuvant. Injections of emulsified vitellogenin in adjuvant were made subcutaneously in eight different sites using approximately 0.1 ml per injection site. Booster immunizations were given at 30-d intervals after the initial immunization. This procedure for booster injections was the same as for the initial injection; except that Freund's incomplete adjuvant was used. Bleeding was performed prior to the initial immunization (test bleed) and 10 d after each immunization. Twenty ml of blood was taken from the marginal ear vein and allowed to clot at room temperature for 4 h. Serum was then prepared by centrifugation. Serum was stored at –20°C until use.

Coomassie Protein Assay

BSA was used as the protein standard for protein determination (stored at –20° C). The protein concentration was determined by the methods of the Bradford assay (Bradford, 1976).

The Coomassie stain was made by dissolving 100 mg Coomassie Blue G-250 (available from several sources) in 50 ml 95% ethanol, adding 100 ml 85% (w/v) phosphoric acid to this solution and diluting the mixture to 1 liter with water.

A series of protein dilutions were prepared in duplicate. For serum, between 2 and 5 µg and 1998 – 1995 µl of nanopure water showed the closest range to the standard curve; for cytosol, between 10 – 13 µg and 1990 – 1982 µg of nanopure water showed the closest range to the standard curve. This mixture was put into 13 X 100 Eppendorf tubes. Two ml of the Coomassie stain was added to each tube so that the final tube volume was 4 ml. The tubes were vortexed and allowed to stand for five minutes. In this time the Spectronic 20 spectrophotometer was warmed up and blanked with a tube containing 2 ml of nanopure water and 2 ml of Coomassie stain. The absorbencies of the sample tubes were read at 620 nm, and plotted against the standard curve. The protein concentration of each tube was determined using the standard curve to find the concentration of standard that would have the same absorbance as the sample.

Gel Electrophoresis

Ambystoma tigrinum sera and DEAE fractions were electrophoresed under denaturing conditions in polyacrylamide gels using sodium dodecysulfate-polyacrylamide gel electrophoresis (SDS-PAGE). BioRad precast 4-15% gradient gels were used. Protein-containing samples (DEAE fractions or serum, diluted appropriately) were mixed 1:1 v/v with Laemmli sample buffer (5% w/v 2-mercaptoethanol, 2.3%SDS, 62.4 mM Tris-HCl, pH 6.7, 0.02% w/v bromophenol blue, 10% w/v glycerol) and denatured by heating for 4 min in a boiling water bath. Wide range SigmaMarker standards were used in all SDS-PAGE gels. The proteins and their molecular weights in the marker are as follows: Myosin, rabbit muscle 205,000; β-

Galactosidase, *E.coli* 116,000; Phosphorylase b, rabbit muscle 97,000; Fructose-6-phosphate Kinase, rabbit muscle 84,000; Albumin, bovine serum 66,000; Glutamic Dehydrogenase, bovine liver 55,000; Ovalbumin, chicken egg 45,000; Glyceraldehyde-3-phosphate Dehydrogenase, rabbit muscle 36,000; Carbonic Anhydrase, bovine erythrocytes 29,000; Trypsinogen, bovine pancreas 24,000; Trypsin Inhibitor, soybean 20,000; _-Lactalbumin, bovine milk 14,200; Aprotinin, bovine lung 6,500 (Sigma Aldrich, Saint Louis, MO). Samples were run at a constant current of 20 mA at room temperature until the dye front reached the bottom of the gels. Gels were stained with Coomassie dye or were used for immunoblotting.

Immunoblotting

Proteins separated by SDS-PAGE were transferred to polyvinylidene fluoride (PVDF) membranes (BioRad) for immunoblotting. Kaleidoscope Prestained Standards were used in all SDS-PAGE gels for immunoblotting. The proteins and their molecular weights in the marker are as follows: Myosin 210,000; β-Galactosidase 135,000; Bovine serum albumin 82,000; Carbonic anhydrase 38,700; Soybean trypsin inhibitor 31,900; Lysozyme 18,100; Aprotinin 7,400 (BioRad Laboratories, Hercules, CA). Transfers were performed in a BioRad Trans-Blot apparatus packed in ice, at 70V for 3 h at 4°C. The transfer buffer contained 25 mM Tris, 192 mM glycine, 20% methanol, 10 ml SDS, pH 8.3. After transfer, the PVDF membrane was incubated (blocked) in 5% nonfat dry milk for 1h at room temperature, with shaking. This solution was replaced with 5% nonfat dry milk containing the primary antibody, (rabbit anti-*Ambystoma* vitellogenin serum) diluted appropriately, and was incubated for 16 h at room temperature with shaking. A listing of all potential primary antibodies is listed in Appendix 1. The PVDF membrane was then washed (15 min, with shaking) once with 50 mM Tris-HCl (pH 7.5), 0.9%

NaCl, 0.05% Tween-20, and twice with 50 mM Tris-HCl, 0.9% NaCl. The PVDF membrane as then incubated in 5% nonfat dry milk containing the secondary antibody, (peroxidase-coupled goat anti-rabbit serum (BioRad), diluted 1:3,000, and was incubated for 2 h at room temperature, with shaking. The PVDF membrane was again washed once with 50 mM Tris-HCl, 0.9% NaCl, 0.05% Tween-20, and twice with 50 mM Tris-HCl, 0.9% NaCl, and then was developed for 5 min with peroxidase substrate (diaminobenzidine and urea-hydrogen peroxide tablets, Sigma Chemical) until bands appeared. The reaction was then stopped by pouring the peroxidase-substrate off and rinsing the PVDF membrane with 50 mM Tris-HCl, 0.9% NaCl.

Native Gel Electrophoresis

Ambystoma tigrinum sera and DEAE fractions were electrophoresed under native (non-denaturing) conditions in polyacrylamide gels using native gel electrophoresis. BioRad precast 4 to 15% gradient gels were used. Protein-containing samples (DEAE fractions or serum, diluted appropriately) were mixed 1:1 v/v with native sample buffer. Samples were run at 20 mA at room temperature in sample buffer prepared without SDS, until the dye front reached the bottom of the gels. Gels were stained with Coomassie dye and were used for immunoblotting (without using SDS).

Periodic Acid Schiffs Test

In order to detect glycoproteins, a procedure was adapted from Covens et al., (1988). After performing the gel electrophoresis as usual, the gel was fixed overnight in a solution of methanol: acetic acid: water (50:7:43). The following day, oxidation of carbohydrate moieties in

periodic acid and sodium acetate (1%/1.6% v/w) was performed for 20 h at room temperature on a shaker. The third day, the gel was incubated in undiluted Schiffs reagent for electrophoresis for 1 h at 4°C in the dark on the shaker. The gel was destained in a freshly prepared solution of 5% $N_2S_2O_5$ in 1 N HCl for 15-30 min. The background staining was further decreased in a 10% acetic acid solution.

Enzyme-linked Immunosorbent Assay (ELISA)

Serum samples were diluted with phosphate-buffered saline (PBS) (136 mM NaCl, 1.5 mM KH_2PO_4, 8.2 mM Na_2HPO_4, 2.7 mM KCl, pH 7.2), and 100 μl was added to the individual wells of a polystyrene microtiter plate. Phosphate-buffered saline alone was added to wells designated as blanks. Antigen was allowed to bind to the plates overnight at 4° C in a covered glass dish with water to create a humidity chamber. The plate was then washed four times with PBS. Next, 200 μl PBS-blotto (5 g nonfat dry milk in 100 ml PBS) was used to block for 1 h at room temperature, covered, with shaking. The PBS-blotto was replaced with 100 μl of PBS-blotto containing primary antibody (rabbit anti-*Ambystoma* vitellogenin serum) and the plate was incubated covered for 2 h on the shaker at room temperature. A listing of all potential primary antibodies is listed in Appendix 1. The plate was then washed four times with PBS and incubated covered for 2 h on the shaker at room temperature, with the secondary antibody, (goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (BioRad)) diluted 1:1,000 in PBS-blotto. The plate was then washed four times with PBS, then developed using a TMB Peroxidase EIA Substrate Kit (BioRad) and then incubated for 5 min at room temperature. Absorbance's of the wells was read by a BioRad model 3550 microplate reader at 650 nm at 5

min at room temperature and at 10 min. The reaction was stopped with 1 N H_2SO_4 and the absorbencies were read at 450 nm with background subtraction at 650 nm.

Liver Homogenization Protocol: Preparation of Salamander Liver Cytosol

The low-, mid-, and high-speed centrifuges were chilled to 4° C. Livers were obtained from storage in the –80° C refrigerator and kept on dry ice. The entire liver (contents of approximately 0.3 g), were weighed out and their exact weight recorded. The livers were placed into 10ml beakers, to which 4.5 ml of buffer TE (composed of 50 ml Tris Stock (1 M) and 10 ml of EDTA Stock (0.1 M) brought up to 1 L with nanopure water) was added at a ratio of 1:15 g/ml. The liver mixture was finely diced using scissors and a tissue terror each three times at 10 seconds apiece. This mixture was placed into 15 ml conical tubes, which were centrifuged at 2500 X g for 10 minutes at 4° C in the low-speed centrifuge. The supernanent was placed into Corex tubes with a heavy plastic holder. These were then centrifuged at 9200 RPM for 30 minutes at 4° C in the mid-speed centrifuge. The supernanent was then placed into Beckman tubes and centrifuged at 31,200 RPM for 1 h at 4° C in the high-speed centrifuge. The supernanent was taken and placed into the –20° C freezer until further analysis.

Statistical Analysis

Various treatment groups were compared by Student's T-Test and one-way analysis of variance (ANOVA) with repeated measures. The statistical program used was Prism (GraphPad, Inc., San Diego, CA). Data are presented in the results as the Mean +/- 1 SEM (Standard Error of the Mean). Densitometry was conducted using the National Institute of Health program NIH Image Version 1.61. A sample of densitometry plots is illustrated in Appendix 8.

Results

Experiment 1: 17β-estradiol Injection of Male Ambystoma tigrinum

Estrogen treatment significantly reduced the body weight of the salamanders. The mean body weight of the vehicle salamanders was 173.9 g +/- 7.887. The mean body weight of the estrogen-treated salamanders was 152.1 g +/- 4.613. A t-test showed this difference to be significant ($t = 2.56$; 13 df; $p < 0.05$). Data are from fifteen salamander samples (five vehicle-controls, and ten estrogen-treated). Appendix 2 shows the final weights of each salamander.

Mean serum protein concentration was significantly increased for the estrogen-treated group compared with the vehicle-control group, indicating that estrogen treatment was associated with an increase in total serum protein concentration. The mean serum protein concentration for the control salamanders was 20.03 µg/µl +/- 2.66 while the mean for the estrogen-treated salamanders was 38.40 µg/µl +/- 3.99. The significance was demonstrated by a t-test ($t = 3.044$; 13 df; $p < 0.01$). Appendix 3 shows the total protein concentrations for each salamander.

Differences between 17β-estradiol-treated and untreated males were examined for salamander liver cytosol using SDS-PAGE (Figure 1). No obvious differences were observed between the experimental and control groups. There was no indication of a 200-kDa protein (the expected size of vitellogenin) in either group.

Differences between 17β-estradiol-treated and untreated males were also examined for salamander serum using SDS-PAGE (Figure 2). A 66-kDa protein appeared to decrease upon exposure to estrogen. Although the amounts of albumin were depressed in the estrogen-treated

salamanders as compared to the control group, the results of a t-test ($t = 1.509$; 8 df; $p > 0.05$) determined that there is no statistical significance between the two groups when using densitometry. There was no indication of the presence of a 200-kDa protein (the expected size of vitellogenin) in either group.

Native PAGE was run to evaluate differences in serum proteins under non-denaturing conditions (Figure 3). No clear differences were observed between the experimental and control salamanders sera.

Experiment 2: Ethinyl-estradiol Injection of Ambystoma tigrinum

Single Injection, Males and Females

Mean serum protein concentration was not significantly different between the estrogen-treated group and the vehicle-control group. The mean value for the control salamanders was 29.90 µg/µl +/- 6.005 while the estrogen-treated salamanders was 30.74 µg/µl +/- 2.739. No significant difference was found by a t-test ($t = 0.1275$; 6 df; $p > 0.05$). Data shown are representative of eight salamander samples (after ten deaths due to red-legged disease). Appendix 4 shows the total protein concentrations for each salamander.

SDS-PAGE was used to evaluate the differences between ethinyl-estradiol single-injection-treated and untreated male and female sera. Both estrogen-treated male and female salamanders revealed the presence of a 200kDa protein (the expected size of vitellogenin) (Figure 4). This protein was not evident in untreated male and female sera and was determined to be statistically increased in the estrogen-treated groups as compared to the controls by densitometry readings ($t = 2.436$; 6 df; $p < 0.05$).

Multiple Injection, Males

Mean serum protein concentration was reduced for the estrogen-treated group compared with the vehicle-control group. The mean value for the control salamanders was 30.39 µg/µl +/- 3.94 while that for the high dose estrogen-treated salamanders was 21.72 µg/µl +/- 3.39. These values were not significantly different, as demonstrated by a t-test ($t = 1.668$; 4 df; $p > 0.05$). Data shown are representative of six salamander samples (after four deaths due to red-legged disease). Appendix 5 shows the total protein concentrations for each salamander.

SDS-PAGE was used to examine differences in cytosol between multi-injected ethinyl-estradiol-treated and untreated males. There were no obvious differences seen between the high, low, and control groups. There was no evidence of a 200kDa protein (the expected size of vitellogenin) on SDS-PAGE (Figure 5).

SDS-PAGE was also used to assess differences in serum proteins between ethinyl-estradiol multi-injection-treated and untreated males (Figure 6). A 66kDa protein appeared to decrease upon exposure to estrogen, however, again the results of a t-test ($t = 1.225$; 6 df; $p > 0.05$) determined that there was no statistical significance between the two groups using densitometry. Also, high and low dose ethinyl-estradiol-treated salamanders contained high levels of a 200kDa protein (the size of the protein vitellogenin). This protein was not present in the control serum samples. A periodic acid Schiff's test for glycoproteins was inconclusive as to whether there were glycoproteins present or not (Figure not shown).

An SDS-PAGE gel was run and transferred to a PVDF membrane for use in N-terminal amino acid sequencing. The blot was sent off to ProSeq, Inc. (Boxford, MA) for sequencing of the salamander presumptive vitellogenin. The first sequence obtained was "(S)QYN(S)E(S)VF(S)()(A)". The letters represented in parenthesis are the most likely amino

acids, but they cannot be deemed conclusive. Since this result did not seem very accurate, we had them re-sequence the amino acids, and determined from the two that the more confident sequence was the following: "CQYNHEPVFSESKSY(V)YN(Y)(E)A(L)I". The result was a sequence closely resembling the *Xenopus* vitellogenin N-terminal sequence (Table 7).

Table 7: Comparison of the N-Terminal Amino Acid Sequences of Vitellogenin Proteins in Amphibians

X. laevis[a]	**EPVFSESKTSVYNYEAVI**
A. tigrinum	**CQYNHEPVFSESKSYVYNYEALI**
C. alleganiensis	**EQYNYEPVFSEIKTYVYNYEA**

Black = Identical
Pink = Conserved
Blue = Different

[a] Sequence from Genbank Accession No. X01168.1.

DEAE chromatography was performed on sera from ethinyl-estradiol multi-injection-treated and untreated males to further evaluate protein differences (Figures 7, 8, 9, 10). Three distinct peaks were evident in fractions from the DEAE column. The first peak eluted during the wash and represented proteins that did not adhere to the column. The second and third peaks eluted during the salt gradient, with peak two at low salt and peak three at high salt. Analysis of the peaks by SDS-PAGE revealed that the second peak consisted largely of proteins in the 70kDa range, probably representing albumin. This peak was consistently larger for control sera as compared to estrogen-treated sera. Densitometry showed the control proteins to be more abundant than the estrogen treated proteins. Statistics could not be run between fractions, since

only one serum sample was used for estrogen and control profiles. The third peak contained abundant amounts of a 200kDa protein. This protein was presumed to be vitellogenin, because it had the appropriate molecular weight for *Xenopus laevis* vitellogenin and because vitellogenin is known to elute at high salt from DEAE. Also, the 200kDa protein was present in DEAE fractions from estrogen-treated salamander sera but was absent in the corresponding fractions from control salamander sera, indicating that it was estrogen-induced.

Experiment 3: Ethinyl-estradiol Injection of Juvenile Ambystoma tigrinum

Estrogen treatment had no significant effect on the body weight of the experimental salamanders. The mean body weight of the vehicle salamanders was 7.427 g +/- 0.009. The mean body weight of the estrogen-treated salamanders were 6.753 g +/- 0.2404. A t-test showed this difference was not significant ($t = 1.362$; 13 df; $p > 0.05$). Data shown are representative of fifteen (three controls and twelve ethinyl-estradiol treated) salamander samples after deaths (one vehicle and four ethinyl-estradiol treated) due to red-legged disease. Appendix 6 shows the final weights of each salamander.

SDS-PAGE was used to examine differences between ethinyl-estradiol-treated and untreated juveniles liver cytosol (Figure 11). There were no obvious differences observed between the experimental and control groups. There was no evidence of a 200kDa protein (the expected size of vitellogenin).

SDS-PAGE was also used to evaluate the differences between ethinyl-estradiol-treated and untreated juvenile sera. A 66kDa protein appeared to decrease upon exposure to estrogen, and this difference was statistically significant when evaluated by densitometry ($t = 2.781$; 13 df;

$p < 0.05$). The sera of ethinyl-estradiol salamanders contained high levels of a 200kDa protein (the size of the protein vitellogenin) on SDS-PAGE (Figure 12). This protein resembling vitellogenin was not present in the control samples of salamander serum, and was shown to be significantly higher when evaluated by densitometry ($t = 4.035$; 13 df; $p < 0.01$).

A native PAGE was run to evaluate adult and juvenile samples in their non-denatured form (salamanders from Experiments 2 and 3). No clear differences were evident between the experimental and control salamanders (Figure 13).

DEAE chromatography was performed to further evaluate the differences in serum proteins for juveniles (Figure not shown). Three distinct peaks were evident in fractions for the DEAE column. The first peak eluted during the wash and represented proteins that did not adhere to the column. The second and third peaks eluted during the salt gradient with the second peak at low salt and the third at high salt. Analysis of the peaks by SDS-PAGE revealed that the second peak consisted largely of proteins in the 70kDa range, presumably albumin. This peak was consistently larger for control sera as compared to estrogen-treated sera. The third peak contained abundant amounts of a 200kDa protein. This protein was presumed to be vitellogenin, because it had the appropriate molecular weight for *Xenopus laevis* vitellogenin, and because vitellogenin is known to elute at high salt from DEAE. Also, the 200kDa protein was present in DEAE fractions from estrogen-treated salamander sera but was absent in the corresponding fractions from control salamander sera, indicating that it was estrogen-induced.

Experiment 4: Ethinyl-estradiol Injection and Endocrine Disrupting Chemicals of Juvenile Ambystoma maculatum

Ethinyl-Estradiol Treatment

Estrogen treatment apparently increased the body weight of the salamanders. The mean body weight of the vehicle salamanders was 12.07 g +/- 0.7602. The mean body weight of the estrogen-treated salamanders were 13.08 g +/- 0.4126. An ANOVA showed no significant differences between the groups for body weight ($F = 0.2901$; 3, 35 df; $p > 0.05$). The groups included in the ANOVA were vehicle salamanders, estrogen-treated salamander, atrazine-treated salamanders, and aroclor 1221-treated salamanders. Data shown are representative of sixteen (seven controls and nine ethinyl-estradiol treated), after deaths (three vehicle, eight ethinyl-estradiol treated) due to natural causes. Appendix 7 shows the final weights of each salamander.

SDS-PAGE was used to examine differences between ethinyl-estradiol-treated and untreated sera. A 66kDa protein appeared to decrease upon exposure to estrogen, however this difference was not statistically significant using densitometry ($F = 1.164$; 4, 33 df; $p > 0.05$) (Figure 14). The sera of ethinyl-estradiol-treated salamanders contained high levels of a 200kDa protein on SDS-PAGE (the expected size of vitellogenin). This protein, resembling vitellogenin, was not present in control salamander sera, and the increase was shown to be statistically significant by a densitometry ($t = 6.467$; 14 df; $p < 0.0001$).

Atrazine Injection

Atrazine exposure had no apparent effect on the body weight of salamanders. The mean body weight of the vehicle salamander was 12.07 g +/- 0.76. The mean body weight of the high- and low-dose atrazine-treated salamanders was 12.63 g +/- 0.95. Again, the ANOVA showed no

significant differences between the groups (data shown above). Data shown are representative of eight atrazine (six low dose and two high dose) salamander samples after deaths (four atrazine high dose treated) due to natural causes (same controls as previous). Appendix 6 shows the final weights of each salamander.

SDS-PAGE was used to examine the differences between atrazine-treated and untreated sera. A 66kDa protein appeared to decrease upon exposure to atrazine, however the difference was not statistically significant using densitometry ($F = 1.164$; 4, 33 df; $p > 0.05$) (Figure 15). The atrazine serum samples did not show the presence of the 200kDa protein (the expected size of vitellogenin).

Aroclor 1221 Injection

Aroclor exposure also had no apparent effect on the body weight of salamanders. The Aroclor high-dose exposure appeared to initiate a slight decrease in the body weight of the salamanders, while the low-dose exposure increased salamander body weight. The mean body weight of the vehicle salamander was 12.07 g +/- 0.76. The mean body weight of the high- and low-dose Aroclor-treated salamanders was 12.36 g +/- 0.79. Again, the ANOVA showed no significant differences between the groups (data shown above). Data shown are representative of 12 salamander samples (6 aroclor 1221 high dose, 6 aroclor 1221 low dose). Appendix 6 shows the final weights of each salamander.

SDS-PAGE was used to evaluate the differences between Aroclor 1221-treated and untreated sera. A 66kDa protein appeared to decrease upon exposure to Aroclor 1221, however this difference was not statistically significant using densitometry ($F = 1.164$; 4, 33 df; $p > 0.05$) (Figure 16). The Aroclor 1221 serum samples did not show the presence of the 200kDa protein

visually (the expected size of vitellogenin) on SDS-PAGE, these samples did appear to show weak bands in the 200kDa protein range when evaluated using densitometry.

Hellbender Experiment (Cryptobranchus alleganiensis*)*

SDS-PAGE was used to evaluate the differences between 17-β estradiol-treated and pre-injection male sera. A 66kDa protein appeared to decrease upon exposure to estrogen (Figure 17). This protein appeared to be depressed in the first injection samples as compared with controls, and was even more depressed in the second injection animals. The sample sizes were not large enough to run statistics. The treated hellbenders contained high levels of a 200kDa protein (the expected size of vitellogenin). This protein, resembling vitellogenin, was not present in the pre-injection samples of hellbender serum. Again the sample sizes were not large enough to run statistics.

An SDS-PAGE gel was run and transferred to a PVDF membrane to be used for N-terminal amino acid sequencing. The blot was sent off to ProSeq, Inc. (Boxford, MA) for sequencing of the hellbender presumed vitellogenin. The result was a sequence closely resembling vitellogenin in *Xenopus*, and the sequence received for salamanders (EQYNYEPVFSE(I)KTYVYNYEA) (see Table 7).

DEAE-agarose chromatography was performed to further evaluate differences between serum proteins of estrogen-treated and pre-treated males. Three distinct peaks were evident in treated fractions from the DEAE column. The first peak eluted during the wash and represented proteins that did not adhere to the column. The second and third peaks eluted during the salt gradient with peak two at low salt and peak three at high salt. Analysis of the peaks by SDS-

PAGE revealed that the second peak consisted largely of proteins in the 70kDa range, representing albumin (Figure 18, 19). This peak was consistently larger for control sera as compared to estrogen-treated sera. Densitometry readings of the peak two fractions showed this decrease to be significantly different between the experimental and pre-treatment groups ($t = 3.440$; 6 df; $p < 0.05$). The third peak contained abundant amounts of a 200kDa protein. This was presumed to be vitellogenin, because it had the appropriate molecular weight for *Xenopus laevis* vitellogenin, and because vitellogenin is known to elute at high salt from DEAE. Also, the 200kDa protein was present in DEAE fractions from estrogen-treated male hellbender sera but was absent in the corresponding fractions from pre-injection male hellbender sera.

Crocodile Experiment (Crocodylus moreletii)

Male and female crocodile sera were used to test for the presence of vitellogenin in wild-caught crocodiles from Belize. SDS-PAGE revealed the presence of an approximately 200kDa protein (the size of the protein vitellogenin) in female samples that was absent in male samples (Figure 20). This protein resembling vitellogenin was not present in the male samples of crocodile serum, and the difference was shown to be significant when evaluated by densitometry readings ($t = 12.48$; 10 df; $p < 0.0001$). These female samples were pre-selected from all of the females sampled having the highest estradiol levels, as determined by RIA.

An SDS-PAGE gel was run and transferred to a PVDF membrane to be used for N-terminal amino acid sequencing. The blot was sent off to ProSeq, Inc. (Boxford, MA) for sequencing of the crocodile presumed vitellogenin. The resulting sequence could not be determined after two attempts.

The Western blot using vitellogenin antibody #498 showed cross-reactivity with a protein of approximately 200kDa in the female sera, and showed no cross-reactivity at all in the male sera (Figure 21). The Western blot also shows reactivity with a few other bands, at approximately 77kDa and 64kDa, however, no bands show up in any of the male lanes.

An ELISA was performed using pooled samples to create a titration curve of broadly cross-reactive antibody #498 in order to find the correct antibody dilution to use when evaluating crocodile samples (Figure 22). This titration curve illustrated that the samples reacted best against the 1:1000 dilution. A second ELISA was run using a 1:1000 dilution of broadly cross-reactive antibody #498 against all samples. This ELISA showed much greater cross-reactivity with the female sera than with the male sera, suggesting that wild-caught females contain vitellogenin whereas the males do not (Figure 23). The mean optical density of the females was 0.7010 +/-0.0469 where the mean of the males was 0.1277 +/- 0.00438. A t-test shows this difference to be significant ($t = 12.17$; 22 df; $p < 0.0001$).

DEAE-agarose chromatography was performed to further evaluate the serum proteins of male and female crocodiles (Figure 24, 25). Three distinct peaks were evident in fractions from the DEAE column. The first peak eluted during the wash and represented proteins that did not adhere to the column. The second and third peaks eluted during the salt gradient with peak two at low salt and peak three at high salt. Analysis of the peaks by SDS-PAGE revealed that the second peak consisted largely of proteins in the 70kDa range, resembling albumin. This peak was consistently larger for control sera as compared to estrogen-treated sera. Densitometry showed the male peak being larger than the female peak, although statistics could not be run between fraction numbers. The third peak contained abundant amounts of a 200kDa protein, presumably vitellogenin, since this protein had the appropriate molecular weight for *Xenopus*

laevis vitellogenin and because vitellogenin is known to elute at high salt from DEAE. Although very light, the 200kDa protein was present in DEAE fractions from female crocodiles sera but was absent in the corresponding fractions from male crocodile sera, (this difference was clearly demonstrated in densitometry readings). The densitometry values for these samples are as follows, for lane 29: female = 813 pixels, male = 481, lane 30: female = 1137 pixels, male = 581 pixels.

Rana aurora *Experiment*

A number of wild caught *Rana aurora* samples (13 females, 3 non-distinguishable of sex, 81 sub-adults (juveniles), and 58 males) from California were screened for vitellogenin. An initial SDS-PAGE analysis was done using five estrogen-treated samples and three wild-caught (juveniles), revealing the presence of an approximately 200kDa protein (the expected size of vitellogenin) in both the wild-caught and estrogen-treated *Rana aurora* samples (Figure 26).

The corresponding Western blot using Rana-anti-vitellogenin-antibody #92 revealed cross-reactivity against a 200kDa protein in three of the five estrogen-treated samples, but did not react with anything in the wild-caught samples (Figure 26).

An ELISA using five *Rana aurora* wild-caught males, five estrogen-injected samples as a positive control, and a previous *Rana aurora* male (negative-control) and estrogen-treated sample (positive control), were evaluated in a screen of antisera from five different rabbits (#498, #547, #90, #92, and anti-turtle). This was done as a means for assessing which antibody would be best suited for screening all of the wild-caught *Rana aurora* samples. The five wild-caught samples showed no reactivity to any antibody (suggesting that no vitellogenin was present). Out of the five estrogen-treated samples, two showed no reactivity to any antibody, but the other

three showed reactivity against antibodies #90 and #92 (*Rana*), which was comparable to the reactivity of estrogen-treated serum positive controls against only antibody #90 and #92. The negative control serum showed no reactivity to any antibody. An ELISA screen of all *Rana aurora* wild-caught samples was then evaluated using antibody #92. Only 9 of the 13 female samples, 2 of the 3 non-distinguishable of the sex, 2 of the 81 sub-adults (juveniles), and 0 of the 58 male samples reacted with the antibody, i.e., most samples in the population were non-vitellogenic (Table 8). Of the total population in the lake, only 13 individuals showed positive for vitellogenin out of the 155 total members of the population, representing that only 8.39% of the population are vitellogenic.

Table 8: Vitellogenic Population of *Rana aurora* from California

Sexual Type	***Total Number in Population***	***Number shown positive for Vitellogenin***
Females	13	9
Males	58	0
Subadults (juveniles, unable to sex yet, could be males or females)	81	2
Non-distinguishable	3	2
TOTAL Population	155	13

Polyclonal Antibody Production

Ambystoma tigrinum

In salamander experiment #1, Western blotting, using a rabbit-anti-vitellogenin antiserum, confirmed that there was no reactivity to any proteins resembling vitellogenin (approximately 200kDa) (Figure 27). This Western did reveal the reaction of a protein of

approximately 100kDa with the salamander serum, but because it occurred in both the 17β-estradiol-treated and control salamanders, it did not appear to be vitellogenin. However, because the SDS-PAGE of estrogen-treated samples did not reveal the presence of vitellogenin (approximately 200kDa), the absence of reactivity was not pursued further (See Figure 2).

In salamander experiment #2, Western blotting with a broadly cross reactive anti-vitellogenin antiserum (#498) did not reveal cross-reactivity against vitellogenin in the female sera. It appeared to again react with a 100kDa protein in both male and female sera. The same antibody was also tested against DEAE fractions and still showed no cross-reactivity for any fractions (male or female) (Figure 28). A Western blot antibody screen was conducted to assess cross-reactivity to the 200-kDa protein using samples from estrogen-treated and control male samples. The only reaction from the sera was the same 100-kDa protein with equal strength to antibodies #498, #547, #90, and #92, but no reaction was seen at all to anti-turtle antiserum (Figure 29). There was very little difference between the appearance of the male and female sera on the Western blot, again suggesting the antibodies did not recognize vitellogenin.

Due to the lack of reactivity in the Western blot, an ELISA was run to attempt to detect serum vitellogenin. In all cases, the ELISA was inconclusive for male, female, ethinyl-estradiol-treated, and control salamanders. However, all estrogen-treated and control samples reacted with antibodies #498, #547, #90, and #92, but did not react to anti-turtle. This again suggested the antibodies were not recognizing vitellogenin.

Another ELISA was run to attempt to detect serum vitellogenin from the salamanders in experiment #3 against antibodies #498, #547, #90, and #92, and anti-turtle antiserum. In all cases, the ELISA was inconclusive (data not shown).

Cryptobranchus alleganiensis

Western blotting using a rabbit-anti-vitellogenin antiserum (#498) in hellbenders again confirmed that there was no reactivity of any proteins resembling vitellogenin (approximately 200kDa) (Figure not shown). A Western blot antibody screen was conducted to assess cross-reactivity to the 200-kDa protein using samples from pre-treated and estrogen-treated hellbenders. The only reaction from the sera was the same 100-kDa protein with equal strength to antibodies #498, #547, #90, and #92, but no reaction was seen at all to anti-turtle antiserum (Figure 30).

The lack of reactivity prompted the performance of an ELISA. In all cases, the ELISA was inconclusive for male-treated and pre-injection hellbenders. Everything reacted with antibodies #498, #547, #90, and #92 and did not react to anti-turtle.

The lack of a positive reaction between an obvious 200kDa protein, resembling vitellogenin, seen in the SDS-PAGE and DEAE chromatography of these three experiments and the hellbender experiment was evidence enough that an antibody specific for salamander vitellogenin was necessary in order to detect the presence of this 200kDa protein in this species.

However, to first rule out the possibility of the mystery protein being albumin, a Western blot was used to test if the 100kDa protein was possibly antigenically similar to albumin. No commercial antibody against salamander serum albumin was found, but polyclonal antibodies raised against chicken serum albumin was available. The antisera crossreacted with a band from salamander sera at 66kDa. The intensity of 66kDa staining in the Western blots was greater for the control salamander sera compared to the estrogen-treated salamander sera, indicating a greater abundance of the 66kDa protein in control animals. All animals again reacted also with

the 100kDa protein, and so it was established that the mystery protein was not albumin (Figure 31).

Polyclonal antibodies were produced against the conserved amino acid sequence of vitellogenin we obtained according to standard procedures. After a BLAST search of this sequence against various other vitellogenin amphibian species, it was decided that a little alteration of the sequence was necessary before antibody production. Therefore, the following sequence was the one sent to have the antibody made against: "EPVFSESKISVYNYEAVI". Antibodies against salamander vitellogenin were raised in rabbits using MAP technology by Invitrogen (Carlsbad, CA). The antibodies (#371 and #358), arrived and were analyzed using ELISA and Western blotting against a number of salamander samples (Figure 32). Reactivity was only seen in estrogen-treated samples one time out of four tries. In conclusion, the Western also showed no reactivity to either antibody. An ELISA using the peptide as antigen revealed no crossreactivity against the antibodies.

After this series of inconclusive ELISAs, the antibodies against salamander vitellogenin were remade by a new company (Sigma Genosys) using KLH technology. The antibodies (#129 and #130), arrived and were analyzed using ELISA and Western blotting against a number of salamander samples. Western blotting showed only reactivity to either antibody against the 100kDa band seen previously (Figure not shown). Another Western was performed using Alkaline Phosphatase methods to establish if a reaction would be seen, however, the only reaction again was against the 100kDa band seen previously (Figure 33). Reactivity against estrogen-treated salamander samples was only seen when running the ELISA using Alkaline Phosphatase methods in creating a titration curve against both antibodies #129 and #130 (Figure 34, 35).

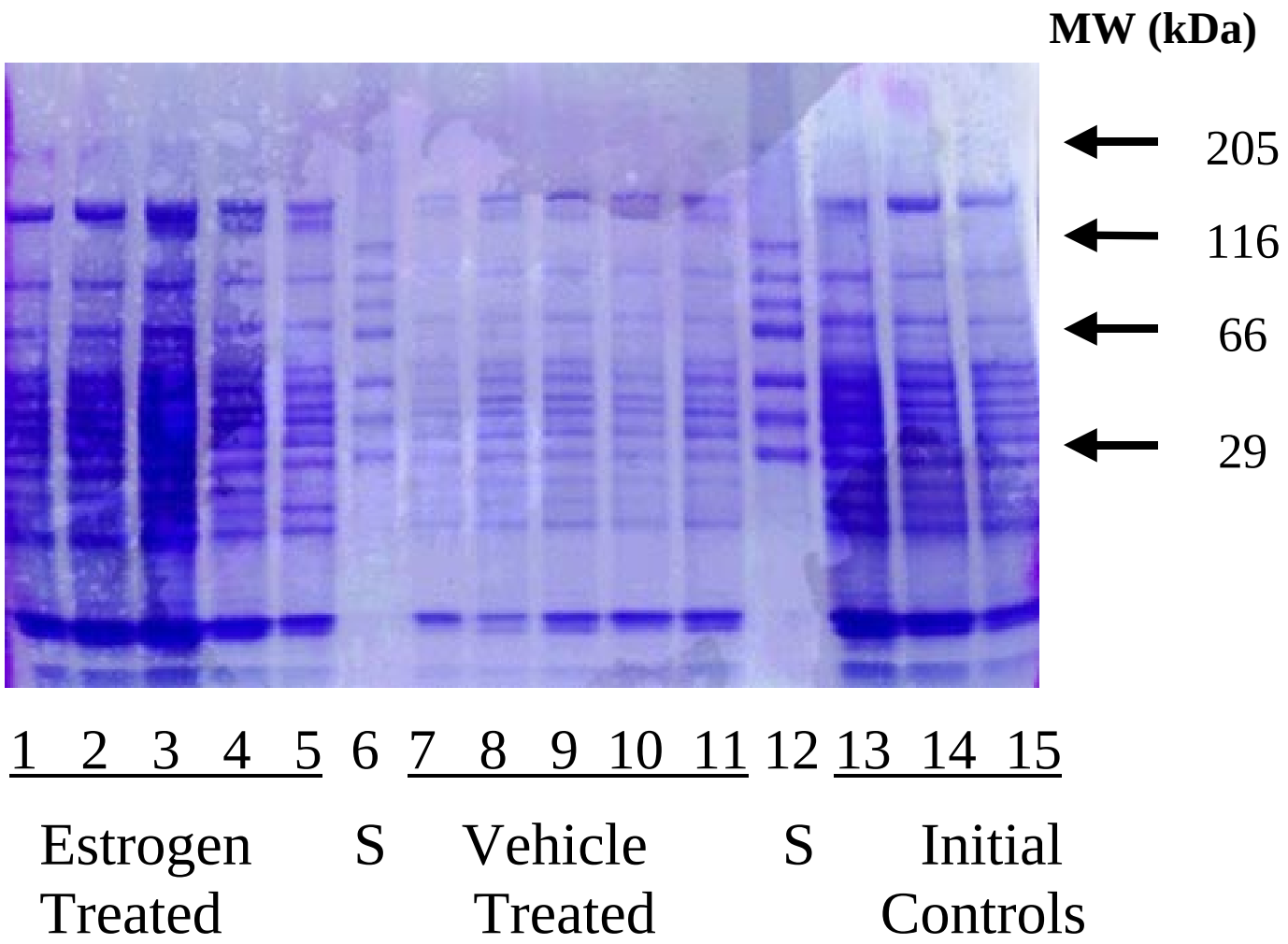


Figure 1. Denaturing polyacrylamide gel (SDS-PAGE) of cytosol from control and estrogen-treated *Ambystoma tigrinum*. Lane assignments are as follows: 1-5 = estrogen-treated; 6, 12 = molecular weight standards (S); 7-11 = vehicle-treated; 13-15 = initial controls. See methods for treatment regimens. Cytosol was separated on a 4 to 15% polyacrylamide gradient gel and stained with Coomassie blue.

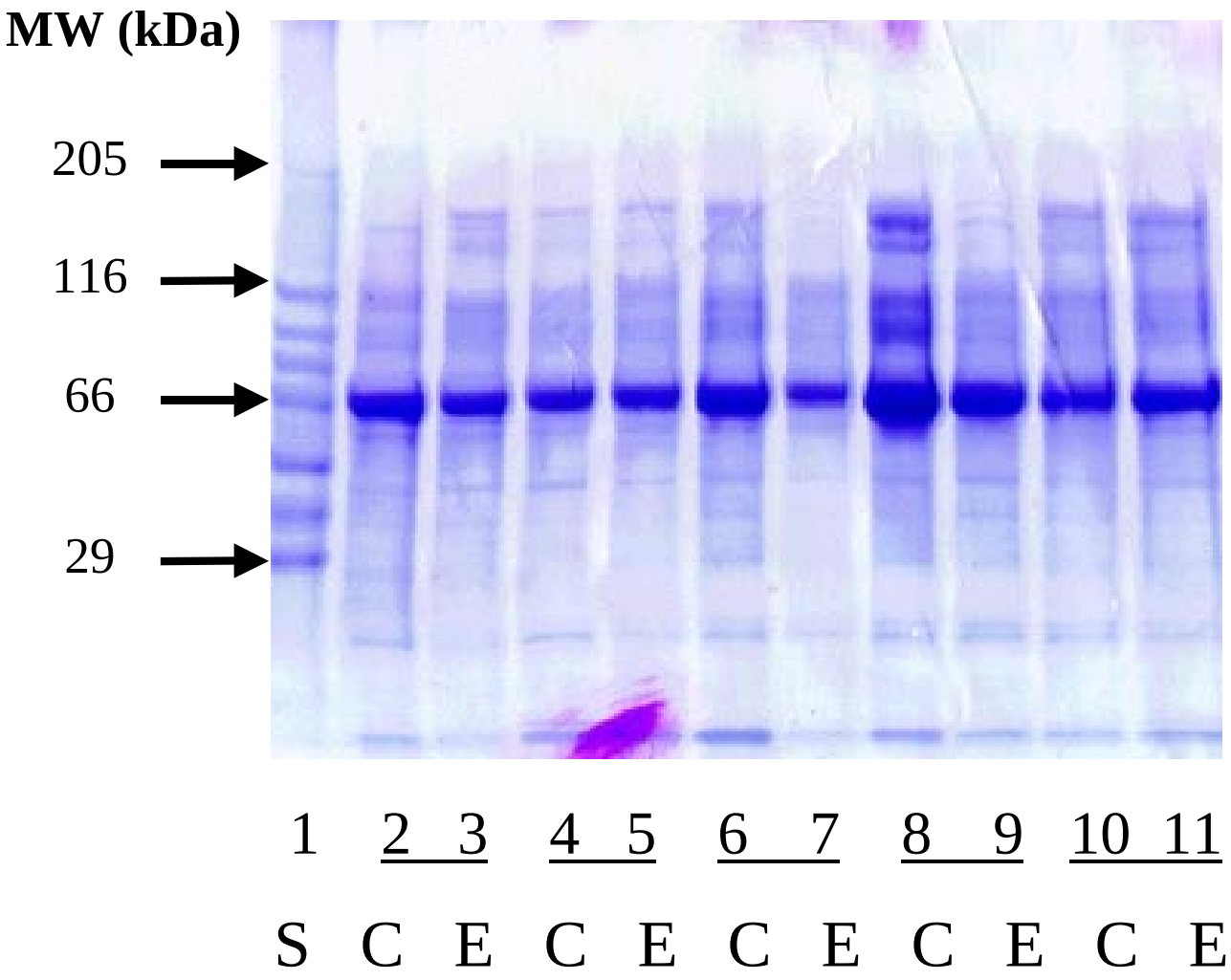


Figure 2: Denaturing polyacrylamide gel (SDS-PAGE) of sera from control and estrogen-treated *Ambystoma tigrinum*. Lane assignments are as follows: 1 = molecular weight standards (S); 2, 4, 6, 8, 10 = control animals (C) #1-5, respectively; 3, 5, 7, 9, 11 = estrogen-treated animals (E) #1-5, respectively. See methods for treatment regimens. Sera were separated on a 4 to 15% polyacrylamide gradient gel and stained with Coomassie blue.

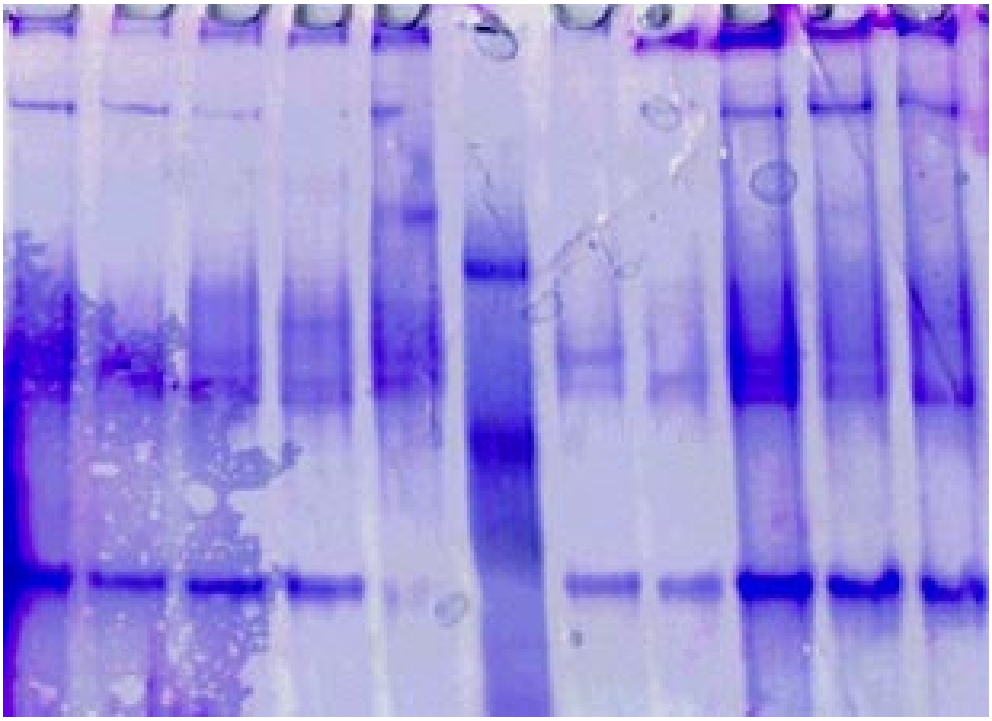


Figure 3: Non-denaturing polyacrylamide gel (PAGE) of sera from control and estrogen-treated *Ambystoma tigrinum*. Lane assignments are as follows: 1-5 = estrogen-treated animals; 6 = molecular weight standards (S); 7-11 = control animals.

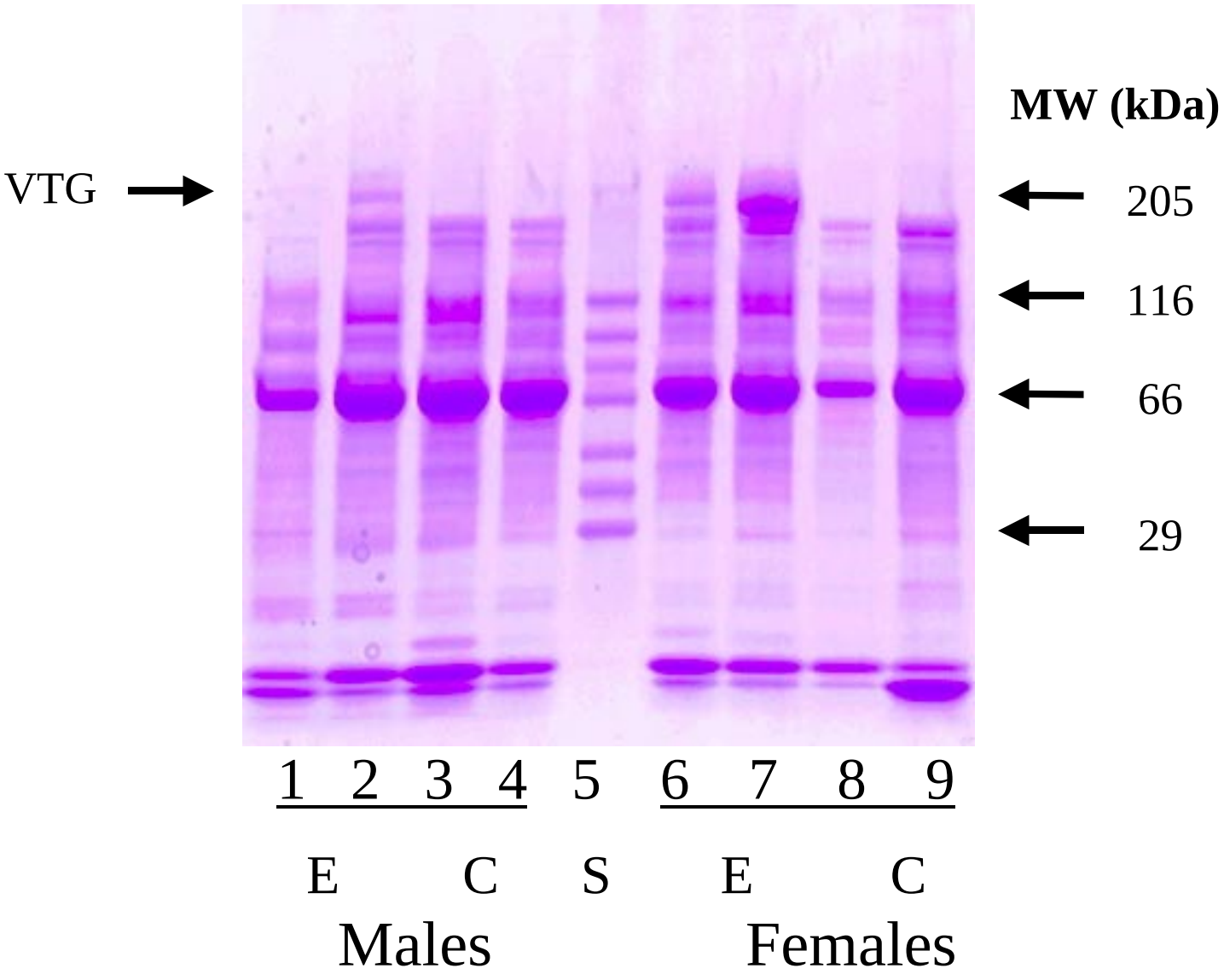


Figure 4: Denaturing polyacrylamide gel (SDS-PAGE) of single-injection sera from control and estrogen-treated *Ambystoma tigrinum*. Lane assignments are as follows: 1-2 = male estrogen-treated (E); 3-4 = male control animals (C); 5 = molecular weight standards (S); 6-7 = female estrogen-treated(E); 8-9 = female control animals (C); VTG = presumptive vitellogenin. See methods for treatment regimens. Sera were separated on a 4 to 15% polyacrylamide gradient gel and stained with Coomassie blue.

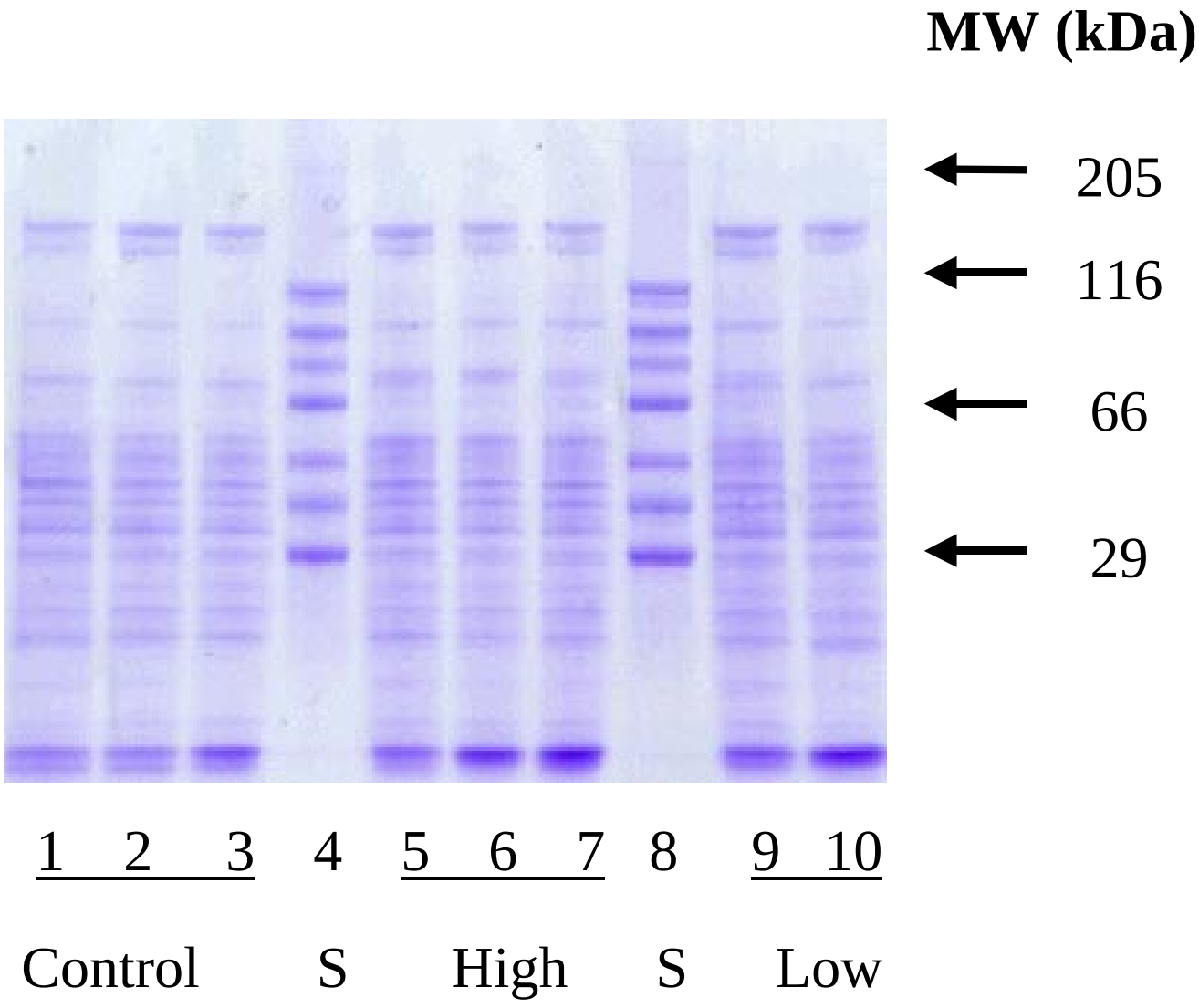


Figure 5: Denaturing polyacrylamide gel (SDS-PAGE) of multi-injection cytosol from control and estrogen-treated *Ambystoma tigrinum*. Lane assignments are as follows: 1-3 = controls; 4, 8 = molecular weight standards (S); 5-7 = high dose estrogen-treated; 9-10 = low dose estrogen-treated. See methods for treatment regimens. Cytosol was separated on a 4 to 15% polyacrylamide gradient gel and stained with Coomassie blue.

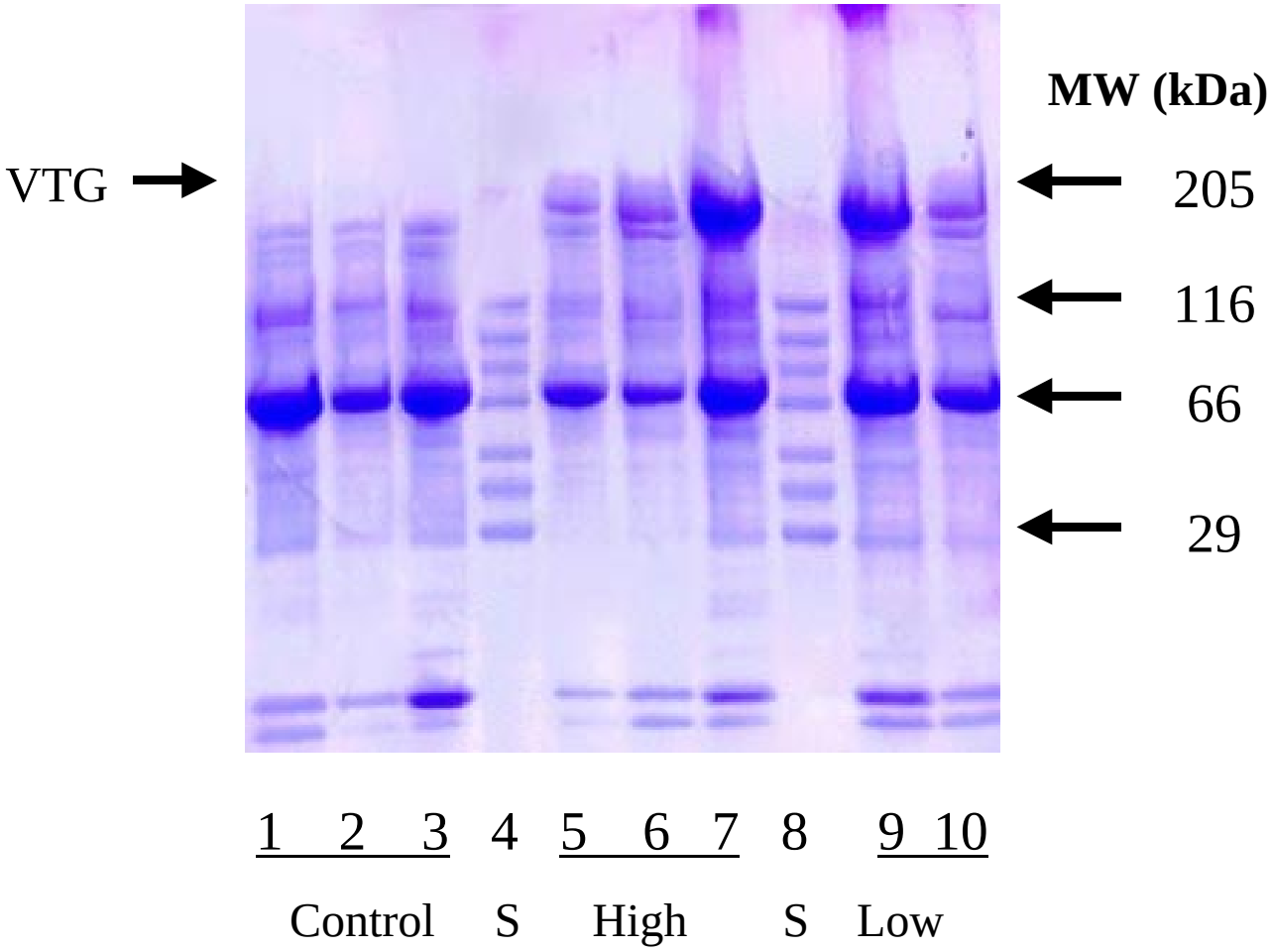


Figure 6: Denaturing polyacrylamide gel (SDS-PAGE) of multi-injection sera from control and estrogen-treated *Ambystoma tigrinum*. Lane assignments are as follows: 1-3 = controls; 4, 8 = molecular weight standards (S); 5-7 = high dose estrogen-treated; 9-10 = low dose estrogen-treated; VTG = presumptive vitellogenin. See methods for treatment regimens. Sera were separated on a 4 to 15% polyacrylamide gradient gel and stained with Coomassie blue.

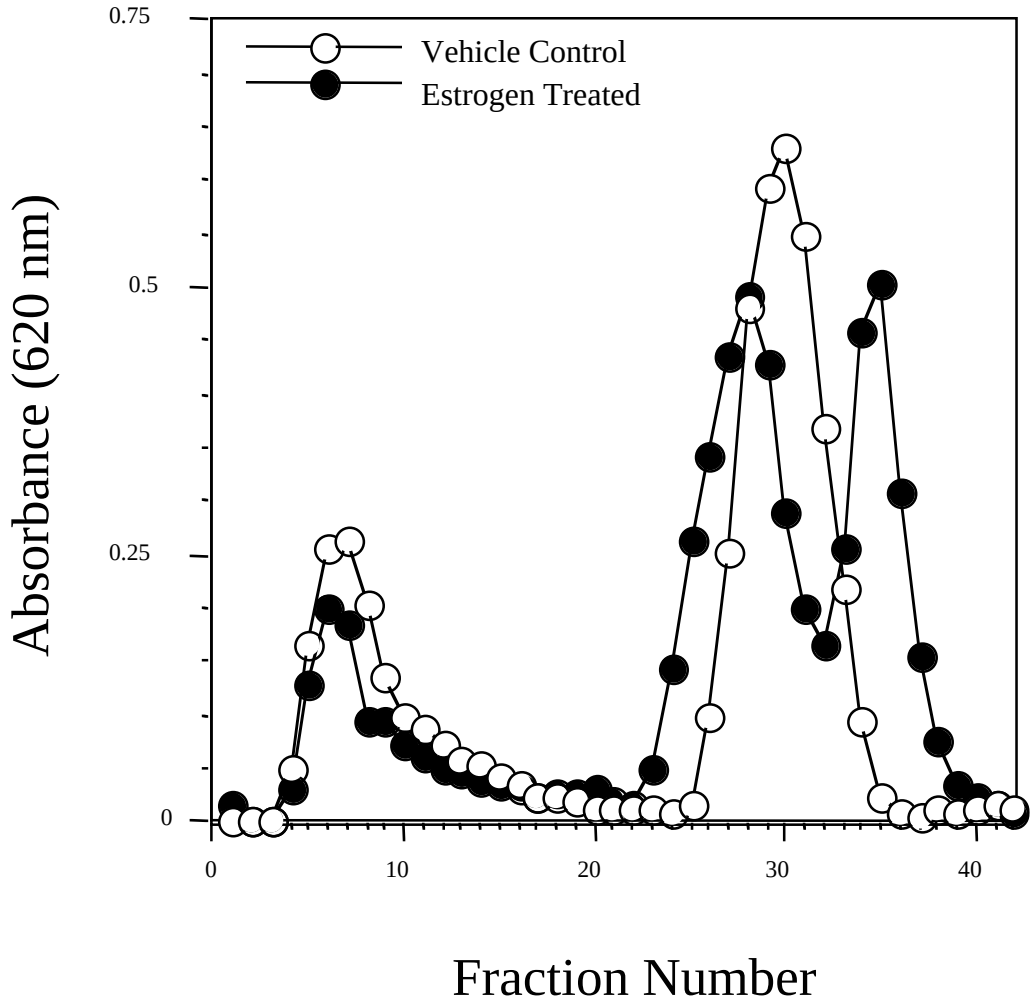


Figure 7: DEAE profile for sera from control and estrogen-treated *Ambystoma tigrinum*. Serum proteins were separated on a DEAE-agarose column using a Bio-Rad Econo Chromatography System. Proteins were subsequently measured by the Coomassie method for each fraction.

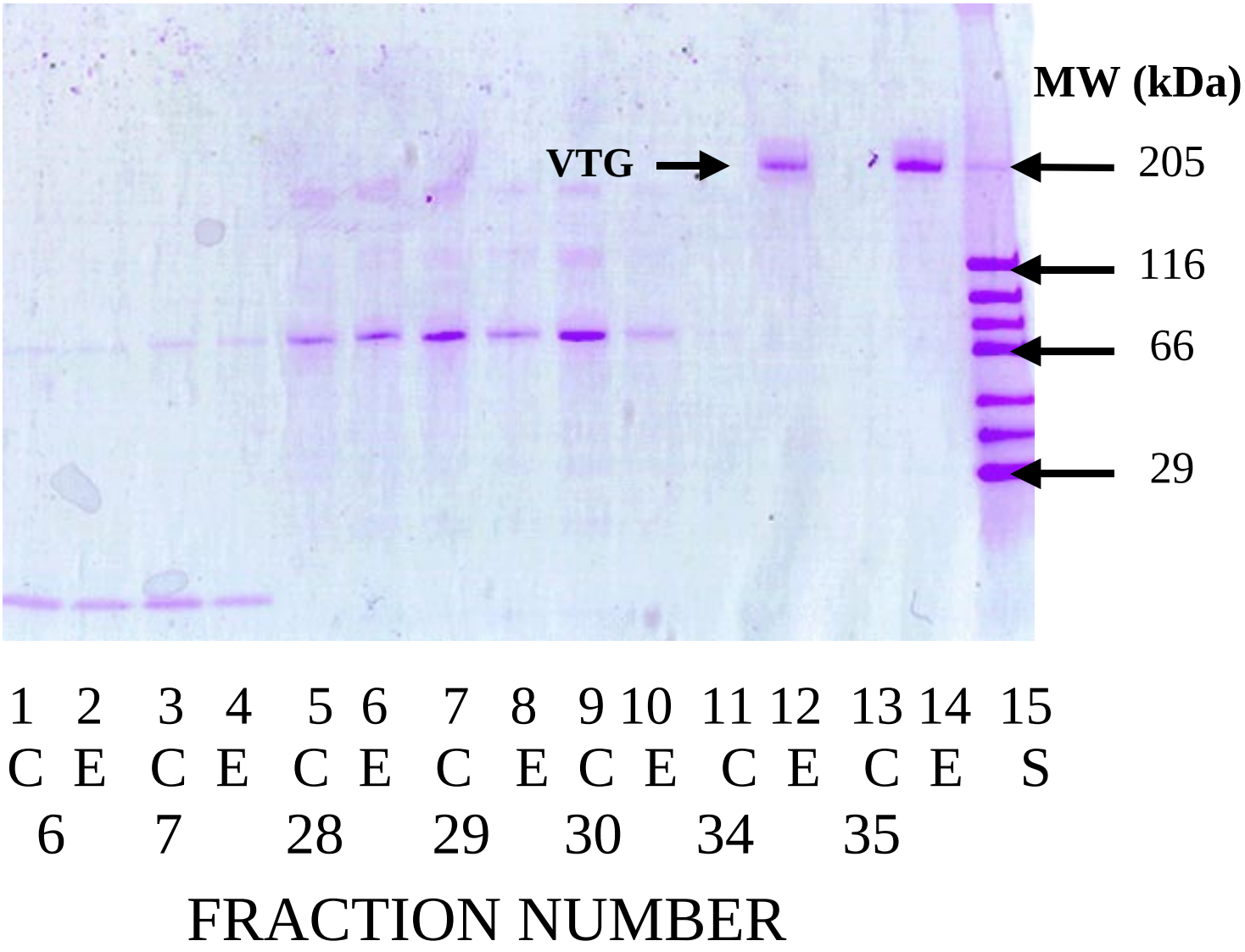


Figure 8: Denaturing polyacrylamide gel (SDS-PAGE) of DEAE fractions from control and estrogen-treated *Ambystoma tigrinum*. Lane assignments are as follows: 1-13 (odd lanes) = fractions from control animal sera (C); 2-14 (even lanes) = fractions from estrogen-treated animal sera (E); 15 = molecular weight standards (S). See methods for treatment regimens. Bottom numbers refer to fraction numbers from the DEAE separation. Fractions were separated on a 4 to 15% gradient gel and stained with Coomassie blue.

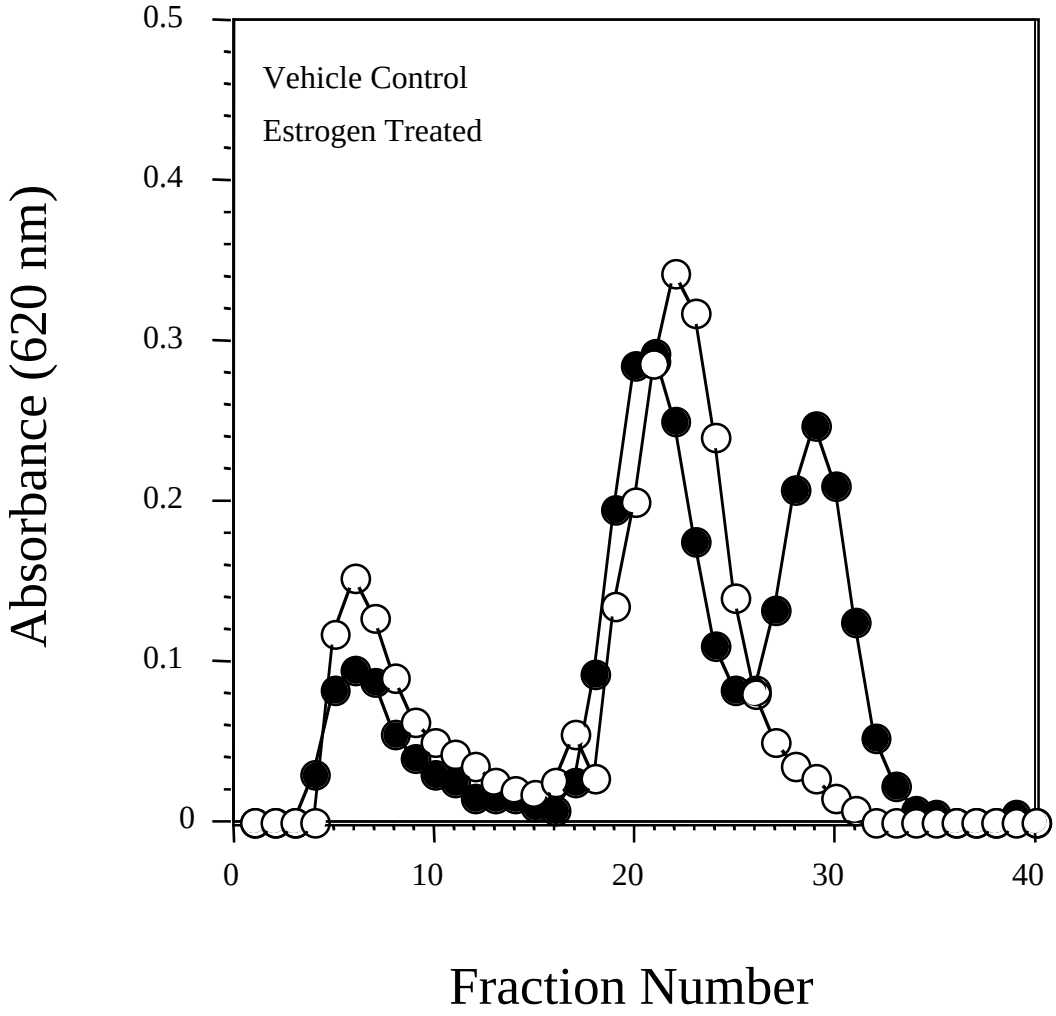


Figure 9: DEAE profile for sera from control and estrogen-treated *Ambystoma tigrinum*. Serum proteins were separated on a DEAE-agarose column using a Bio-Rad Econo Chromatography System. Proteins were subsequently measured by the Coomassie method for each fraction.

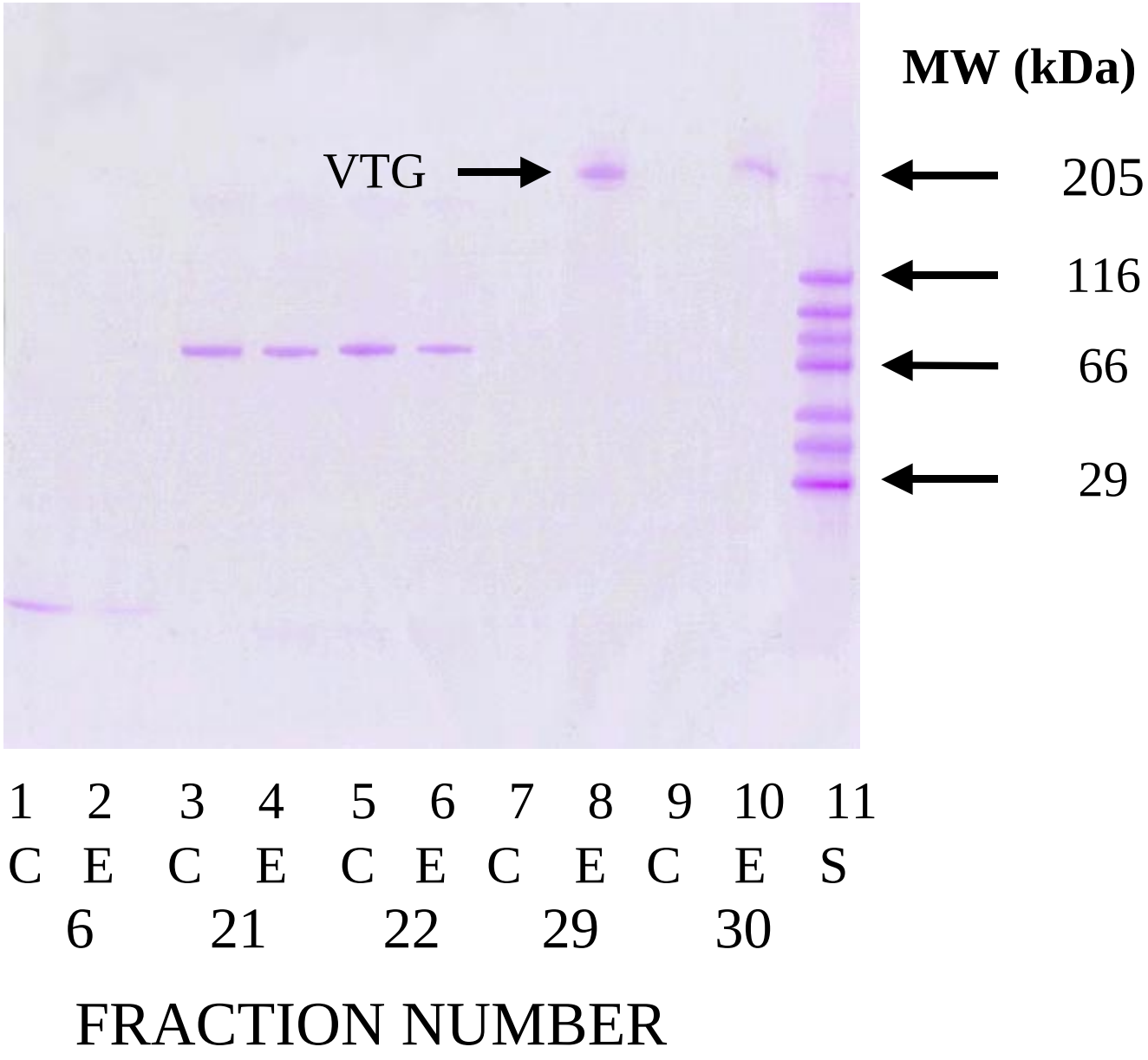


Figure 10: Denaturing polyacrylamide gel (SDS-PAGE) of DEAE fractions from control and estrogen-treated *Ambystoma tigrinum*. Fractions were separated on a 4 to 15% polyacrylamide gradient gel and stained with Coomassie blue. Lane assignments are as follows: 1-9 (odd lanes) = fractions from control animal sera; 2-10 (even lanes) = fractions from estrogen-treated animal sera; 11 = molecular weight standards (S). See methods for treatment regimens. Abbreviations are C = control; E = estrogen treated. Bottom numbers refer to fraction numbers from the DEAE separation.

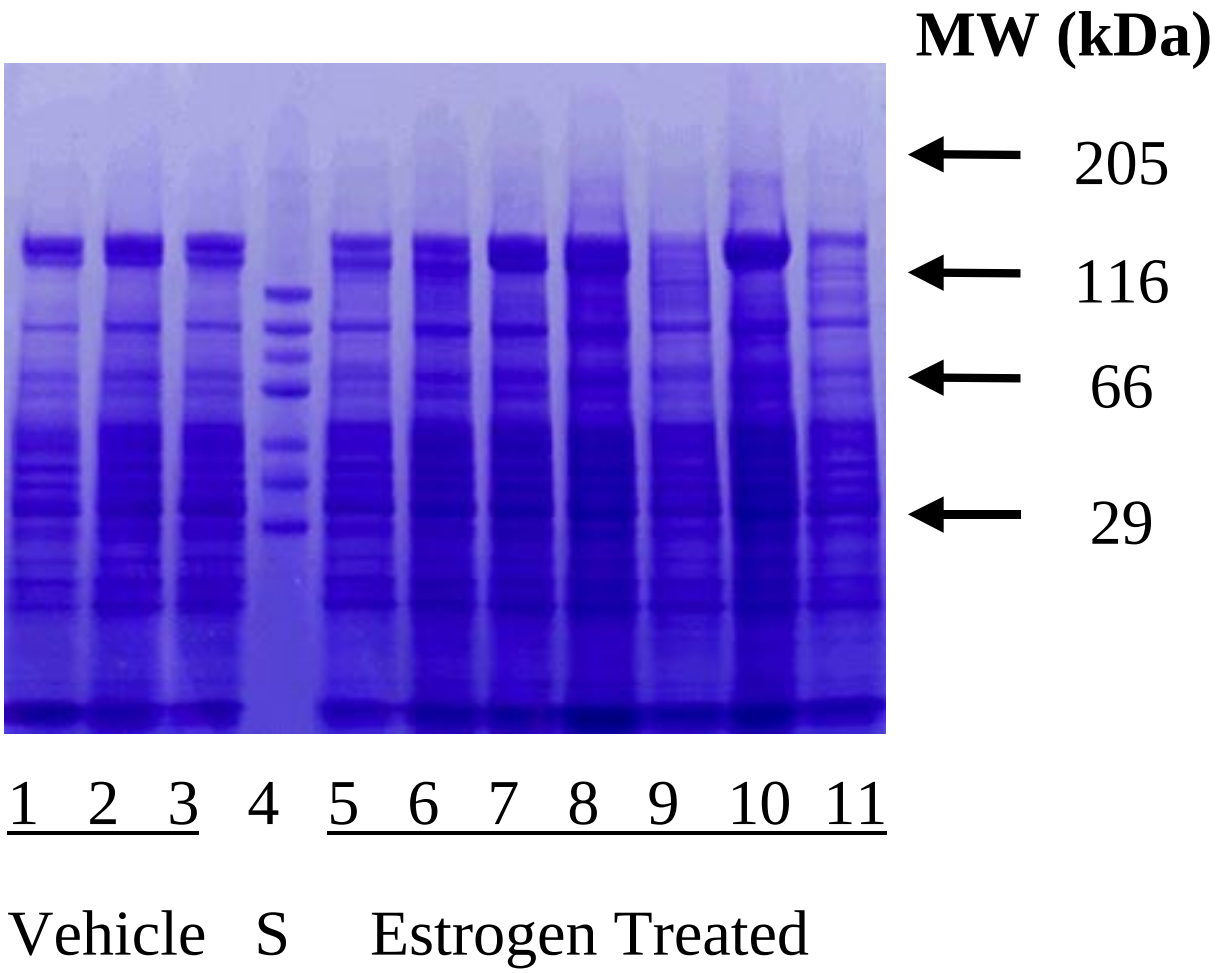


Figure 11: Denaturing polyacrylamide gel (SDS-PAGE) of cytosol from control and estrogen-treated *Ambystoma tigrinum*. Lane assignments are as follows: 1-3 = control; 4 = molecular weight standards (S); 5-11 = estrogen treated. See methods for treatment regimens. Cytosol was separated on a 4 to 15% gradient gel and stained with Coomassie blue.

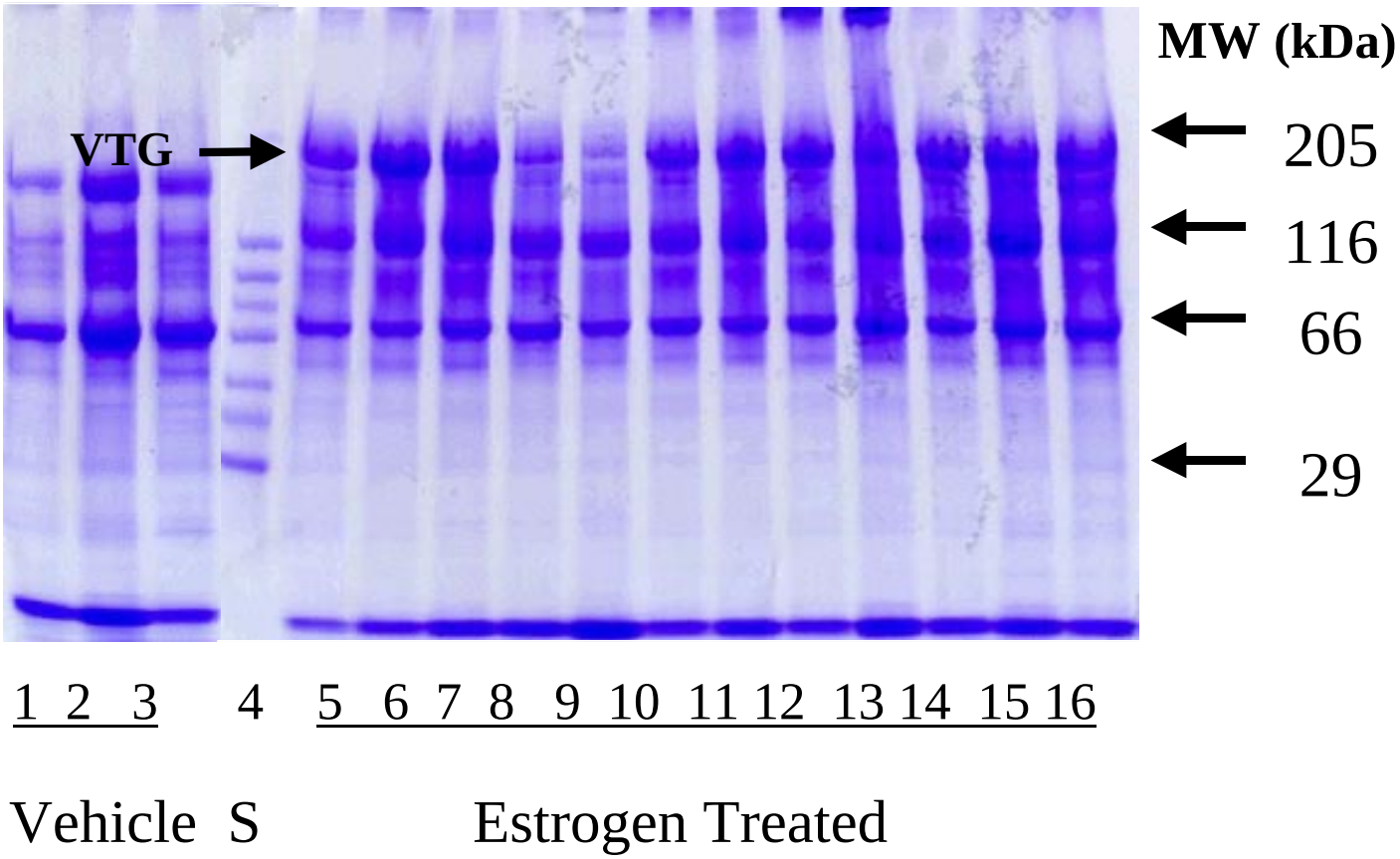


Figure 12: Denaturing polyacrylamide gel (SDS-PAGE) of sera from control and estrogen-treated *Ambystoma tigrinum*. Lane assignments are as follows: 1-3 = vehicle; 4 = molecular weight standards (S); 5-16 = estrogen-treated; VTG = Size of presumptive vitellogenin. See methods for treatment regimens. Sera were separated on a 4 to 15% gradient gel and stained with Coomassie blue.

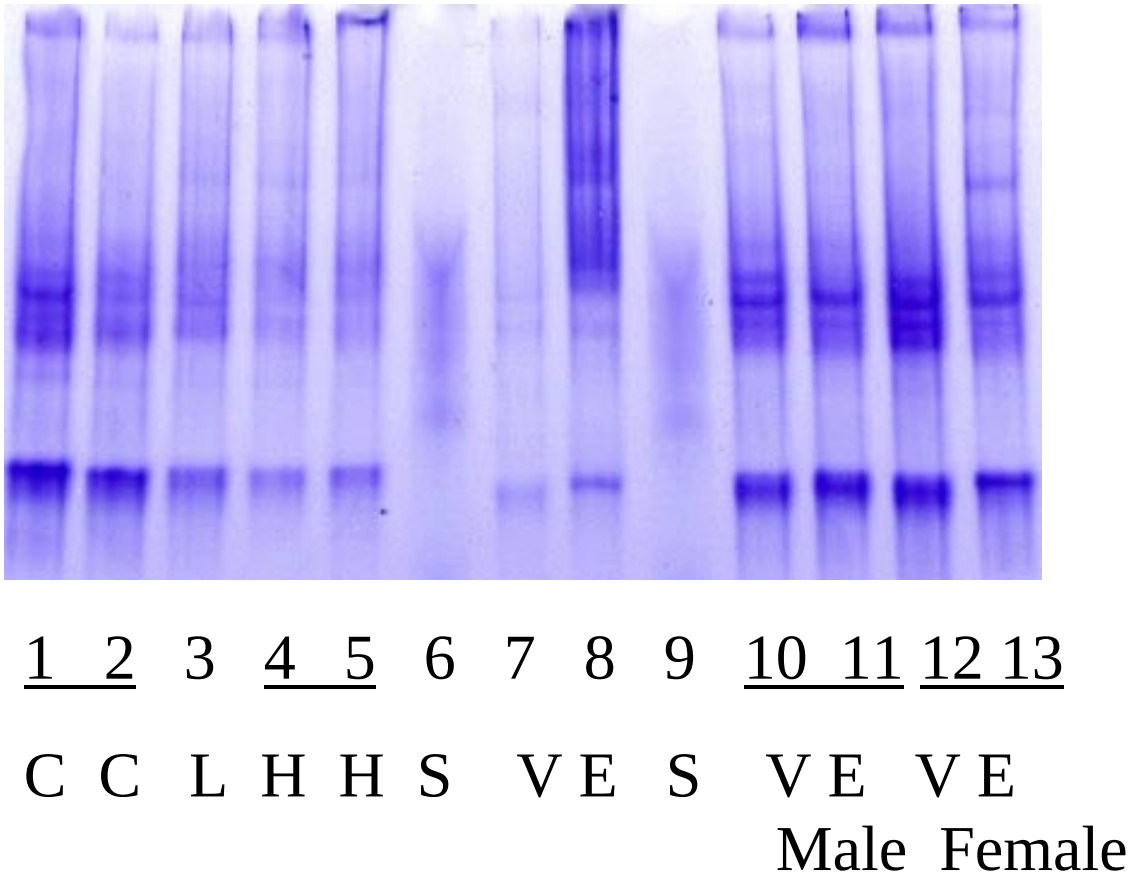


Figure 13: Non-denaturing polyacrylamide gel (PAGE) of sera from control and estrogen-treated *Ambystoma tigrinum*. Lane assignments are as follows: 1-2 = multi-injection control-treated animals (C); 3 = multi-injection low dose estrogen treated (L); 4-5 = multi-injection high dose estrogen treated (H); 6, 9 = molecular weight standards (S); 7 = multi-injection juvenile vehicle (V); 8 = multi-injection juvenile estrogen-treated (E); 10 = single-injection male control animals (V); 11 = single-injection male estrogen animals (E); 12 = single-injection female control animals (V); 13 = single-injection female estrogen-treated (E).

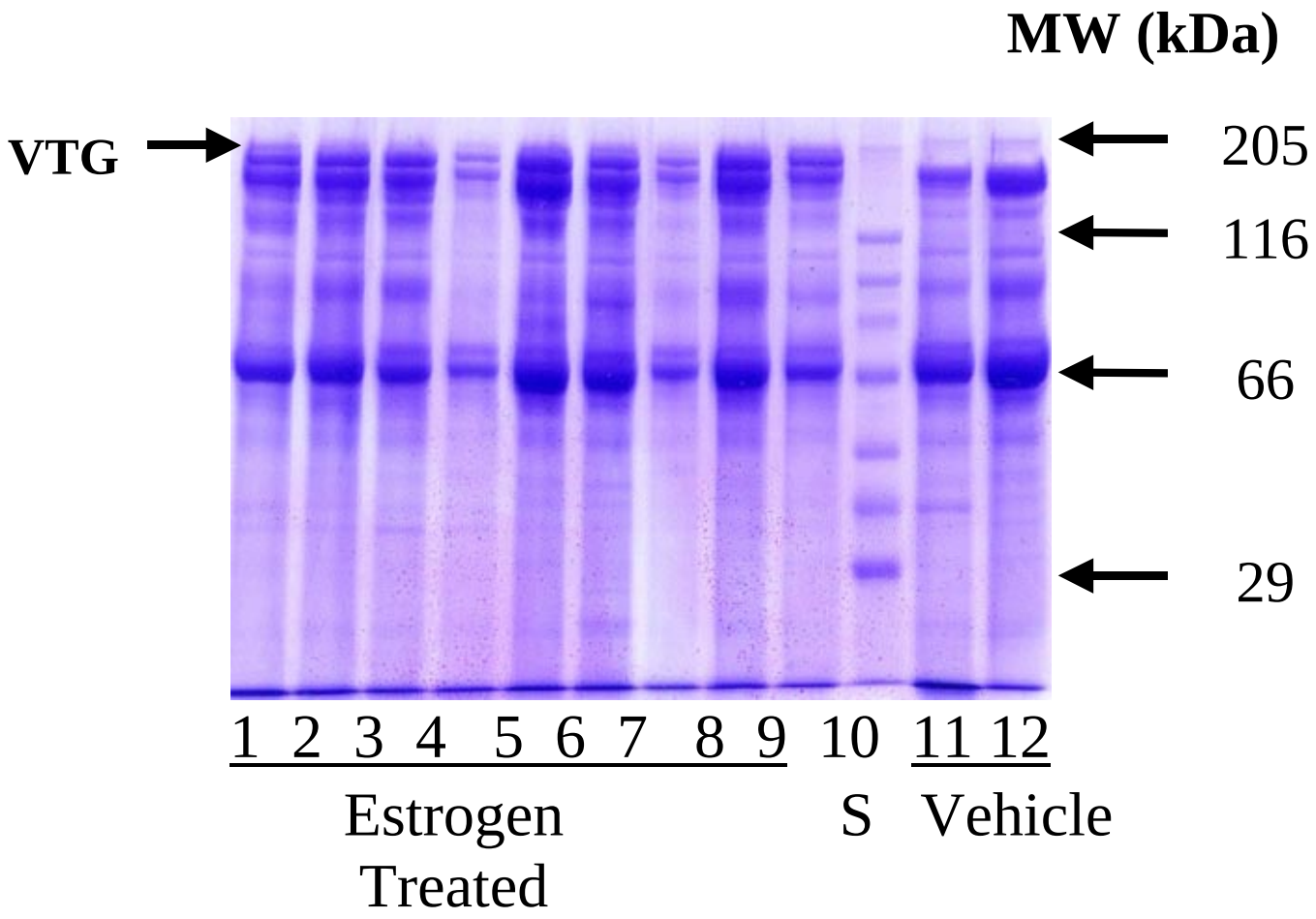


Figure 14: Denaturing polyacrylamide gel (SDS-PAGE) of sera from control and estrogen-treated *Ambystoma maculatum*. Lane assignments are as follows: 1-9 = estrogen-treated; 10 = molecular weight standards (S); 11-12 = vehicles; VTG = Size of presumptive vitellogenin. See methods for treatment regimens. Sera were separated on a 4 to 15% gradient gel and stained with Coomassie blue.

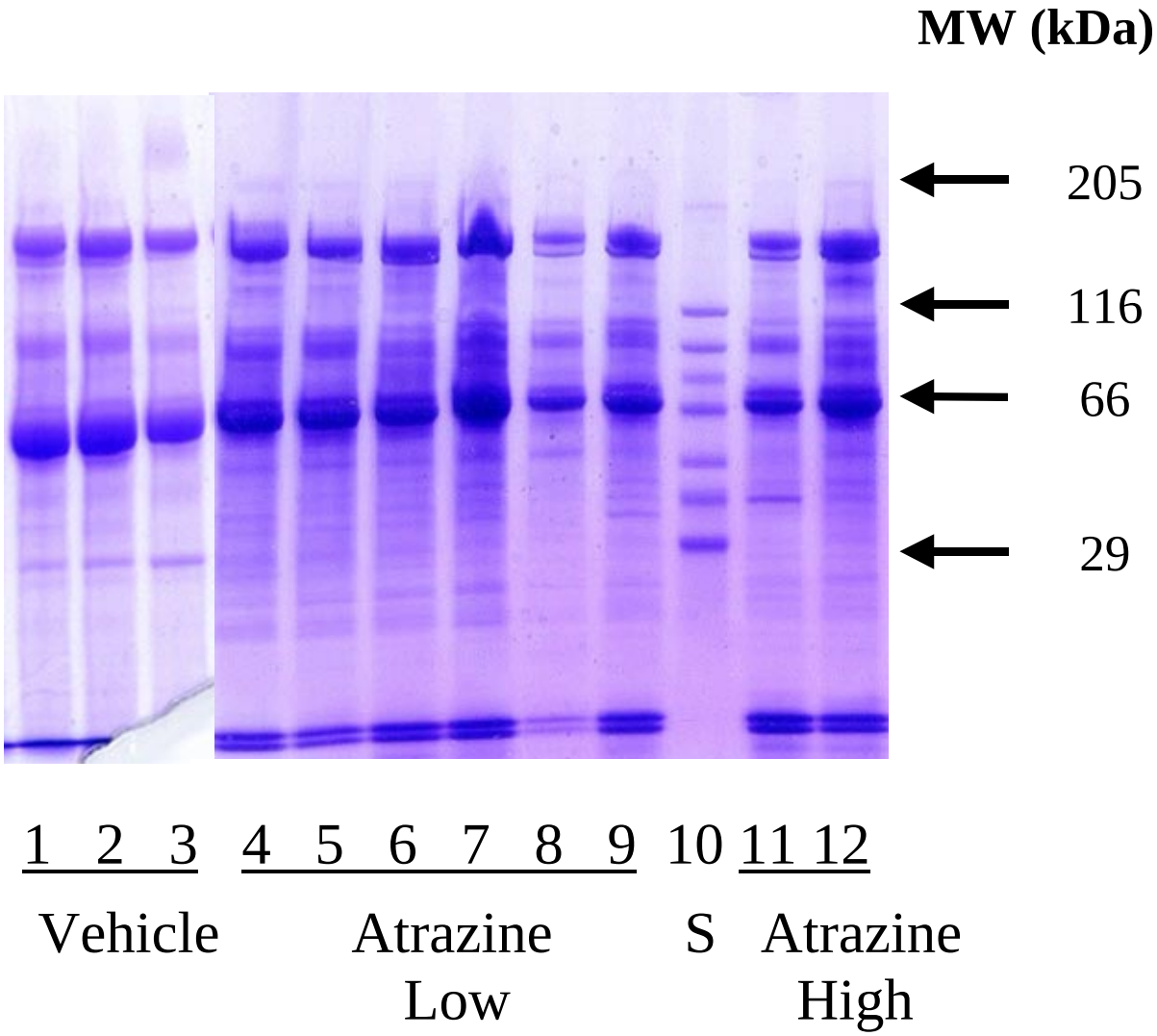


Figure 15: Denaturing polyacrylamide gel (SDS-PAGE) of sera from high and low atrazine-treated *Ambystoma maculatum*. Lane assignments are as follows: 1-3 = vehicles from same experiment; 4-9 = atrazine low dose; 10 = molecular weight standards (S); 11-12 = atrazine high dose. See methods for treatment regimens. Sera were separated on a 4 to 15% gradient gel and stained with Coomassie blue.

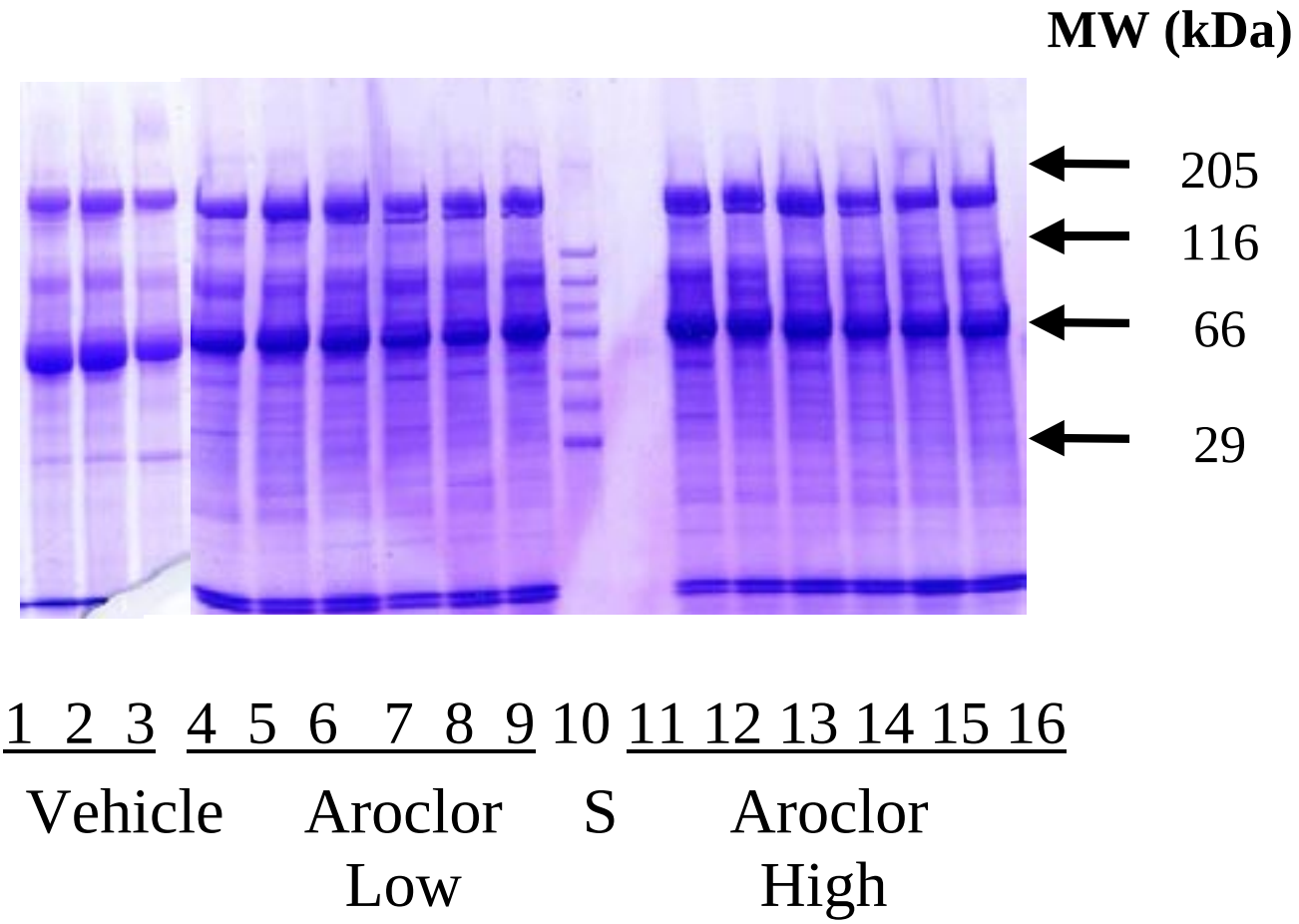


Figure 16: Denaturing polyacrylamide gel (SDS-PAGE) of sera from high and low aroclor-treated *Ambystoma maculatum*. Lane assignments are as follows: 1-3 = vehicles from same experiment; 4-9 = aroclor low dose; 10 = molecular weight standards (S); 11-16 = aroclor high dose. See methods for treatment regimens. Sera were separated on a 4 to 15% gradient gel and stained with Coomassie blue.

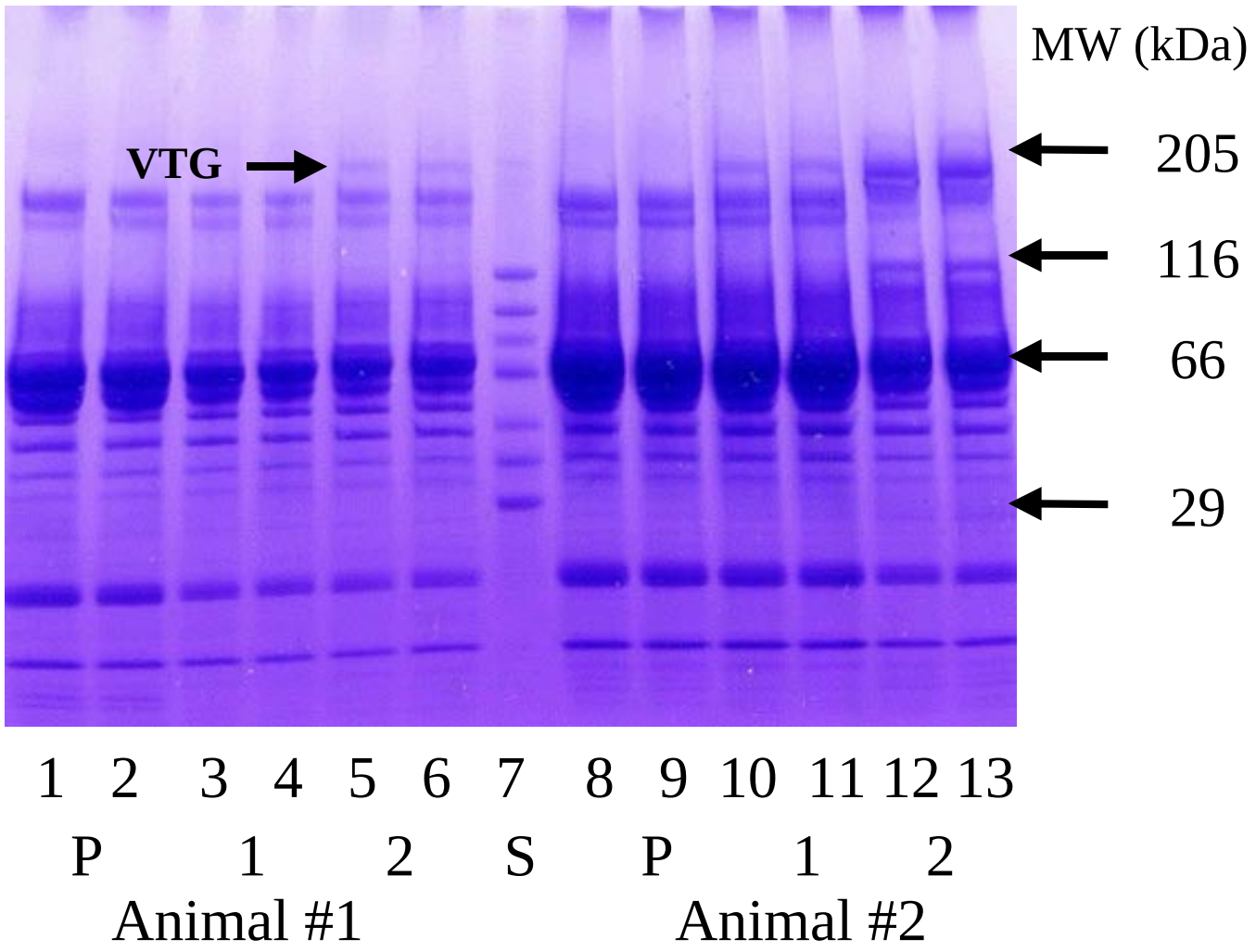


Figure 17: Denaturing polyacrylamide gel (SDS-PAGE) of sera from pre-treated and 17β-estradiol-treated male *Cryptobranchus alleganiensis*. Lane assignments are as follows: 1-2 = animal #1, pre-treatment (P); 3,4 = animal #1, 1st bleed; (1) 5,6 animal #1, 2nd bleed (2); 7 = molecular weight standards (S); 8, 9 = animal #2, pre-treatment (P); 10, 11 = animal #2, 1st bleed (1); 12, 13 animal #2, 2nd bleed (2); VTG = Presumptive vitellogenin. See methods for treatment regimen. Sera were separated on a 4 to 15% gradient gel and stained with Coomassie blue.

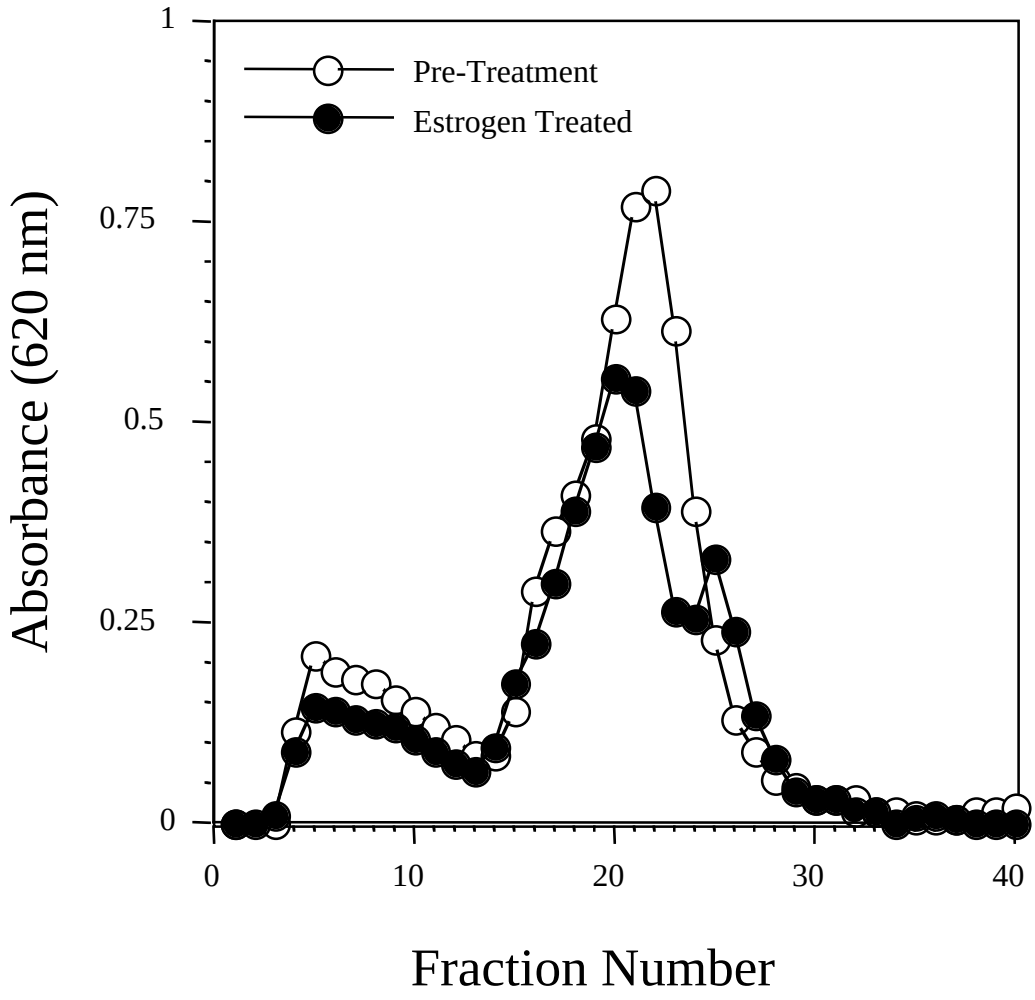


Figure 18: DEAE profile for sera from control and estrogen-treated *Cryptobranchus alleganiensis*. Serum proteins were separated on a DEAE-agarose column using a Bio-Rad Econo Chromatography System. Proteins were subsequently measured by the Coomassie method for each fraction.

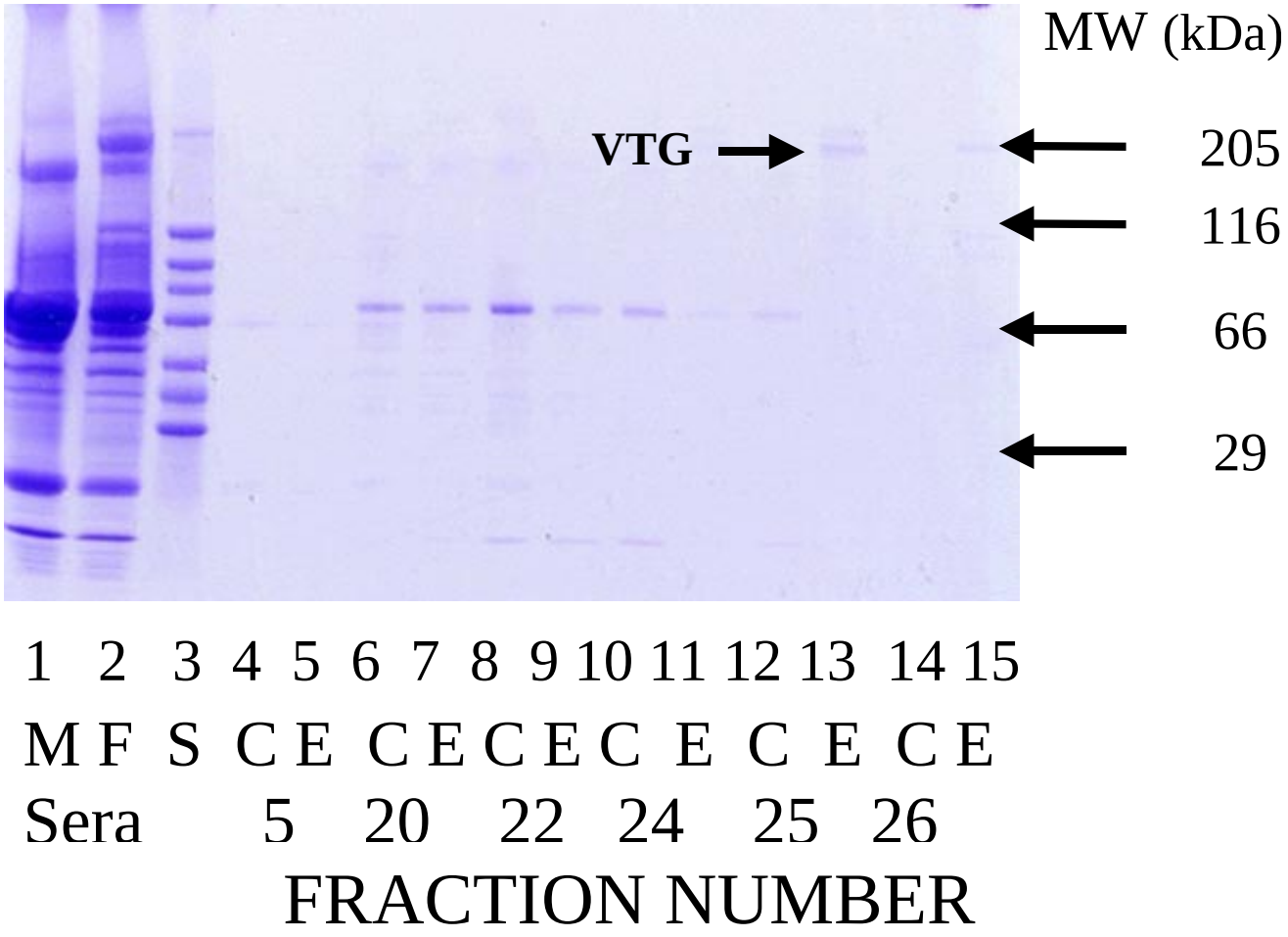


Figure 19: Denaturing polyacrylamide gel (SDS-PAGE) of DEAE fractions from control (C) and estrogen-treated (E) *Cryptobranchus alleganiensis*. Fractions were separated on a 4 to 15% gradient gel and stained with Coomassie blue. Lane assignments are as follows: 1 = Male serum (M); 2 = Female serum (F); 3 = molecular weight standards (S); 4-14 (even lanes) = control treated animals; 5-15 (odd lanes) = estrogen-treated animals. Abbreviations are C=control; E=estrogen treated; VTG = Presumptive vitellogenin. Numbers refer to fraction numbers from the DEAE separation.

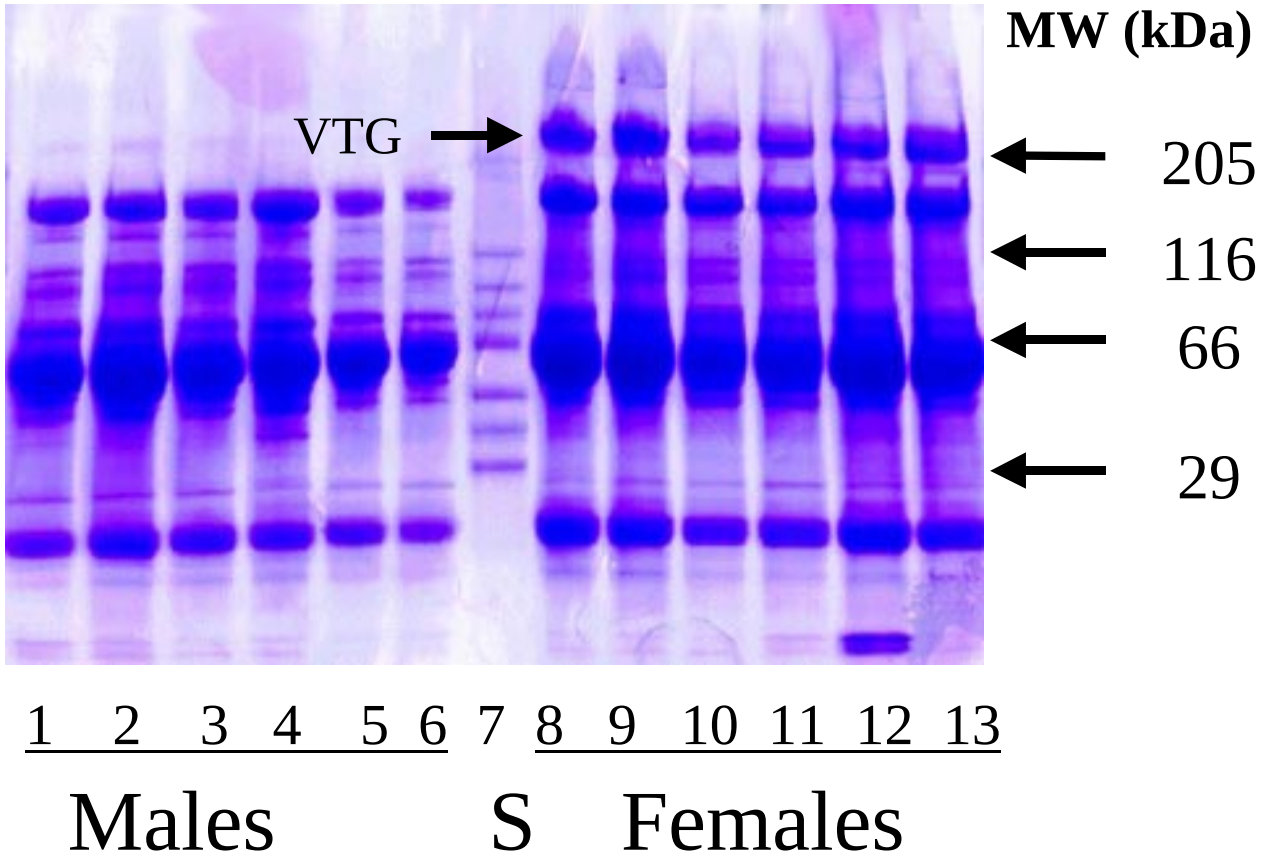


Figure 20: Denaturing polyacrylamide gel (SDS-PAGE) of sera from male and female *Crocodylus moreletii*. Lane assignments are as follows: 1-6 = male wild caught animals; 7 = molecular weight standards (S); 8-13 = female wild caught animals; VTG = Presumptive vitellogenin. See methods for treatment regimens. Sera were separated on a 4 to 15% gradient gel and stained with Coomassie blue.

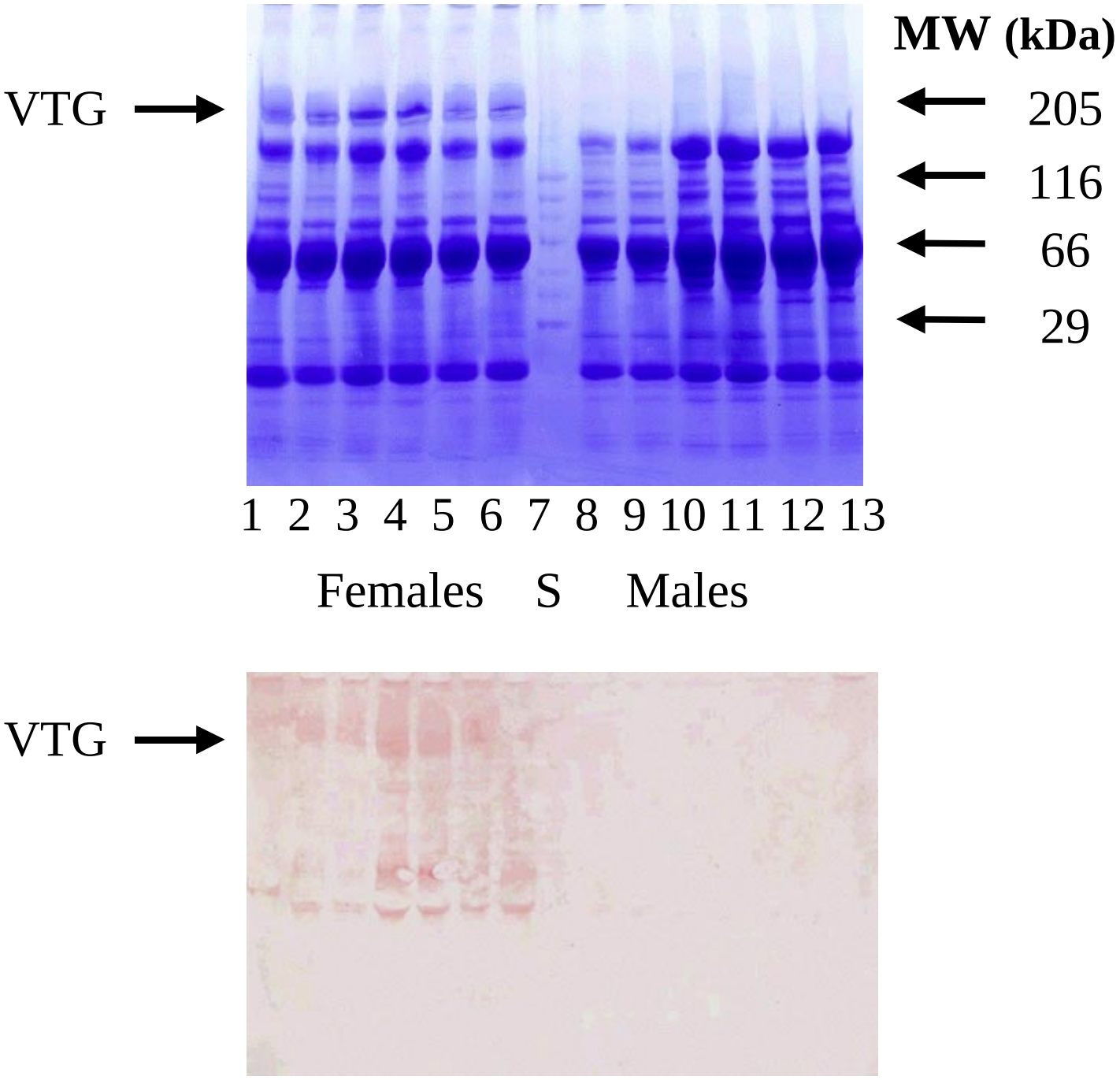


Figure 21: Denaturing polyacrylamide gel (SDS-PAGE) (top panel) and Western blot (bottom panel) of sera from male and female *Crocodylus moreletii*. Lane assignments are as follows: 1-6 = female wild caught animals; 7 = molecular weight standards (S); 8-13 = male wild caught animals; VTG = Presumptive vitellogenin. See methods for treatment regimens. Sera were separated on a 4 to 15% gradient gel and stained with Coomassie blue. For Western blotting, the primary antiserum (polyclonal antiserum #498) was used at a 1:100 dilution and the second antibody was used at a 1:1000 dilution.

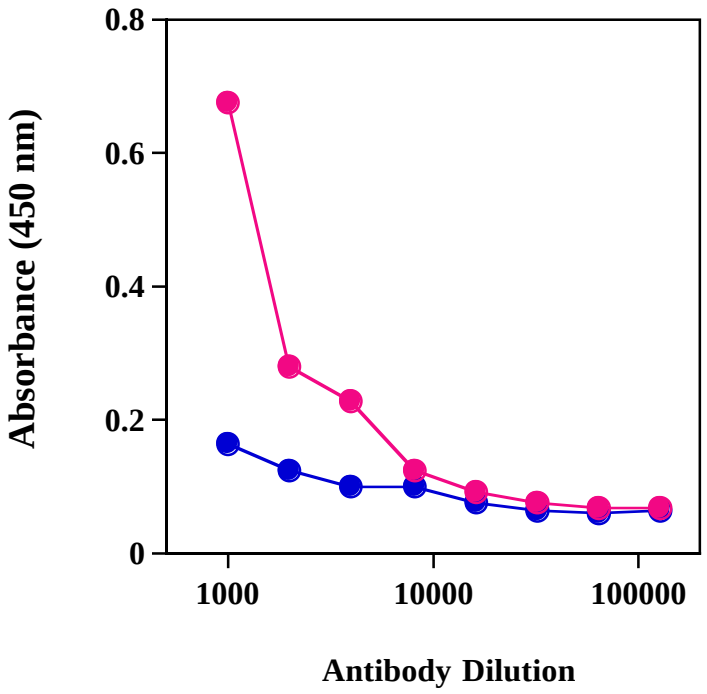


Figure 22: Vitellogenin enzyme linked immunosorbent assay (ELISA) of pooled male (blue circles) and female (pink circles) *Crocodylus moreletii* sera. See methods for treatment regimens. Samples were diluted 1:1000 and the primary antiserum (polyclonal #498) was serially diluted from 1:1000 to 1:128,000, as indicated. The secondary antibody was used at a 1:1000 dilution.

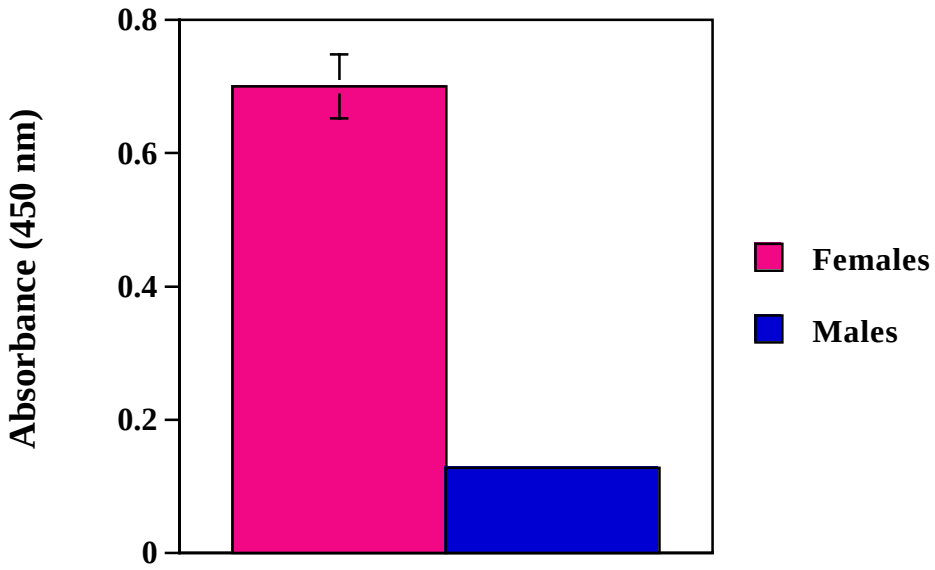


Figure 23: Vitellogenin enzyme linked immunosorbent assay (ELISA) of pooled male and female *Crocodylus moreletii* sera. See methods for treatment regimens. Bars represent the mean of eight samples. Samples were diluted 1:1000 and the primary antiserum (polyclonal #498) was diluted 1:1000. The secondary antibody was used at a 1:1000 dilution.

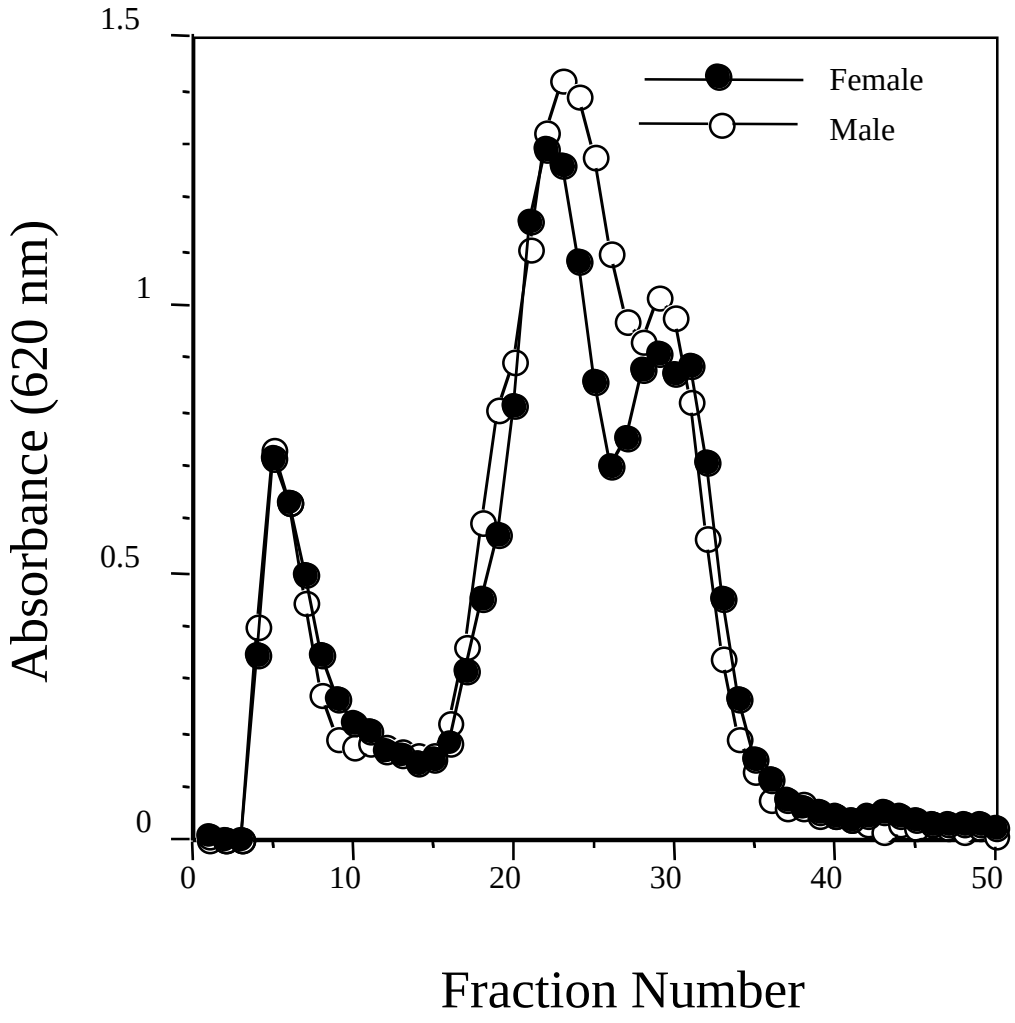


Figure 24: DEAE profile for sera from male and female *Crocodylus moreletii*. Serum proteins were separated on a DEAE-agarose column using a Bio-Rad Econo Chromatography System. Proteins were subsequently measured by the Coomassie method for each fraction.

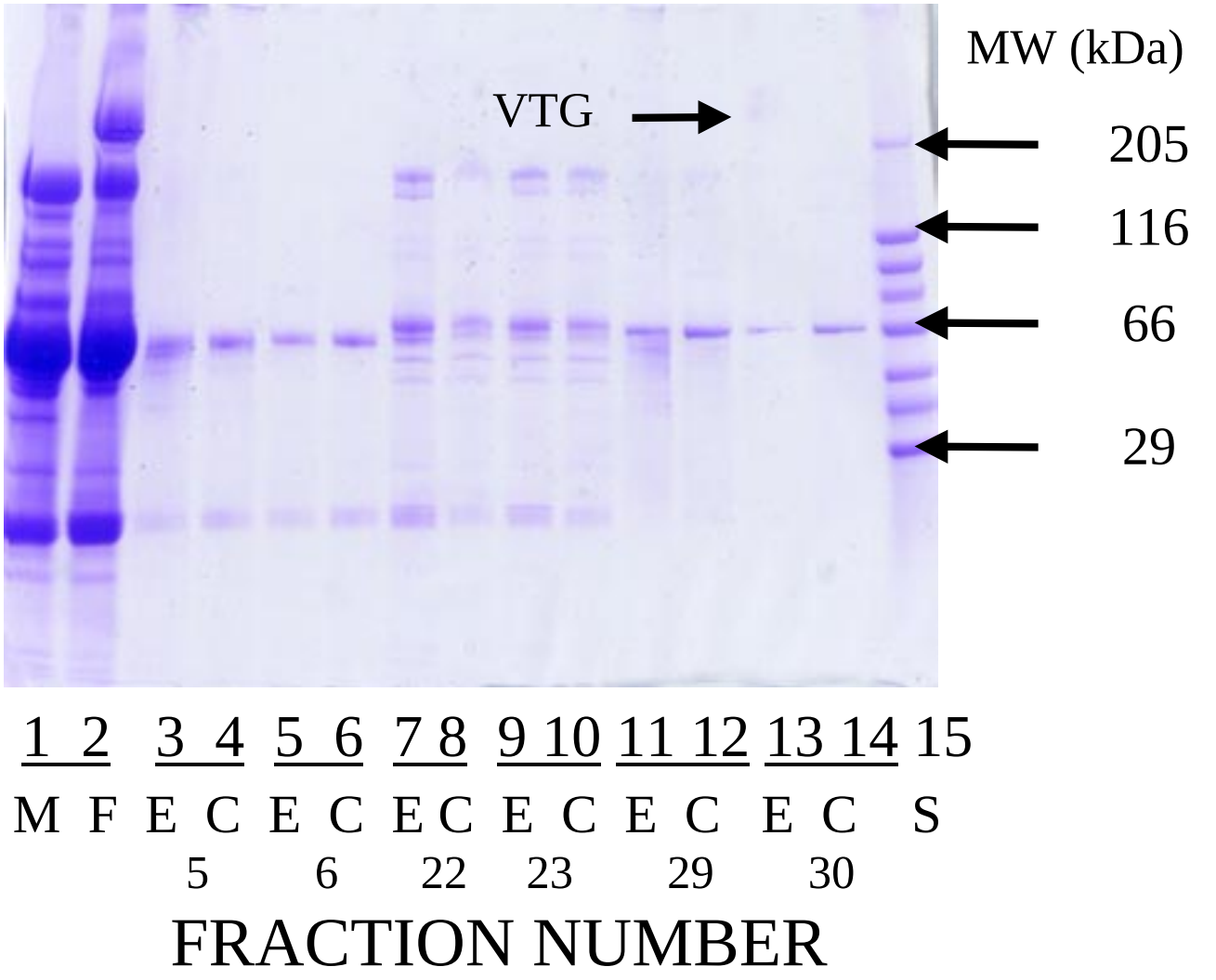


Figure 25: Denaturing polyacrylamide gel (SDS-PAGE) of DEAE fractions from male and female *Crocodylus moreletii*. Lane assignments are as follows: 1 = Male serum; 2 = Female serum; 3-13 (odd lanes) = estrogen-treated animals (E); 4-14 (even lanes) = control treated animals (C); 15 = molecular weight standards (S). Abbreviations are M=male; F=female treated; VTG = Presumptive vitellogenin. Numbers refer to fraction numbers from the DEAE separation. Fractions were separated on a 4 to 15% gradient gel and stained with Coomassie blue.

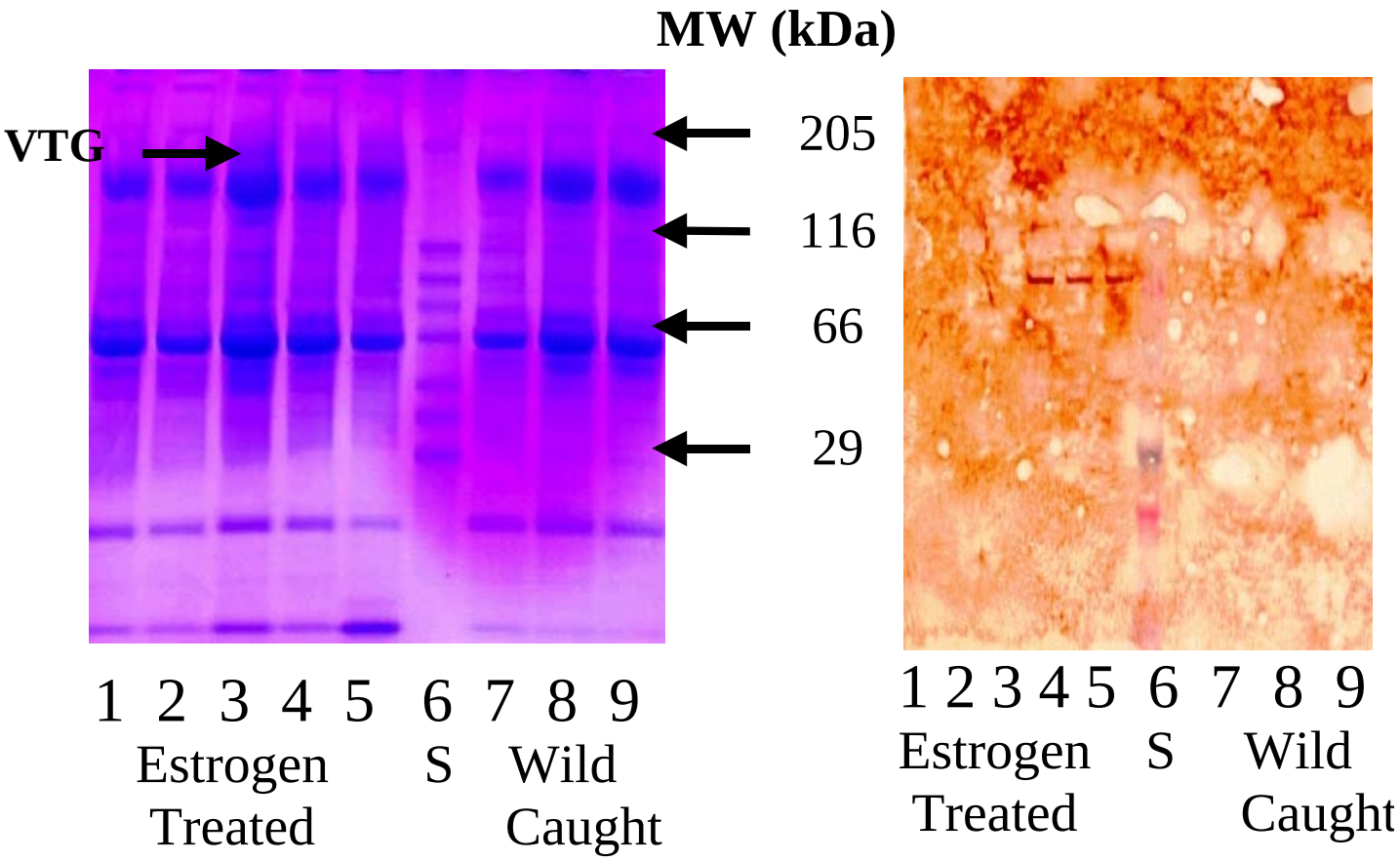


Figure 26: Denaturing polyacrylamide gel (SDS-PAGE) (left) and Western blot (right) of sera from estrogen-treated and wild caught *Rana aurora*. Lane assignments are as follows: 1-5 = estrogen-treated animals; 6 = molecular weight standards (S); 7-9 = wild caught animals; VTG = Presumptive vitellogenin. See methods for treatment regimens. Sera were separated on a 4 to 15% gradient gel and stained with Coomassie blue. For Western blotting, the primary antiserum (polyclonal antiserum #498) was used at a 1:100 dilution and the second antibody was used at a 1:1000 dilution.

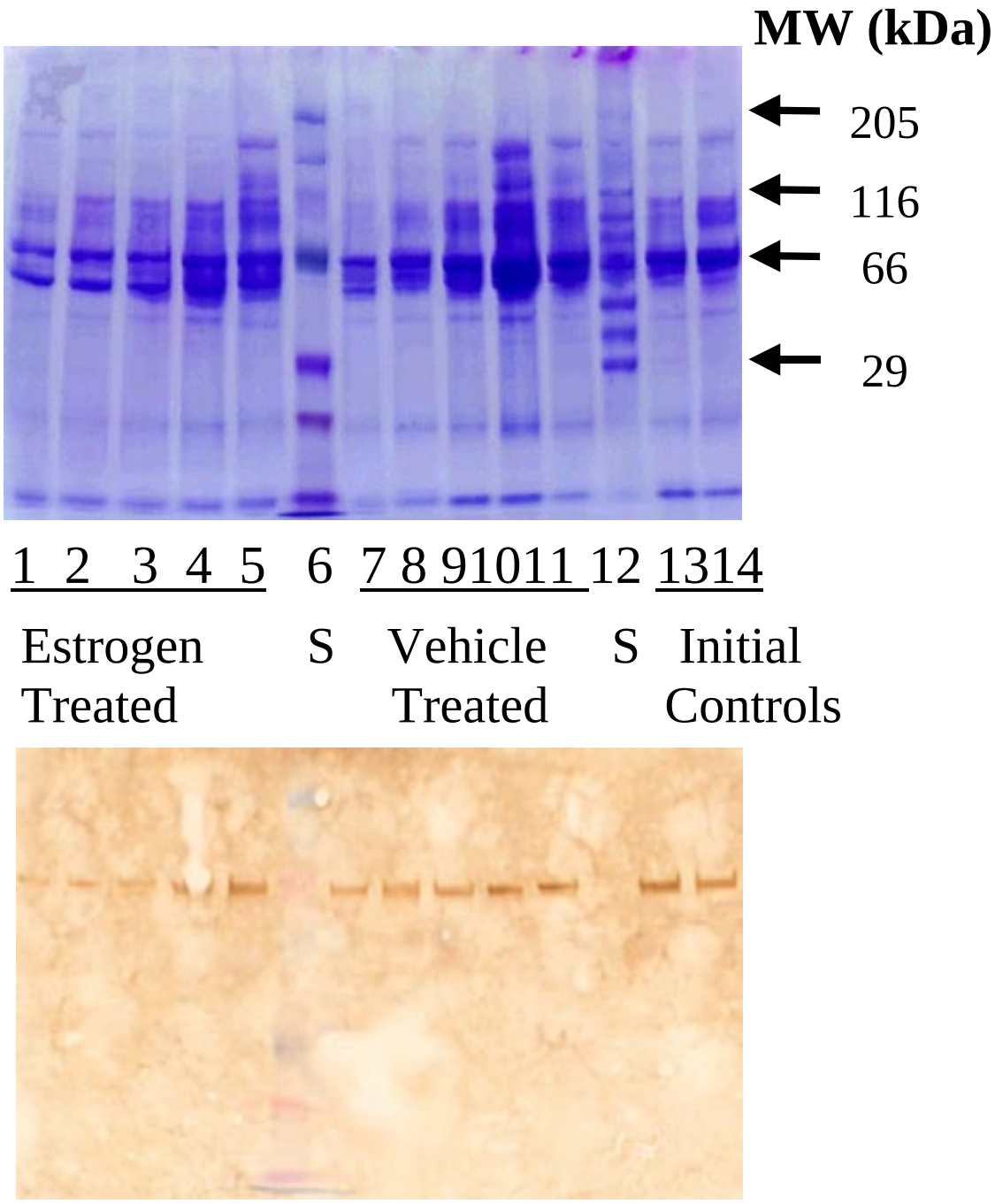


Figure 27: Denaturing polyacrylamide gel (SDS-PAGE) (top panel) and Western blot (bottom panel) of sera from control and estrogen-treated *Ambystoma tigrinum*. Lane assignments are as follows: 1-5 = estrogen-treated animals; 6, 12 = molecular weight standards (S); 7-11 = vehicle treated animals; 13-14 = initial controls. See methods for treatment regimens. Sera were separated on a 4 to 15% gradient gel and stained with Coomassie blue. For Western blotting, the primary antiserum (polyclonal antiserum #498) was used at a 1:100 dilution and the second antibody was used at a 1:1000 dilution.

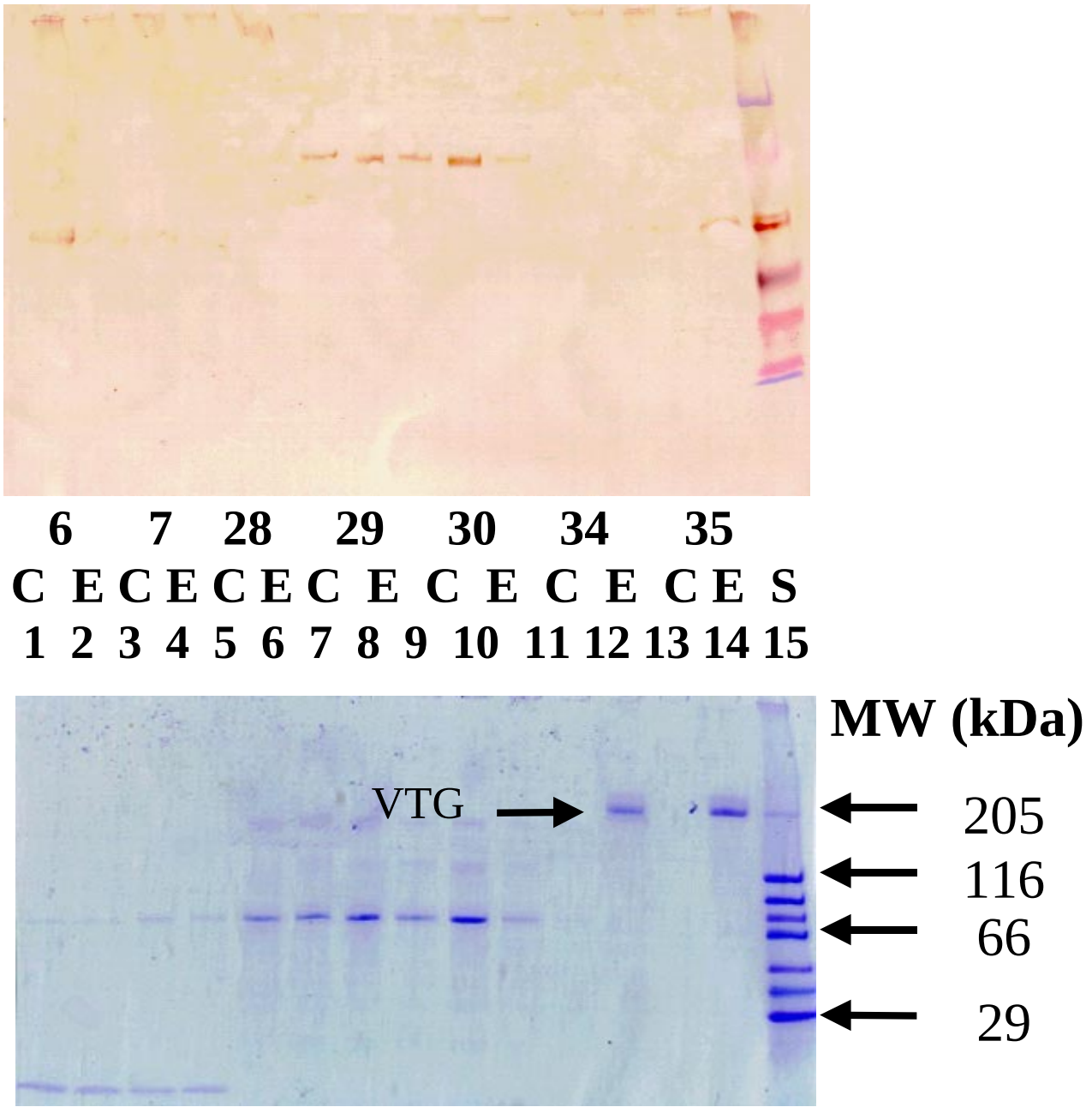


Figure 28: Denaturing polyacrylamide gel (SDS-PAGE) (bottom panel) and Western Blot (top panel) of DEAE fractions from male and female *Ambystoma tigrinum*. Lane assignments are as follows: 1-13 (odd lanes) = control treated animals (C); 2-14 (even lanes) = estrogen-treated animals (E); 15 = molecular weight standards (S). Abbreviations are VTG = Presumptive vitellogenin. Numbers refer to fraction numbers from the DEAE separation. Fractions were separated on a 4 to 15% gradient gel and stained with Coomassie blue. For Western blotting, the primary antiserum (polyclonal antiserum #498) was used at a 1:100 dilution and the second antibody was used at a 1:1000 dilution.

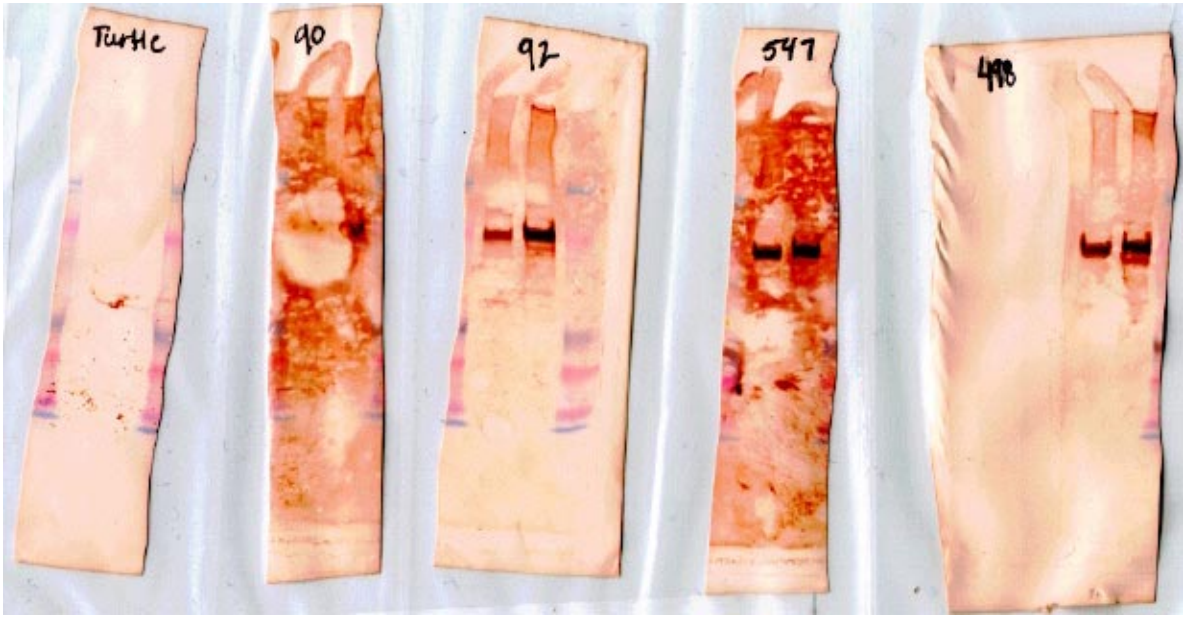
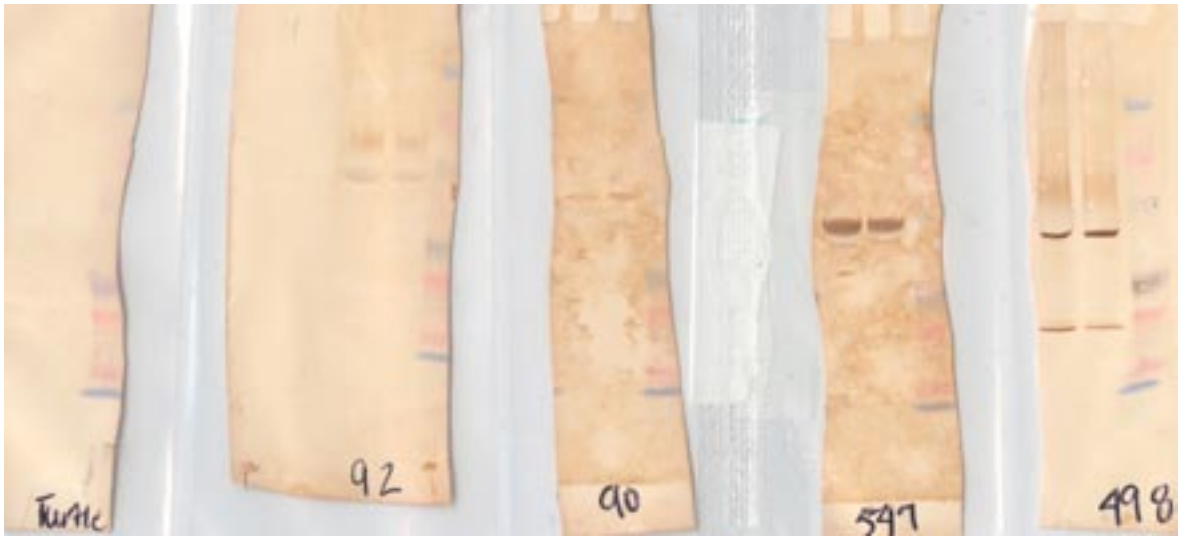


Figure 29: Denaturing polyacrylamide gel (SDS-PAGE) (not shown) and Western blots of sera from control and estrogen-treated *Ambystoma tigrinum*. Lane assignments are as follows: 1, 4, 7, 10, 13 = estrogen-treated animals (E); 2, 5, 8, 11, 14 = control animals (C); 3, 6, 9, 12, 15 = molecular weight standards (S). See methods for treatment regimens. Sera were separated on a 4 to 15% gradient gel. For Western blotting, the primary antiserum (noted above the lanes) were used at a 1:1000 dilution and the second antibody was used at a 1:1000 dilution.



1 2 3 **4 5 6** **7 8 9** **10 11 12** **13 14 15**

P 2 S P 2 S P 2 S P 2 S P 2 S

Figure 30: Denaturing polyacrylamide gel (SDS-PAGE) (not shown) and Western blots of sera from control and estrogen-treated *Cryptobranchus alleganiensis*. Lane assignments are as follows: 1, 4, 7, 10, 13 = pre-treated animals (P); 2, 5, 8, 11, 14 = estrogen-treated animals (2); 3, 6, 9, 12, 15 = molecular weight standards (S). See methods for treatment regimens. Sera were separated on a 4 to 15% gradient gel. For Western blotting, the primary antiserum (noted above the lanes) were used at a 1:1000 dilution and the second antibody was used at a 1:1000 dilution.

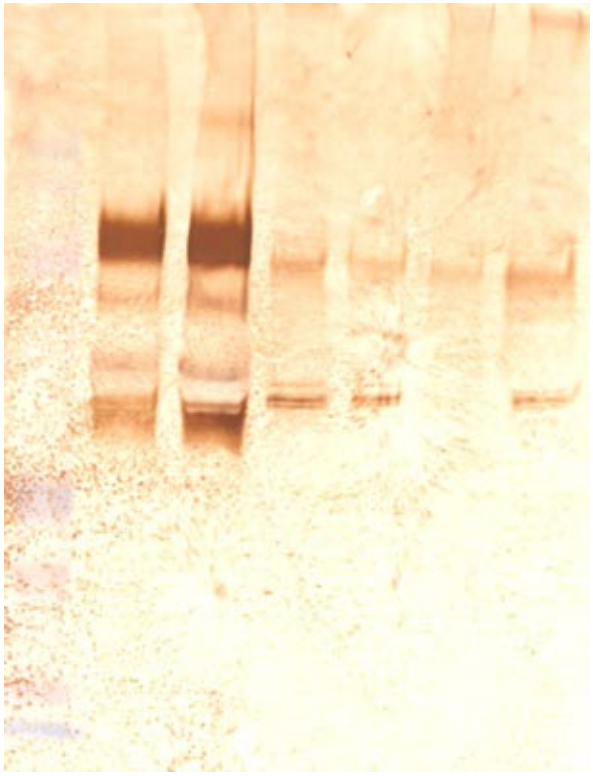


Figure 31: Denaturing polyacrylamide gel (SDS-PAGE) (not shown) and Western blots of sera from control, estrogen-treated *Ambystoma tigrinum, and Cryptobranchus alleganiensis*. Lane assignments are as follows: 1 = molecular weight standards (S); 2 estrogen-treated *Cryptobranchus alleganiensis* (E); 3 = pre-treatment *Cryptobranchus alleganiensis* (P); 4, 6 = estrogen-treated *Ambystoma tigrinum* (E); 5, 7 = vehicle treated *Ambystoma tigrinum* (C). See methods for treatment regimens. Sera were separated on a 4 to 15% gradient gel. For Western blotting, the primary antiserum (rabbit-anti-chicken-anti-albumin) was used at a 1:1000 dilution and the second antibody was used at a 1:1000 dilution.

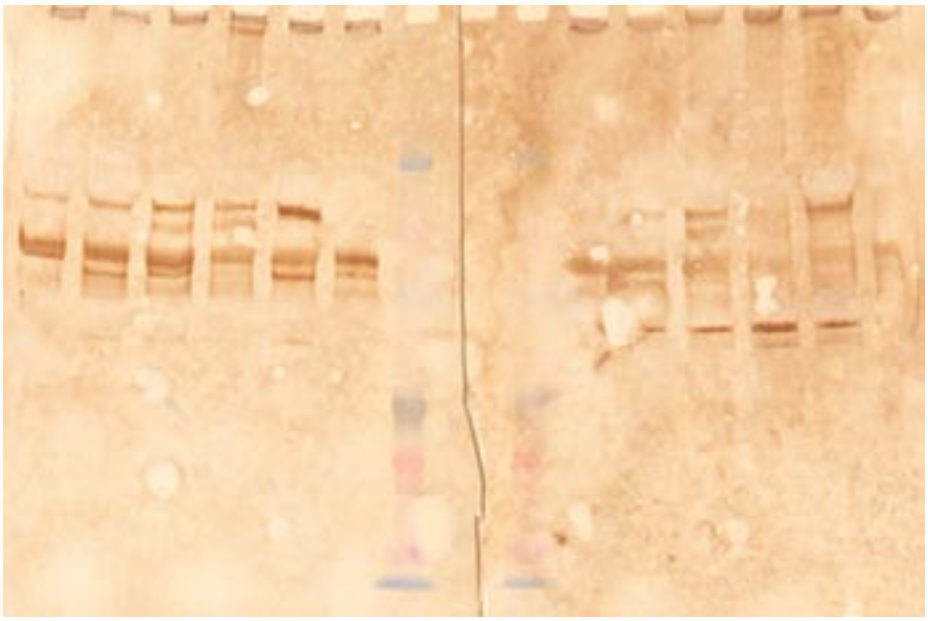


Figure 32: Denaturing polyacrylamide gel (SDS-PAGE) (not shown) and Western blots of sera from control, estrogen- and xenobiotic-treated *Ambystoma tigrinum*. Lane assignments are as follows: 1-2, 9-10 = estrogen-treated animals (E); 3-4, 11-12 = vehicle treated animals (V); 5,13 = atrazine-treated animals (A); 6, 14 = aroclor-treated animals (P); 7-8 = molecular weight standards (S). See methods for treatment regimens. Sera were separated on a 4 to 15% gradient gel. For Western blotting, the primary antiserum (left side of gel is #371; right side of the gel was #358) was used at a 1:1000 dilution and the second antibody was used at a 1:1000 dilution.

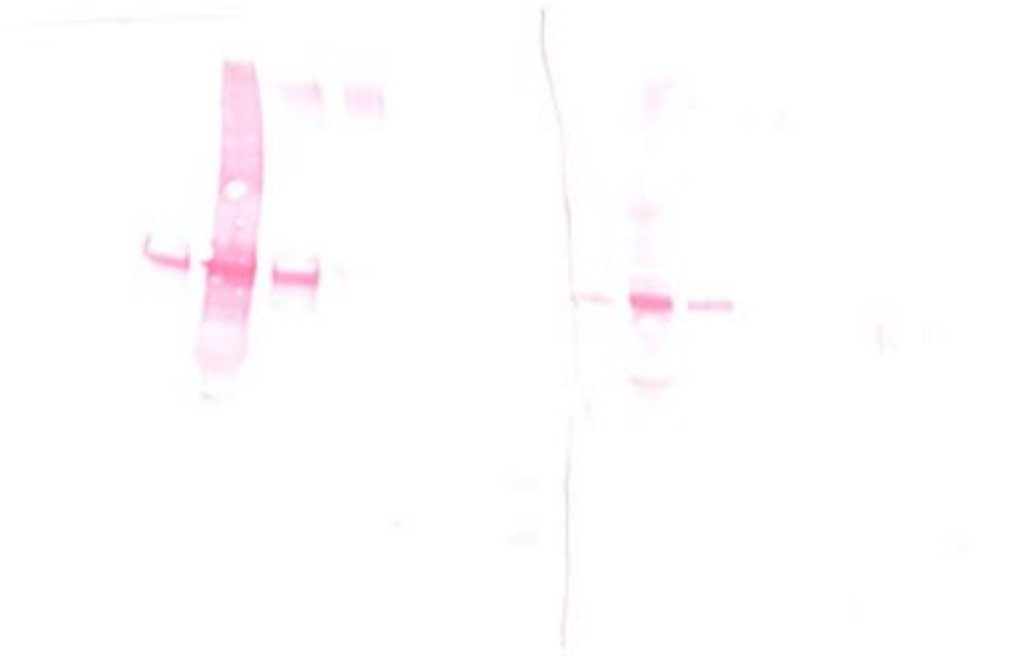


Figure 33: Denaturing polyacrylamide gel (SDS-PAGE) (not shown) and Western blots of sera from control, estrogen-treated *Ambystoma tigrinum, and Cryptobranchus alleganiensis*. Lane assignments are as follows: 1, 3, 9, 11 = vehicle treated *Ambystoma tigrinum* (C); 2, 4, 10, 12 = estrogen-treated *Ambystoma tigrinum* (E); 5, 12 = pre-treatment *Cryptobranchus alleganiensis* (P); 6, 13 estrogen-treated *Cryptobranchus alleganiensis* (E); 7, 14 = atrazine-treated animals (A); 6, 14 = aroclor-treated animals (P); 7-8 = molecular weight standards (S). See methods for treatment regimens. Sera were separated on a 4 to 15% gradient gel. For Western blotting, the primary antiserum (left side of gel is #130; right side of the gel was #129) was used at a 1:1000 dilution and the second antibody was used at a 1:1000 dilution.

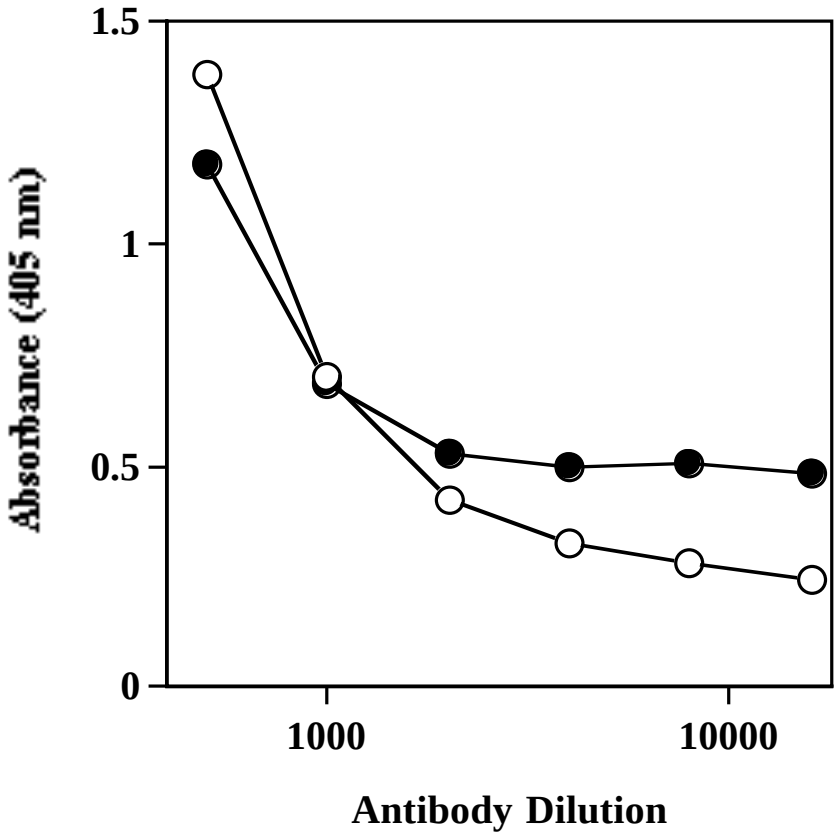


Figure 34: Vitellogenin enzyme linked immunosorbent assay (ELISA) of estrogen-treated (black circles) and control (white circles) *Ambystoma tigrinum* juvenile sera. See methods for treatment regimens. Samples were diluted 1:3000 and the primary antiserum (#129) was serially diluted from 1:500 to 1:16,000, as indicated. The secondary antibody was used at a 1:1000 dilution.

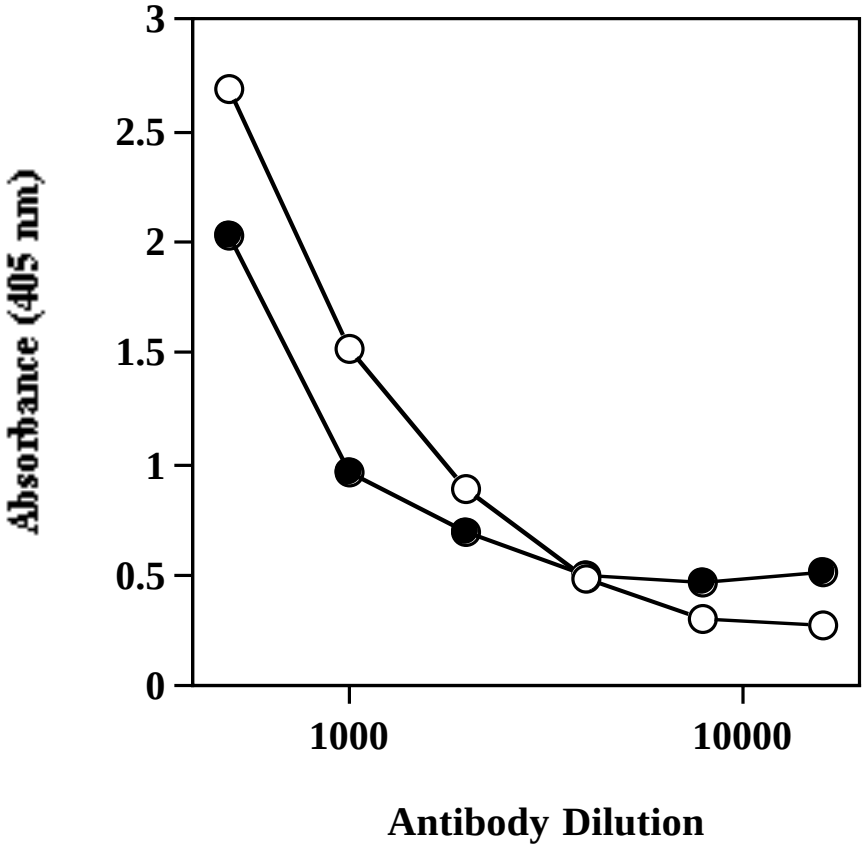


Figure 35: Vitellogenin enzyme linked immunosorbent assay (ELISA) of estrogen-treated (black circles) and control (white circles) *Ambystoma tigrinum* juvenile sera. See methods for treatment regimens. Samples were diluted 1:3000 and the primary antiserum (#130) was serially diluted from 1:500 to 1:16,000, as indicated. The secondary antibody was used at a 1:1000 dilution.

Discussion

A substantial number of chemicals in the environment possess estrogenic activity (McLachlan, 1993). These chemicals include a wide range of natural and manufactured compounds, such as plant and fungal products, plasticizers, industrial chemicals, and pesticides (Danzo, 1998). Most of these chemicals eventually enter the water, making aquatic organisms more susceptible to their effects. Many wildlife species, in particular amphibians, are experiencing population declines. Endocrine disruption is suspected to play a role in this, although there is insufficient data to prove this assumption (Carey and Bryant, 1995). Amphibians are ideal biological indicators, because their semi-permeable epidermis and complex life cycle expose them to multiple stressors in both aquatic and terrestrial environments (Wyman, 1990). A reliable method of detecting and classifying xenobiotic estrogens in wildlife species is needed due to the major concern that these estrogenic chemicals persisting in the environment may be adversely affecting the health of wildlife and humans (Blaustein and Wake, 1990; Raloff, 1994; Wake, 1991; Carey and Bryant, 1995; Colborn et al., 1993). Vitellogenin induction has shown promise as a sensitive and useful biomarker of wildlife exposure to xenobiotic estrogens, predominantly in aquatic systems (Palmer and Palmer, 1995; Sumpter and Jobling, 1995; Folmar et al., 1996; Purdom et al., 1994). There have been no previous studies using salamanders as a model species to test for the presence of endocrine disruption, or to assess estrogenicity using vitellogenin as a biomarker. Therefore, in the present study, vitellogenin was used as a biomarker for environmental estrogens in the amphibian model *Ambystoma tigrinum*.

The first objective of this research was to characterize the changes in serum proteins associated with estrogen exposure. The second objective was to develop immunoassays for detecting changes in vitellogenin and perhaps other proteins for the future use in studies of endocrine disruption. The third objective was to employ these assays to evaluate responses to specific endocrine-disrupting chemicals.

The specific type of *Ambystoma tigrinum* that we wished to use in this study were adult male tiger salamanders. We chose males because we wanted to evaluate the effects of being exposed to estrogen without having an endogenous supply, as would be the case in females. We did use females, though, to obtain positive controls. However, adult tiger salamanders were not always available, so when they were unavailable, we used juveniles, which also lack endogenous estrogens. The only problem with using juveniles was that we were unable to determine their gender. Another problem was availability of the salamander species of choice. We intended to use *Ambystoma tigrinum* consistently throughout all studies. However, there were long periods of time where *Ambystoma tigrinum* was unavailable, so in order to proceed, we were forced to use *Ambystoma maculatum*, the spotted salamander.

The method of delivering the estrogen was also changed during the project. We first dissolved the steroid into corn oil. However, after the first experiment was unsuccessful and doing further literature searches, it seemed that the most current method of delivering the estrogen was by dissolving it into propylene glycol. The type of estrogen also varied. We began to use the natural salamander estrogen, 17ß-estradiol. However again, after the first experiment was unsuccessful and doing further literature searches it was noted that most studies were using ethinyl-estradiol, the active ingredient in birth control pills. We realized that this was the most relevant option, since women using birth control pills in increasing numbers places an increased amount of estrogen and their breakdown products through sewage treatment plants and into the waterways. This exposes wildlife because these waters serve as nurseries for offspring and breeding ponds for mature animals. Therefore, we switched to using ethinyl-estradiol for the further studies.

Although our studies of vitellogenin induction in salamanders were begun by injection, there have been no studies for vitellogenin induction by immersion in salamanders. While injection and oral dosing are common used methods for delivery of experimental compounds for studies of toxicity, immersion is a more appropriate for testing the effects of waterborne contaminants. Although I did wish to evaluate

vitellogenin induction in salamanders via immersion, the unavailability of aquatic salamanders prohibited this type of exposure.

Salamander Experiments

While previous work in our lab used DES as the stimulant for vitellogenin induction, we chose to first use 17ß-estradiol instead. Interestingly, there was no evident induction of vitellogenin in the male salamanders after four weeks. SDS-PAGE analysis of sera and cytosol *Ambystoma tigrinum* samples revealed no presence of vitellogenin. This was perplexing because most studies have shown vitellogenin induction after estrogen injections. One explanation is that the salamanders that we received were neotenic salamanders. The supplier advised us to keep them in the cold, 3° C, in order to keep them alive. While they lived well for a month, their metabolism was certainly slowed down, and they would not eat. They may not have reacted to estrogen treatments because they could not respond to the injections or because the estrogen could not come out of the oil at that temperature. It has been shown in turtles and fish that steroid receptors do not bind hormones in the cold as efficiently as they do in warmer temperatures (Licht et al., 1990; Tsai et al., 2003). There is a blockage of the potassium channels, which does not allow for proper hormone-receptor binding (Licht et al., 1990). A very recent study also found that an exposure at a lower temperature than room temperature (20° C) resulted in a significant decrease in the expression of estrogen receptor alpha while estrogen receptor beta was unaffected by temperature (Tsai et al., 2003).

Discontentedly to many women beginning birth control, estrogen has been noted to induce weight gain, as an example, DES was administered to livestock in order for them to gain weight (Palmer and Selcer, 1996). On the contrary, endocrine disruptors have been shown to have the opposite effect; they have been shown to induce weight loss in exposed animals, as in the case of atrazine exposure to tiger salamanders (Larson et al., 1998). Salamanders in the current study were expected to have a difference in body weight from the controls when exposed to estrogen treatments. In the first experiment, estrogen did

significantly decrease the body weight of salamanders while at the same time significantly increasing their total serum protein concentration. However, further salamander experiments did not demonstrate this same decrease in body weight and were inconclusive as to whether estrogen treatment had a significant effect on body weight. Further experiments are necessary to determine if there is a linkage between estrogen exposure and a reduction in body weight.

For the next experiment, the estrogen was changed to ethinyl-estradiol, a component of the female birth control pill. The salamanders that we used were fully transformed adult male, female, and juvenile tiger salamanders. Being fully transformed, they were not kept in the cold, but were incabuted at room temperature. These experiments did indeed show that vitellogenin was inducible in both male and female salamanders when exposed to estrogen, and revealed that both adults and juveniles could serve as appropriate models for further experiments on vitellogenin induction by environmental estrogens. SDS-PAGE clearly showed a band in the 200kDa range in the female and estrogen-treated samples. This protein was never present in the control samples. This is consistent with known vitellogenin from amphibian and reptile species, weighing approximately 200kDa, while fish vitellogenins weigh less, approximately 160kDa (Selcer et al., 2001).

DEAE chromatography was performed on *Ambystoma tigrinum* estrogen-treated and control male samples to determine if the estrogen-induced vitellogenin protein has the same characteristics as it does in other species. DEAE revealed the consistent presence of this 200kDa protein in the estrogen-treated samples but not in the control samples. The DEAE profile that was created from the estrogen-treated and control male *Ambystoma tigrinum* were very similar to those created from the red-eared slider turtle, *Trachemys scripta*, (Selcer and Palmer, 1995) and also the African Clawed Frog, *Xenopus laevis* (Palmer and Palmer, 1995). In all three species, there was a very distinct peak that eluted at high salt, which is representative of vitellogenin, in the estrogen-treated samples that was clearly absent in the control or male samples. This difference was also evident in densitometry readings of the SDS-PAGE fractions. Vitellogenin in vertebrates is known to bind to DEAE and only elute at high salt. When fractions from

the DEAE-agarose high salt peak were run on SDS-PAGE, only the 200kDa band was seen in the estrogen-treated samples and no bands were present in the control samples, the same as in *Trachemys scripta*. This demonstrates further that not only is this protein in the treated-male *Ambystoma tigrinum* samples vitellogenin (due to it eluting at high salt), but it also consistently behaves like the protein vitellogenin in many other different species. An ulterior motive in purifying the salamander vitellogenin by DEAE was to prepare it for sequencing to further assure that this protein was vitellogenin. The N-terminal sequencing determined that this protein is closely related to other vertebrate vitellogenins, especially the African clawed frog, *Xenopus laevis*, (Germond, et al., 1984) and the hellbender, *Cryptobranchus alleganiensis*.

In order to test if xenobiotics had an effect on vitellogenin induction, we chose to first use aroclor 1221 (a mixture of PCBs) and atrazine as the representatives of environmental estrogens. Interestingly, there was no evident induction of vitellogenin in the juvenile salamanders. SDS-PAGE analysis of serum *Ambystoma maculatum* samples revealed no presence of vitellogenin in treated individuals. One possibility for the lack of response is that the dose was insufficient, (i.e., it was not high enough to bring them over the threshold necessary to produce vitellogenin). Some studies have reported that aroclor does not induce vitellogenin when administered alone; however, it does produce a vitellogenin response when added with other xenobiotics such as DDT, Bisphenyl A, and nonylphenol (Arukwe et al., 2000). Another recent study found that 4'-MeSO (2)-PCBs are antiestrogenic in vitro and while MeSO (2)-PCB metabolites are persistent and bioaccumulative contaminants, they could be potentially active as environmental antiestrogens in wildlife and humans (Letcher et al., 2002). Another study of atrazine and male carp hepatocytes displayed that none of the triazine herbicides nor their metabolites induced vitellogenin production in male carp hepatocytes, indicating that the estrogenic effects associated with the triazine herbicides *in vivo* are not estrogen receptor-mediated, but may be explained partly by their ability to induce aromatase *in vitro* (Sanderson et al., 2001). Behavioral or developmental effects may prove to be better biomarkers for these compounds than vitellogenin induction. A study looking at the behavioral

effects of atrazine on tiger salamanders using relevant concentrations to ours found that when exposed to atrazine, plasma thyroxine was elevated, plasma corticosterone was depressed, larvae reached developmental stage 4 later, at a smaller size, and lower weight. These results indicate that the herbicide atrazine has the potential to influence tiger salamander life history (Larson et al., 1998). A study done with *Xenopus* and aroclor 1254 showed that exposure caused alterations in gross morphology and swimming behavior and statistically significant decreases in survival and gene expression (Jelaso et al., 2002).

Polyclonal Antibody Production

The utilization of vitellogenin as a biomarker in immunoassays is based on the ability of an antibody to recognize this protein. Several broadly cross-reactive vitellogenin antibodies have been previously developed (Denslow et al., 1996; Heppel et al., 1995). In our lab, a polyclonal broadly-crossreactive antibody for vitellogenins was developed by Phil Foret, 1998 (Selcer et al., 2001). This antibody cross-reacts in immunoassays with representative species of fish, amphibian, and reptile classes. However, this antibody, #498, and a few others, #547, *Rana*-specific (#90, #92), and anti-turtle, did not react against salamander vitellogenin. The puzzling part is that we know that the vitellogenin is present, as we demonstrated previously. In a number of Western blots and ELISAs against salamanders from each experimental design, none reacted against vitellogenin using any of these antibodies.

A further problem was most antibodies we tried reacted with another protein non-specifically in both the control and estrogen-treated salamander samples. This band was always present in the Western blots, and using either detection system (DAB or Alkaline Phosphatase). The only time that the band was not present was when using anti-turtle antibody, and this we feel is because that antibody was affinity purified, so as to alleviate this non-specific binding. We know that this unidentified protein is not albumin, as when we did a Western blot against anti-chicken albumin, the unidentified protein reacted at a weight of approximately 100kDa along with a reaction of albumin at the weight of 66kDa. Since this

unidentified protein was binding so strongly, it was also masking the potential antibody-vitellogenin reactivity in ELISAs. Since this was the case, we decided to manufacture a salamander-specific antibody.

There were two strategies that we considered when making the antibody (Palmer and Selcer, 1996). The first was to purify the protein, and then send it off for production of an antibody. The second was to sequence the protein, and then use the sequence to make a peptide, and then create an antibody against that peptide. Since we had received the salamander vitellogenin N-terminal sequence already, we decided to do the latter (i.e., have a peptide synthesized and raise antibodies to that peptide). The antibody that we received from Invitrogen using the MAP standard technologies was not a salamander-specific antibody. Being over 3 months late, the antibody they sent did not recognize the peptide it was generated against when tested with an ELISA. From these results, we chose to re-produce the antibody. The second time the antibody was constructed, we used the same sequence, but ordered from Sigma Genosys, and used the KLH-coupling approach. This time the antibody at least recognized the peptide when screening with ELISA. The results we have obtained so far are still inconclusive. There have been times that it appeared to work, and times that it did not. We cannot conclude if this is because of the unidentified protein causing interference, or if there is still an issue with dilutions, or that the salamander samples are starting to break-down, since we have had them for so long. Further studies need to be conducted in order to establish the utility of the salamander-specific antibody.

*Hellbender Experiment (*Cryptobranchus alleganiensis)

Pre-treated and estrogen-treated male *Cryptobranchus alleganiensis* serum samples were sent to us to be screened for vitellogenin. SDS-PAGE was used to identify estrogen-induced proteins. SDS-PAGE clearly showed a band in the 200kDa range in the estrogen-treated samples. This protein was not present in the pre-treatment samples. Estrogen-treated *Alligator mississippiensis* samples ran on SDS-PAGE also showed a band at approximately 220kDa in the estrogen-treated samples but were not present in the untreated plasma (Selcer et al., 2001).

DEAE chromatography was performed on *Cryptobranchus alleganiensis* pre-treatment and estrogen-treated male samples to determine if the estrogen-induced vitellogenin protein behaves the same way as it does in previously characterized species. The DEAE revealed the consistent presence of a 200kDa protein in the estrogen-treated samples but not in the pre-treatment samples. The DEAE profile that was created from the pre-treatment and estrogen-treated *Cryptobranchus alleganiensis* were very similar to those created from the red-eared slider turtle, *Trachemys scripta*, (Selcer and Palmer, 1995) *Xenopus laevis* (Palmer and Palmer, 1995) and for *Ambystoma tigrinum*. In all three species, there was a very distinct peak that eluted at high salt, which is representative of vitellogenin, in the estrogen-treated or female samples that was clearly absent in the control or male samples. This demonstrates further that not only is this protein in the estrogen-treated *Cryptobranchus alleganiensis* samples vitellogenin, but that it also consistently behaves like the protein vitellogenin in many other different species. An ulterior motive in purifying the hellbender vitellogenin was to prepare it for sequencing to further assure that this protein was vitellogenin. The sequencing determined that this protein is closely related to other vertebrate vitellogenins, especially the African clawed frog, *Xenopus laevis*, (Germond, et al., 1984), and the salamander, *Ambystoma tigrinum*.

The plasma samples were first tested against polyclonal antiserum #498. This antibody has been shown to be effective at detecting vitellogenin in a number of species, including *Xenopus laevis, Carassius auratus, Sphyrna tiburo, Trachemys scripta*, and *Alligator mississippiensis* (Selcer et al., 2001). This diversity of animals show that the antibody displays broad cross-reactivity against representatives of four vertebrate classes, Chondrichthyes, Osteichthyes, Amphibia, and Reptilia (Selcer et al., 2001). We felt that it would also show reactivity against *Cryptobranchus alleganiensis* vitellogenin.

Reactivity of the presumptive vitellogenin with the antiserum #498 was tested by Western blotting with vitellogenic and nonvitellogenic samples. Western blotting showed that antiserum #498 showed no cross-reactivity with the presumptive vitellogenin protein in any samples. The only band that could be seen was the same unidentified protein found for *Ambystoma tigrinum* in both the pre-treatment and

estrogen-treated samples. The results of the Western blotting are consistent with that of control and estrogen-treated *Ambystoma tigrinum* samples. Even when doing an antibody screen of antibodies, #498, #547, #90, #92, and anti-turtle, there was no reactivity in any lanes except the unidentified protein present in all lanes (estrogen-treated and pre-treatment) except against anti-turtle. Again, the only time that the band was not present was when using anti-turtle antibody, and this we feel is because that antibody was affinity purified, so as to alleviate this non-specific binding. The newly produced salamander antibody also does not appear to react with either the pre-treated or estrogen-treated *Cryptobranchus alleganiensis* samples at this time.

Reactivity of the presumptive vitellogenin with the antiserum was also tested by ELISA to determine the reactivity of the antiserum #498 with vitellogenic and nonvitellogenic samples. The ELISA also demonstrated that this broadly cross-reactive antiserum had no cross-reactivity with the pre-treatment or estrogen-treated males. Again, these results are consistent with other salamander species, control and estrogen-treated *Ambystoma tigrinum* samples. The newly produced salamander antibody still does not appear to react with either the pre-treated or estrogen-treated *Cryptobranchus alleganiensis* samples at this time.

Crocodile Experiment (*Crocodylus moreletii*)

Three-hundred and eighty-one male and female *Crocodylus moreletii* wild-caught samples from potentially contaminated lakes in Belize were sent to us to be screened for vitellogenin. The plasma samples were tested against polyclonal antiserum #498. This antibody has been shown to be effective at detecting vitellogenin in a number of species, *Xenopus laevis, Carassius auratus, Sphyrna tiburo, Trachemys scripta*, and *Alligator mississippiensis*. This diversity of animals show that the antibody displays broadly cross-reactivity against representatives of four vertebrate classes, Chondrichthyes, Osteichthyes, Amphibia, and Reptilia (Selcer et al., 2001). Especially since it showed reactivity with

Alligator mississippiensis, we felt that it would also show reactivity against *Crocodylus moreletii* vitellogenin.

SDS-PAGE was used to identify female-specific or estrogen-induced proteins. SDS-PAGE clearly showed a band in the 210kDa range in the female samples. This protein was not present in the male samples. Estrogen-treated *Alligator mississippiensis* samples ran on SDS-PAGE also showed a band at approximately 220kDa in the estrogen-treated samples that were not present in the untreated plasma (Selcer et al., 2001).

Reactivity of the presumptive vitellogenin with the antiserum was tested by Western blotting to determine the reactivity of the antiserum #498 with vitellogenic and nonvitellogenic samples. Western blotting showed that antiserum #498 strongly cross-reacted with this protein only in the female samples, and showed no reactivity at all with male samples. Multiple bands were seen in the female samples (approximately three), where none were seen in the male samples. The strongest reactivity occurred against the presumptive vitellogenin (approximately 210kDa). The other two bands that showed weak reactivity in the female serum occurred directly above and below where albumin would have been (approximately 66kDa). Since these bands were not present in the control sera, they may represent degradation products of vitellogenin. The results of the Western blotting are again consistent with that of estrogen-treated *Alligator mississippiensis* samples. Western blotting of *Alligator mississippiensis* samples against antiserum #498 showed strong cross-reactivity with the 220kDa band only in the estrogen-treated samples, and also some weak cross-reactivity with a few other bands again around the albumin range (66kDa) (Selcer et al., 2001). Again, these proteins were only present in estrogen-treated serum, and were absent in the controls, suggesting that these proteins may be breakdown proteins of vitellogenin.

Reactivity of the presumptive vitellogenin with the antiserum was also tested by ELISA using vitellogenic and nonvitellogenic samples. The ELISA also demonstrated that this broadly cross-reactive antiserum had much greater reactivity with the females and none with the male sera. These experiments

established the effectiveness of broadly crossreactive antiserum #498 for detection of *Crocodylus moreletii* vitellogenin. The titration curve that was established for *Crocodylus moreletii* looked very similar to that of *Alligator mississippiensis* described previously, again showing that antibody #498 showed reactivity to a female specific protein vitellogenin in multiple species (Selcer et al., 2001).

DEAE chromatography was performed on *Crocodylus moreletii* female and male samples to determine if the female vitellogenin protein behaves the same way as it does in previously characterized species. The DEAE revealed the consistent presence of this 210kDa protein in the female samples but not in the male samples. The DEAE profile that was created from the female and male *Crocodylus moreletii* were very similar to those created from the red-eared slider turtle, *Trachemys scripta,* (Selcer and Palmer, 1995), for *Xenopus laevis* (Palmer and Palmer, 1995) and for *Ambystoma tigrinum*. In all three species, there was a very distinct peak that eluted at high salt, which is representative of vitellogenin, in the estrogen-treated or female samples that was clearly absent in the control or male samples. This demonstrates further that not only is this protein in the female *Crocodylus moreletii* samples vitellogenin, but that it also consistently behaves like the protein vitellogenin in many other different species. These series of experiments also demonstrated that endocrine disruption did not seem to be occurring in the that wild caught animals from this lake despite the fact that the animals were known to have been exposed to pollutants.

Rana aurora *Experiment*

One-hundred and fifty-five *Rana aurora* wild-caught samples were sent to us from California to be screened for vitellogenin. Before screening all samples, an initial screening was conducted to ensure the proper working conditions of the assay. SDS-PAGE, Western blotting, and ELISAs were conducted using *Rana aurora* females as a positive control and wild-caught samples. The SDS-PAGE showed all samples to show the presence of the protein, presumably vitellogenin (180kDa). The Western blot using vitellogenin-antiserum #92 (a *Rana*-specific antibody) only showed cross-reactivity in three of five

estrogen-treated female samples, and showed no reactivity with any of the wild-caught samples. This lack of reactivity with the other two estrogen-treated female samples may have occurred due to the fact that the female samples were very old samples, and had gone through many potential degradation periods due to faulty handling. The ELISA using the five *Rana aurora* wild-caught males, along with positive and negative *Rana aurora* controls in the antibody screen only showed reactivity in the positive control serum against the *Rana* specific antibodies, antibody #90, and more strongly against antibody #92. Antibody #92 was thus selected to be used as the anti-vitellogenin for screening all of the wild-caught *Rana aurora* samples for their presence of vitellogenin.

In the ELISA assays, some females showed a positive response for vitellogenin. Although 2 of the juveniles and 2 of the non-distinguishables did show positive for vitellogenin production, it is unclear whether they were female samples and were not able to be sexed properly. From this study, it can be concluded that only 8.39% of the population of the lake were vitellogenic, meaning that only a subset of the females were reproductive at the time, while no males showed positive for vitellogenin at any time. It can be speculated that the lake is not being exposed to xenobiotic estrogenic agents, since there was no presence of vitellogenin in any samples except females.

Conclusions and Future Directions

It is likely that there will be concern regarding the potential risks of chemical contaminants and possibilities of exposure to the public well into the future. Many wildlife species, in particular amphibians, are experiencing population declines. While many feel that there is no link between human or wildlife health and estrogenic chemicals, few studies have been conducted to investigate these issues. There are insufficient data to prove either assumption.

The salamander vitellogenin experiments have provided valuable background information on vitellogenin induction. The salamander vitellogenin assay developed will be useful in screening for environmental estrogens. After *in vitro* screening, this *in vivo* assay will give more information about the effects of certain chemicals on the entire animal. This assay can also be used to study the effects of anti-estrogenic compounds.

Future directions include performing many more experiments to evaluate the suspected effects of environmental estrogens on salamanders. If more salamander samples are accessible, it would be wise to continue analyzing the antibody to determine the appropriate dilutions necessary to ensure that the assay is working at its optimal capabilities. Perhaps if this antibody does not work at levels that are felt to be optimal, there will be a necessity to produce a new antibody from the purified protein, which would allow for a stronger crossreactivity between the vitellogenin and the primary antibody. Another experiment to pursue after concluding that the assay was working at optimal conditions would be to evaluate the anti-estrogenic effects on amphibians, particularly salamanders, of endocrine disrupting chemicals such as aroclor 1221 and atrazine. This would be an extremely valuable technique for measuring the impact of these compounds on female reproduction and for assessing the detriment to their offspring.

References

Arnold SF, Collins BM, Robinson MK, Guillette LJ, Jr., McLachlan JA. 1996a. Differential interaction of natural and synthetic estrogens with extracellular binding proteins in a yeast estrogen screen. Steroids 61(11):642-646.

Arnold SF, Robinson MK, Notides AC, Guillette LJ, Jr., McLachlan JA. 1996b. A yeast estrogen screen for examining the relative exposure of cells to natural and xenoestrogens. Environmental Health Perspectives 104(5):544-548.

Arukwe A, Celius T, Walther BT, Goksoyr A. 2000. Effects of xenoestrogen treatment on zona radiata protein and vitellogenin expression in Atlantic salmon (*Salmo salar*). Aquatic Toxicology 49(3):159-170.

Baulieu EE. 1984. Pharmacology of steroid receptors: from molecular and cellular studies to efficient antihormonal drugs. Biochemical Pharmacology 33(6):905-912.

Bergeron JM, Crews D, McLachlan JA. 1994. PCBs as environmental estrogens: turtle sex determination as a biomarker of environmental contamination. Environmental Health Perspectives 102(9):780-781.

Bitman J, Cecil HC. 1970. Estrogenic activity of DDT analogs and polychlorinated biphenyls. Journal of Agricultural and Food Chemistry 18(6):1108-1112.

Blais JM, Froese KL, Kimpe LE, Muir DC, Backus S, Comba M, Schindler DW. 2003. Assessment and characterization of polychlorinated biphenyls near a hazardous waste incinerator: analysis of vegetation, snow, and sediments. Environmental Toxicology and Chemistry / SETAC 22(1):126-133.

Blaustein AR, and Wake, D.B. 1990. Declining Amphibian Populations: A Global Phenomenon? Trends in Ecology and Evolution 5:203-204.

Bolander FF. 1994. Molecular Endocrinology. San Diego, California: Academic Press. 601

Borlak J, Thum T. 2002. PCBs alter gene expression of nuclear transcription factors and other heart-specific genes in cultures of primary cardiomyocytes: possible implications for cardiotoxicity. Xenobiotica 32(12):1173-1183.

Bradford, MM. 1976. A rapid and sensitive method microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

Bringolf RB, Summerfelt RC. 2003. Reduction of estrogenic activity of municipal wastewater by aerated lagoon treatment facilities. Environmental Toxicology and Chemistry / SETAC 22(1):77-83.

Bustos S, Denegri JC, Diaz F, Tchernitchin AN. 1988. p,p'-DDT is an estrogenic compound. Bulletin of Environmental Contamination and Toxicology 41(4):496-501.

Carey C, Bryant CJ. 1995. Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. Environmental Health Perspectives 103 Suppl 4:13-17.

Chen HJ, Shapiro DJ. 1990. Nucleotide sequence and estrogen induction of *Xenopus laevis* 3-hydroxy-3-methylglutaryl-coenzyme A reductase. Journal of Biology and Chemistry 265(8):4622-4629.

Clark EJ, Norris DO, Jones RE. 1998. Interactions of gonadal steroids and pesticides (DDT, DDE) on gonaduct growth in larval tiger salamanders, *Ambystoma tigrinum*. General and Comparative Endocrinology 109(1):94-105.

Colborn T. 1995. Environmental estrogens: health implications for humans and wildlife. Environmental Health Perspectives 103(Supplement 7):135-136.

Colborn T, vom Saal FS, Soto AM. 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environmental Health Perspectives 101(5):378-384.

Conant R, and Collins, J. 1999. Petersons Field Guide. Boston: Houghton Mifflin Company. 255

Dankwardt A. 1998. Endocrine Disrupting Pesticides. University of Munich, Germany: World Wildlife Federation.

Danzo BJ. 1998. The effects of environmental hormones on reproduction. Cellular and Molecular Life Sciences: CMLS 54(11):1249-1264.

de Solla SR, Bishop CA, Pettit KE, Elliott JE. 2002. Organochlorine pesticides and polychlorinated biphenyls (PCBs) in eggs of red-legged frogs (*Rana aurora*) and northwestern salamanders (*Ambystoma gracile*) in an agricultural landscape. Chemosphere 46(7):1027-1032.

Denslow ND, Bowman CJ, Ferguson RJ, Lee HS, Hemmer MJ, Folmar LC. 2001. Induction of gene expression in sheepshead minnows (*Cyprinodon variegatus*) treated with 17beta-estradiol, diethylstilbestrol, or ethinylestradiol: the use of mRNA fingerprints as an indicator of gene regulation. General and Comparative Endocrinology 121(3):250-260.

Denslow ND, M.M. Chow, L.C. Folmar, S.L. Bonomelli, S.A. Heppell, and C.V. Sullivan. 1996. Development of antibodies to teleost vitellogenins: potential biomarkers for environmental estrogens. Bengtson DA, and D.S. Henshel, editor. Philadelphia, PA: American Society for Testing and Materials.

Dodson RE, Shapiro DJ. 1997. Vigilin, a ubiquitous protein with 14 K homology domains, is the estrogen-inducible vitellogenin mRNA 3'-untranslated region-binding protein. Journal of Biology and Chemistry. 272(19):12249-12252.

EDSTAC. EDSTAC Executive Summary; 1998 August 1998.

Fairbrother A, Ankley G.T., Birnbaum, L.S., Bradbury, S.P., Francis, B., Gray, L.E., Hinton, D., Johnson, L.L., Peterson, R.E., Van Der Kraak, G. 1999. Reproductive and Developmental Effects of Contaminants in Oviparous Vertebrates. Di Giulio RT, and Tillitt, D.E., editor. Pensacola, Florida: SETAC Press (Society of Environmental Toxicology and Chemistry). 447

Follett BK, and Redshaw, M.R. 1974. The Physiology of Vitellogenesis. Lofts B, editor. New York: Academic Press. 219-308

Folmar LC, Denslow ND, Kroll K, Orlando EF, Enblom J, Marcino J, Metcalfe C, Guillette LJ, Jr. 2001. Altered serum sex steroids and vitellogenin induction in walleye (*Stizostedion vitreum*) collected near a metropolitan sewage treatment plant. Archives of Environmental Contamination and Toxicology 40(3):392-398.

Folmar LC, Denslow ND, Rao V, Chow M, Crain DA, Enblom J, Marcino J, Guillette LJ, Jr. 1996. Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. Environmental Health Perspectives 104(10):1096-1101.

Germond JE, Walker P, ten Heggeler B, Brown-Luedi M, de Bony E, Wahli W. 1984. Evolution of vitellogenin genes: comparative analysis of the nucleotide sequences downstream of the transcription initiation site of four *Xenopus laevis* and one chicken gene. Nucleic Acids Research 12(22):8595-8609.

Gilbertson MK, Haffner GD, Drouillard KG, Albert A, Dixon B. 2003. Immunosuppression in the northern leopard frog (*Rana pipiens*) induced by pesticide exposure. Environmental Toxicology and Chemistry 22(1):101-110.

Ginsburg J. 1996. Tackling environmental endocrine disrupters. Lancet 347(9014):1501-1502.

Gray LE, Jr. 1998. Tiered screening and testing strategy for xenoestrogens and antiandrogens. Toxicology Letters 102-103:677-680.

Gray LE, Jr., Kelce WR, Wiese T, Tyl R, Gaido K, Cook J, Klinefelter G, Desaulniers D, Wilson E, Zacharewski T and others. 1997. Endocrine Screening Methods Workshop report: detection of estrogenic and androgenic hormonal and antihormonal activity for chemicals that act via receptor or steroidogenic enzyme mechanisms. Reproductive Toxicology 11(5):719-750.

Hayes T, Haston K, Tsui M, Hoang A, Haeffele C, Vonk A. 2002a. Herbicides: feminization of male frogs in the wild. Nature 419(6910):895-896.

Hayes TB, Collins A, Lee M, Mendoza M, Noriega N, Stuart AA, Vonk A. 2002b. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Procedures of the National Academy of Science U S A 99(8):5476-5480.

Hayward MA, Brock ML, Shapiro DJ. 1982a. Activation of vitellogenin gene transcription is a direct response to estrogen in *Xenopus laevis* liver. Nucleic Acids Research 10(24):8273-8284.

Hayward MA, Brock ML, Shapiro DJ. 1982b. The role of estrogen receptor in *Xenopus laevis* vitellogenin gene expression. American Journal of Physiology 243(1):C 1-6.

Heppell SA, Denslow ND, Folmar LC, Sullivan CV. 1995. Universal assay of vitellogenin as a biomarker for environmental estrogens. Environmental Health Perspectives 103(Supplement 7):9-15.

Ho SM. 1987. Endocrinology of Vitellogenesis. Plenum Press, New York:145-169.

Ho SM, Danko D, Callard IP. 1981. Effect of exogenous estradiol-17beta on plasma vitellogenin levels in male and female *Chrysemys* and its modulation by testosterone and progesterone. General and Comparative Endocrinology 43(4):413-421.

Ho SM, Kleis, S., McPherson, R., Heisermann, G.J., Callard, I.P. 1982. Regulation of Vitellogenesis in Reptiles. Herpetologica 38:40-50.

Hutchinson TH. 2002. Reproductive and developmental effects of endocrine disrupters in invertebrates: in vitro and in vivo approaches. Toxicology Letters 131(1-2):75-81.

Jackson CL, Shapiro DJ. 1986. Dexamethasone and estrogen regulate *Xenopus laevis* albumin mRNA levels. Biochemical and Biophysical Research Community 136(1):8-15.

Jelaso AM, Lehigh-Shirey E, Predenkiewicz A, Means J, Ide CF. 2002. Aroclor 1254 alters morphology, survival, and gene expression in *Xenopus laevis* tadpoles. Environmental Molecular Mutagen 40(1):24-35.

Kavlock RJ. 1997. EPA Special Report on Endocrine Disruption. Environmental Protection Agency. 1-4

Korach KS. 1994. Insights from the study of animals lacking functional estrogen receptor. Science 266(5190):1524-1527.

Kroft S. 2002. Toxic Secret. In: Bloom A, editor. 60 Minutes. New York, New York: 60 Minutes.

Larson DL, McDonald S, Fivizzani AJ, Newton WE, Hamilton SJ. 1998. Effects of the herbicide atrazine on *Ambystoma tigrinum* metamorphosis: duration, larval growth, and hormonal response. Physiological Zoology 71(6):671-9.

Letcher RJ, Lemmen JG, van der Burg B, Brouwer A, Bergman A, Giesy JP, van den Berg M. 2002. In vitro antiestrogenic effects of aryl methyl sulfone metabolites of polychlorinated biphenyls and 2,2-bis(4-chlorophenyl)-1,1-dichloroethene on 17beta-estradiol-induced gene expression in several bioassay systems. Toxicological Science 69(2):362-372.

Licht P, Pavgi S, Denver RJ. 1990. The role of hormone binding in the cold suppression of hormone stimulation of the pituitary, thyroid, and testis of the turtle. General and Comparative Endocrinology 80(3):381-392.

Lukachko A. 1999. Endocrine Disrupters: A Scientific Perspective. Broadway, New York, NY: American Council on Science and Health. 1-30

May FE, Ryffel GU, Weber R, Westley BR. 1982. Estrogen dramatically decreases albumin mRNA levels and albumin synthesis in *Xenopus laevis* liver. Journal of Biology and Chemistry 257(23):13919-13923.

Mbongwe B, Legrand M, Blais JM, Kimpe LE, Ridal JJ, Lean DR. 2003. Dichlorodiphenyltrichloroethane in the aquatic ecosystem of the Okavango Delta, Botswana, South Africa. Environmental Toxicology and Chemistry 22(1):7-19.

McLachlan JA. 1993. Functional toxicology: a new approach to detect biologically active xenobiotics. Environmental Health Perspectives 101(5):386-387.

McLachlan JA. 2001. Environmental signaling: what embryos and evolution teach us about endocrine disrupting chemicals. Endocrinology Review 22(3):319-341.

McLachlan JA, and Arnold, S.F. 1996. Environmental Estrogens. American Scientist 84:452-461.

McLachlan JA, Newbold RR, Burow ME, Li SF. 2001. From malformations to molecular mechanisms in the male: three decades of research on endocrine disrupters. Apmis 109(4):263-272.

McMaster ME, Jardine JJ, Ankley GT, Benson WH, Greeley MS, Jr., Gross TS, Guillette LJ, Jr., MacLatchy DL, Orlando EF, Van Der Kraak GJ and others. 2001. An interlaboratory study on the use of steroid hormones in examining endocrine disruption. Environmental Toxicology and Chemistry 20(9):2081-2087.

Mosconi G, Carnevali O, Franzoni MF, Cottone E, Lutz I, Kloas W, Yamamoto K, Kikuyama S, Polzonetti-Magni AM. 2002. Environmental estrogens and reproductive biology in amphibians. General and Comparative Endocrinology 126(2):125-129.

Nawaz Z, Tsai MJ, McDonnell DP, O'Malley BW. 1992. Identification of novel steroid-response elements. Gene Expression 2(1):39-47.

O'Malley BW, and Strott, C.A. 1991. Reproductive Endocrinology. Yen SS, and Jaffe, R.B., editor. Philadelphia, PA: W.B. Saunders Company. 1016

O'Malley BW, McGuire WL, Kohler PO, Korenman SG. 1969. Studies on the mechanism of steroid hormone regulation of synthesis of specific proteins. Recent Progress in Hormone Research 25:105-160.

O'Malley BW, Tsai MJ. 1992. Molecular pathways of steroid receptor action. Biology of Reproduction 46(2):163-167.

Palmer BD, and K.W. Selcer. 1996. Vitellogenin as a biomarker for xenobiotic estrogens. Bengtson DA, and D.S. Henshel, editor. West Conshohocken, PA: American Society for Testing and Materials. 3-22

Palmer BD, Huth, L.K., Pieto, D.L., Selcer, K.W. 1998. Vitellogenin as a Biomarker for Xenobiotic Estrogens in an Amphibian Model System. Environmental Toxicology and Chemistry 17(1):30-36.

Palmer BD, Palmer SK. 1995. Vitellogenin induction by xenobiotic estrogens in the red-eared turtle and African clawed frog. Environmental Health Perspectives 103(Supplement 4):19-25.

Palmer JR, Hatch EE, Rao RS, Kaufman RH, Herbst AL, Noller KL, Titus-Ernstoff L, Hoover RN. 2001. Infertility among women exposed prenatally to diethylstilbestrol. American Journal of Epidemiology 154(4):316-321.

Pastori RL, Moskaitis JE, Schoenberg DR. 1991. Estrogen-induced ribonuclease activity in *Xenopus* liver. Biochemistry 30(43):10490-10498.

Pelissero C, Flouriot G, Foucher JL, Bennetau B, Dunogues J, Le Gac F, Sumpter JP. 1993. Vitellogenin synthesis in cultured hepatocytes; an in vitro test for the estrogenic potency of chemicals. The Journal of Steroid Biochemistry and Molecular Biology 44(3):263-272.

Pfister G, Juttner I, Severin GF, Schramm KW, Kettrup A. 2003. Controlled release experiments with nonylphenol in aquatic microcosms. Environmental Toxicology and Chemistry 22(1):182-188.

Purdom CE, Hardiman, P.A., Bye, V.J., Eno, N.C., Tyler, C.R., and Sumpter, J.P. 1994. Estrogenic Effects of Effluents from Sewage Treatment Works. Chemical Ecology 8:275-285.

Raloff J. 1994. The Gender Benders: Are Environmental "Hormones" Emasculating Wildlife? Science News 145:24-27.

Read LD, Greene GL, Katzenellenbogen BS. 1989. Regulation of estrogen receptor messenger ribonucleic acid and protein levels in human breast cancer cell lines by sex steroid hormones, their antagonists, and growth factors. Molecular Endocrinology 3(2):295-304.

Riegel AT, Jordan VC, Bain RR, Schoenberg DR. 1986. Effects of antiestrogens on the induction of vitellogenin and its mRNA in *Xenopus laevis*. Journal of Steroid Biochemistry 24(6):1141-1149.

Ryseck RP, Macdonald-Bravo H, Mattei MG, Ruppert S, Bravo R. 1989. Structure, mapping and expression of a growth factor inducible gene encoding a putative nuclear hormonal binding receptor. The EMBO Journal 8(11):3327-3335.

Sanderson JT, Letcher RJ, Heneweer M, Giesy JP, van den Berg M. 2001. Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitellogenin production in male carp hepatocytes. Environmental Health Perspectives 109(10):1027-1031.

Schoenberg DR, Moskaitis JE, Smith LH, Jr., Pastori RL. 1989. Extranuclear estrogen-regulated destabilization of *Xenopus laevis* serum albumin mRNA. Molecular Endocrinology 3(5):805-814.

Selcer KW, Leavitt WW. 1991. Progesterone downregulates progesterone receptor, but not estrogen receptor, in the estrogen-primed oviduct of a turtle (*Trachemys scripta*). General and Comparative Endocrinology 83(2):316-323.

Selcer KW, Nagaraja, S., Foret, P, Wagner, D., Williams, L., and Palmer, B.D. 2001. Vitellogenin as a Biomarker for Estrogenic Chemicals: Development of Antibodies and Primers with Broad Species Applications. In: Robertson LaLGH, editor. PCBs: Recent Advances in Environmental Toxicology and Health Effects.: The University Press of Kentucky. p 285-292.

Selcer KW, Palmer BD. 1995. Estrogen downregulation of albumin and a 170-kDa serum protein in the turtle, *Trachemys scripta*. General and Comparative Endocrinology 97(3):340-352.

Shapiro DJ, Barton MC, McKearin DM, Chang TC, Lew D, Blume J, Nielsen DA, Gould L. 1989. Estrogen regulation of gene transcription and mRNA stability. Recent Progress in Hormone Research 45:29-58.

Shimasaki Y, Kitano T, Oshima Y, Inoue S, Imada N, Honjo T. 2003. Tributyltin causes masculinization in fish. Environmental Toxicology and Chemistry 22(1):141-144.

Smith CL, O'Malley BW. 1999. Evolving concepts of selective estrogen receptor action: from basic science to clinical applications. Trends in Endocrinology and Metabolism: TEM 10(8):299-300.

Soto AM, Justicia H, Wray JW, Sonnenschein C. 1991. p-Nonyl-phenol: an estrogenic xenobiotic released from "modified" polystyrene. Environmental Health Perspectives 92:167-173.

Soto AM, Lin, T., Justicia, H., Silvia, R., and Sonnenschein, C. 1992. An "in culture" bioassay to assess the estrogenicity of xenobiotics (E-SCREEN). Princeton Scientific Publications, Princeton, NJ:296-309.

Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO. 1995. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environmental Health Perspectives 103(Supplement 7):113-122.

Stancel GM, Boettger-Tong HL, Chiappetta C, Hyder SM, Kirkland JL, Murthy L, Loose-Mitchell DS. 1995. Toxicity of endogenous and environmental estrogens: what is the role of elemental interactions? Environmental Health Perspectives 103(Supplement 7):29-33.

Sumpter JP, Jobling S. 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environmental Health Perspectives 103(Supplement 7):173-178.

Suzuki N, Kambegawa A, Hattori A. 2003. Bisphenol A influences the plasma calcium level and inhibits calcitonin secretion in goldfish. Zoological Science 20(6):745-748.

Tata JR, Smith DF. 1979. Vitellogenesis: a versatile model for hormonal regulation of gene expression. Recent Progress in Hormone Research 35:47-95.

Toft D, Shyamala G, Gorski J. 1967. A receptor molecule for estrogens: studies using a cell-free system. Proceedings of the National Academy of Sciences of the United States of America 57(6):1740-1743.

Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette LJ, Jr., Jegou B, Jensen TK, Jouannet P, Keiding N and others. 1996a. Male reproductive health and environmental xenoestrogens. Environmental Health Perspectives 104 Supplement 4:741-803.

Tsai CL, Chang SL, Wang LH, Chao TY. 2003. Temperature influences the ontogenetic expression of aromatase and oestrogen receptor mRNA in the developing tilapia (*Oreochromis mossambicus*) brain. Journal of Neuroendocrinology 15(1):97-102.

Turner KJ, Sharpe RM. 1997. Environmental oestrogens--present understanding. Reviews of Reproduction 2(2):69-73.

Wake DB. 1991. Declining Amphibian Populations. Science 253:860.

Wangh LJ. 1982. Glucocorticoids act together with estrogens and thyroid hormones in regulating the synthesis and secretion of *Xenopus* vitellogenin, serum albumin, and fibrinogen. Developmental Biology 89(2):294-298.

Wangh LJ, Schneider W. 1982. Thyroid hormones are corequisites for estradiol-17beta in vitro induction of *Xenopus* vitellogenin synthesis and secretion. Developmental Biology 89(2): 287-293.

Wyman RL. 1990. What's Happening to the Amphibians? Conservation Biology 4:350-352.

Appendix 1 – Listing of all Potential Anti-Vitellogenin Antibodies Used in this Study

Antibody	***Description***
#498	Broadly-cross reactive polyclonal antibody generated against a conserved vitellogenin peptide
#547	Broadly-cross reactive polyclonal antibody generated against a conserved vitellogenin peptide
#90	Antibody generated against a purified *Rana pipiens* vitellogenin protein
#92	Antibody generated against a purified *Rana pipiens* vitellogenin protein
Anti-Turtle	Antibody generated against a purified *Trachemys scripta* vitellogenin protein
#371	Antibody generated against a *Ambystoma tigrinum* specific vitellogenin peptide
#358	Antibody generated against a *Ambystoma tigrinum* specific vitellogenin peptide
#129	Antibody generated against a *Ambystoma tigrinum* specific vitellogenin peptide
#130	Antibody generated against a *Ambystoma tigrinum* specific vitellogenin peptide

Appendix 2 *- Body Weights of Salamanders from Experiment 1*

Vehicle 1	188.8
Vehicle 2	184.2
Vehicle 3	166.9
Vehicle 4	183.6
Vehicle 5	146.1
Average	173.92
St Dev	17.635
St Error	7.887
	T Test
	0.024
Estrogen 1	153.6
Estrogen 2	170
Estrogen 3	153.6
Estrogen 4	172.7
Estrogen 5	135.6
Estrogen 6	150.2
Estrogen 7	135.5
Estrogen 8	131.8
Estrogen 9	151.3
Estrogen 10	166.3
Average	152.06
St Dev	14.587
St Error	4.613

Appendix 3 *- Total Protein Concentrations of Single Injection Salamanders from Experiment 1*

			Averages
E1	25.84	24.58	25.21
E2	22.5	35.78	29.14
E3	25.62	37.41	31.515
E4	43.8	64.36	54.08
E5	41.37	55.27	48.32
E6	50.08	63.06	56.57
E7	24.37	22.47	23.42
E8	44.34	55.91	50.125
E9	35.19	38.22	36.705
E10	30.11	27.66	28.885

Average	38.397
Stand Dev	13.574
Stand Error	3.035

			0.00025235T test of E/C
C1	19.13	16.14	17.635
C2	17.12	13.38	15.25
C3	22.1	38.54	30.32
C4	21.02	17.76	19.39
C5	17.56	1.042	9.301

Average	18.379
Stand Dev	9.196
Stand Error	2.908

Appendix 4 *- Total Protein of Salamanders from Experiment 2 - Single-Injection*

	Trial 1	Trial 2	Trial 3	Averages	T-test
E1M	23.53	33.84	23.07	26.813	
E2M	25.26	30.91	27.64	27.937	
E1F	34.56	32.2	21.35	29.370	
E2F	32.09	49.52	34.79	38.800	0.978
					(Females)
V1M	28.99	12.82	26.34	22.717	
V2M	30.89	31.86	24.45	29.067	0.667
					(Males)
V1F	23.53	12.3	26.17	20.667	
V2F	40.94	50.12	50.2	47.087	
					0.841
					Control

Standard Dev		Stand Error	
4.293	EM	1.753	
2.837	VM	1.158	
7.045	EF	2.876	
9.041	VF	3.691	

All Estrogen/Control

Appendix 5 *- Total Protein Concentration of Salamanders from Experiment 2 - Multiple Injection*

L1	31.29	29.96	42.97	34.740	St Dev		St Error
L2	33.76	31.34	40.29	35.130	5.396	L1-2	2.203
M1	21.24	25.83	28.84	25.303			
M2	24.22	22.21	28.32	24.917			
M3	15.78	12.99	16.01	14.927	5.722	M1-3	1.907
C1	38.47	37.37	31.51	35.783	7.695	C1-3	2.565
C2	12.91	26.34	28.93	22.727			
C3	29.16	34.96	33.84	32.653			

0.0154 t-test

very significantly different (m1-3 & C1-3)

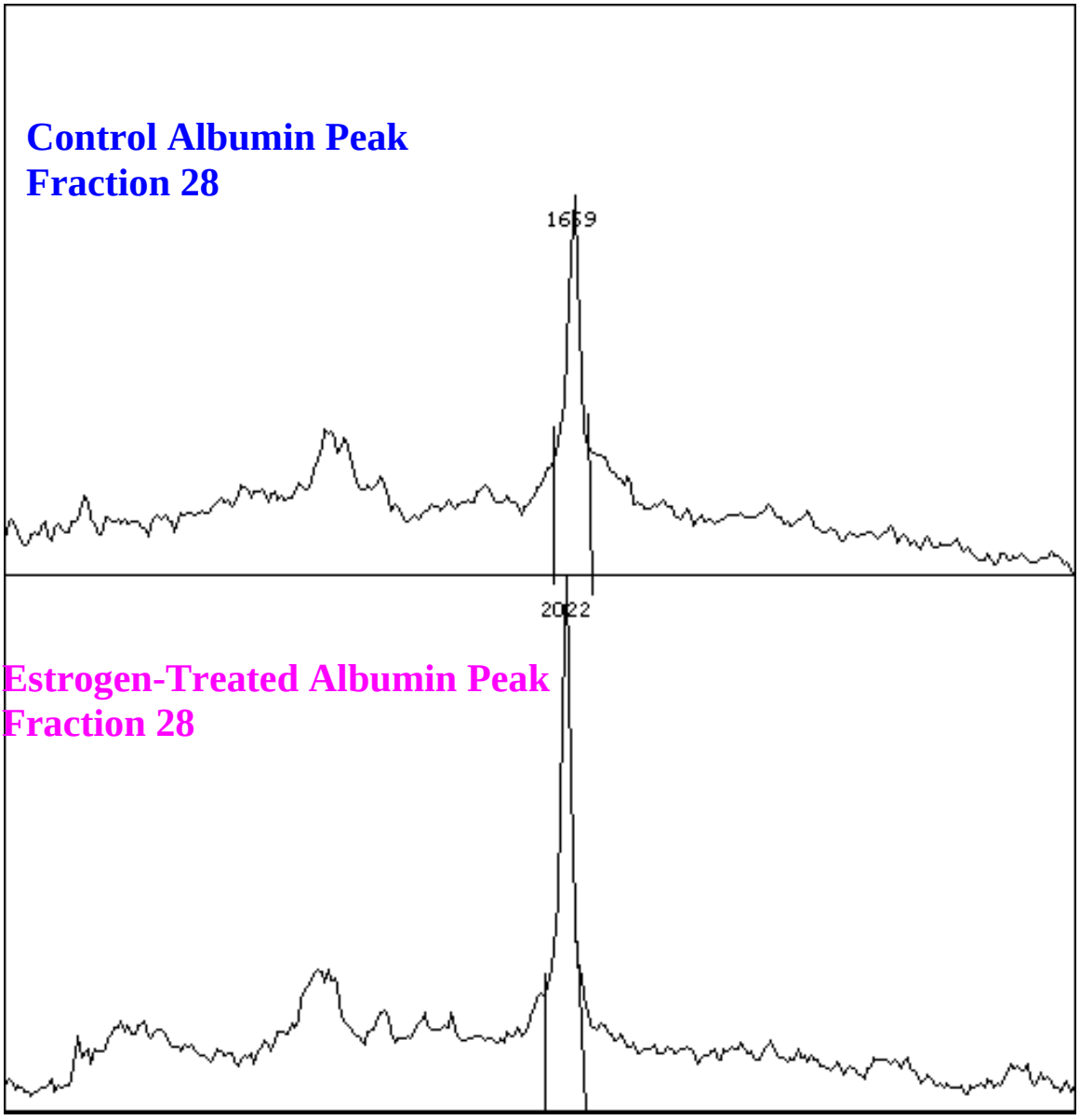
Appendix 6 *- Body Weights of Salamanders from Experiment 3*

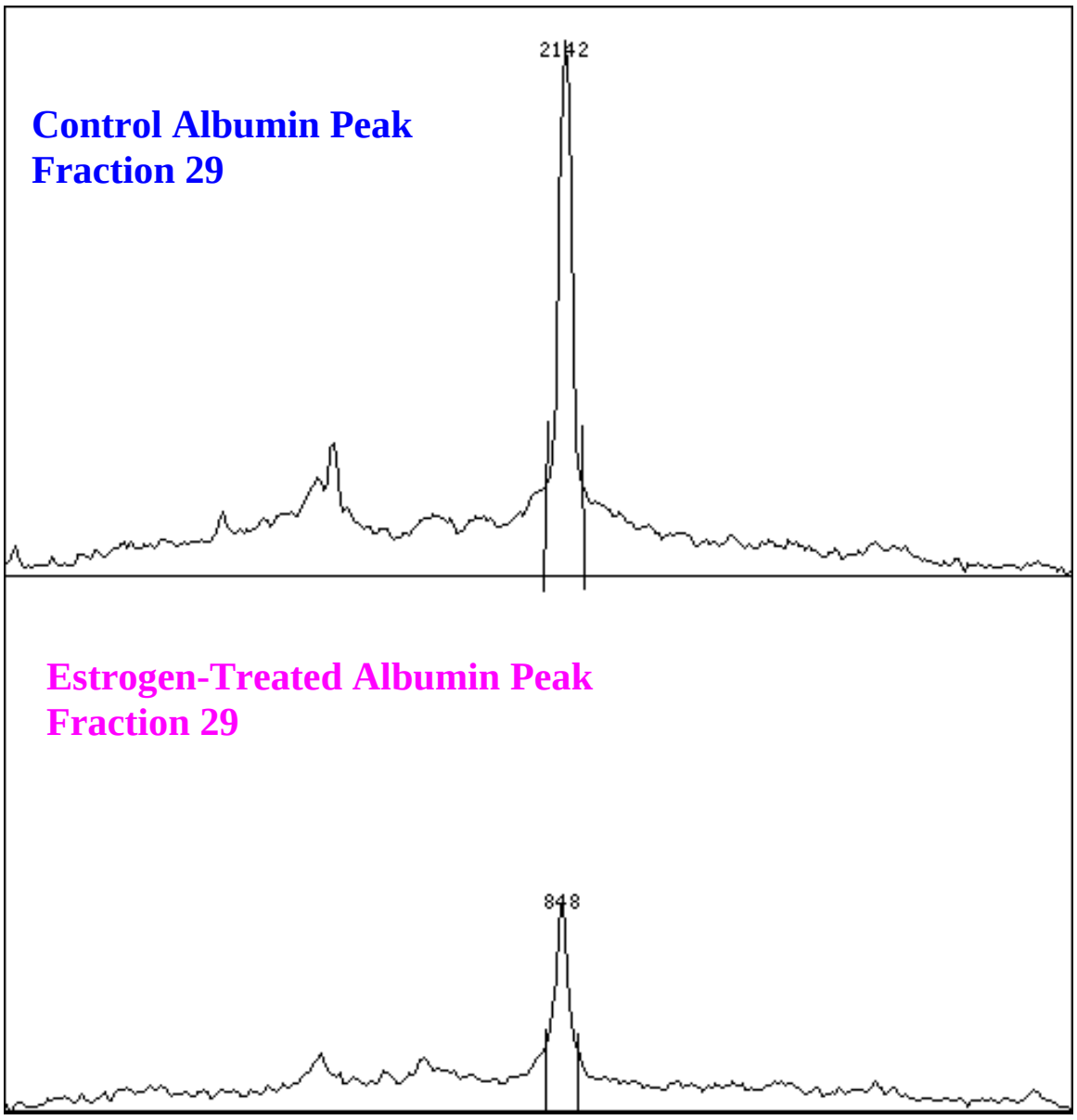
Vehicle 1	7.43
Vehicle 2	7.43
Vehicle 3	7.43
Average	7.43
St Dev	0
St Error	0
	T Test
	0.1943
Estrogen 1	6.14
Estrogen 2	5.13
Estrogen 3	6.94
Estrogen 4	7.44
Estrogen 5	7.65
Estrogen 6	7.46
Estrogen 7	6.63
Estrogen 8	7.83
Estrogen 9	6.3
Estrogen 10	7.03
Estrogen 11	6.89
Estrogen 12	5.6
Average	6.753
St Dev	0.833
St Error	0.240

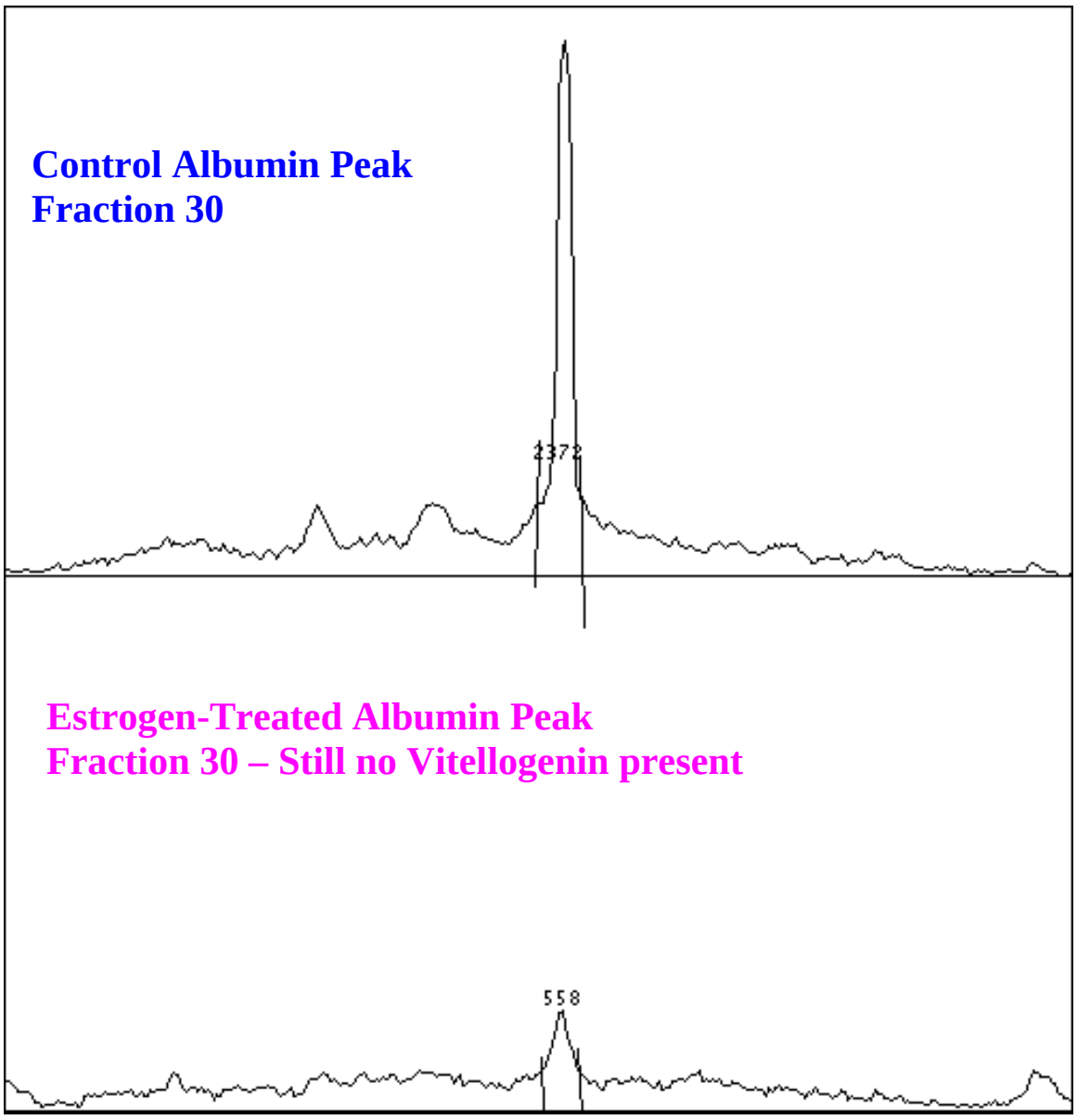
Appendix 7 *- Body Weights of all Salamanders from Experiment 4*

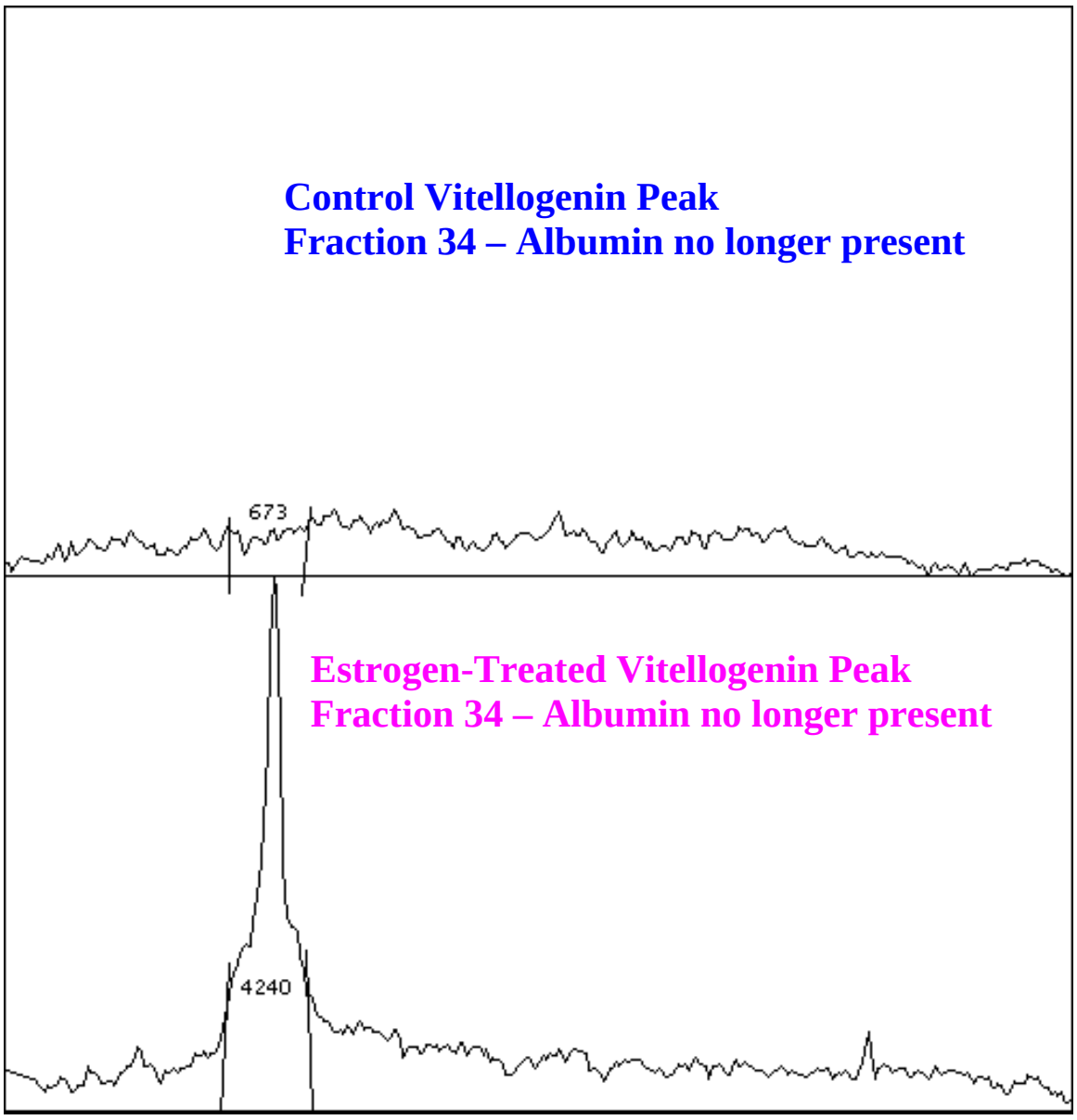
Initial Vehicle 1	10.6		Atrazine High 1	12.2	Avg	12.3	High			
Initial Vehicle 2	11.5		Atrazine High 2	12.4	St Dev	0.141		T Test		
Initial Vehicle 3	15.1		Atrazine Low 1	12.9	St Error	0.1		Atr H/L		
Initial Vehicle 4	12		Atrazine Low 2	9.2				0.860		
Initial Vehicle 5	8.9		Atrazine Low 3	12.8						
										Atrazine High/Init
Vehicle 6	13.2		Atrazine Low 4	18.5	Avg	12.733	Low	0.706		Controls
										Atrazine Low/Init
Vehicle 7	13.2		Atrazine Low 5	12.3	St Dev	3.165		0.529		Controls
			Atrazine Low 6	10.7	St Error	1.292				
	12.07	Initial	All							
Avg	1	Avg	Vehicles							
St Dev	2.011	11.62	12.071							
St Error	0.760		Avg	12.625		0.663	(all a/e)			
	T Test		St Dev	2.683						
	0.237	0.141	St Error	0.949						
	E/C	InitC/E								
Estrogen 1	13.3									
Estrogen 2	13.8									
Estrogen 3	12.3		Aroclor 1221 High 1	14.5						
Estrogen 4	14.1		Aroclor 1221 High 2	11.1						
Estrogen 5	13.1		Aroclor 1221 High 3	9.2	Avg	11.1	High			
Estrogen 6	10.7		Aroclor 1221 High 4	11	St Dev	2.353				
Estrogen 7	11.9		Aroclor 1221 High 5	8	St Error	0.961		T Test		
								Aroclor		
Estrogen 8	13.9		Aroclor 1221 High 6	12.8				H/L		
Estrogen 9	14.6		Aroclor 1221 Low 1	15.5				0.113		
			Aroclor 1221 Low 2	15.8						
										Aroclor High/Init
	13.07									
Avg	8		Aroclor 1221 Low 3	16	Avg	13.617	Low	0.720		Controls
										Aroclor Low/Init
St Dev	1.238		Aroclor 1221 Low 4	12.1	St Dev	2.657		0.219		Controls
St Error	0.413		Aroclor 1221 Low 5	9.3	St Error	1.085				
			Aroclor 1221 Low 6	13						
						0.812	(AllA/V)			
					Avg	12.358	St Dev	2.73		
							St Error	0.78		

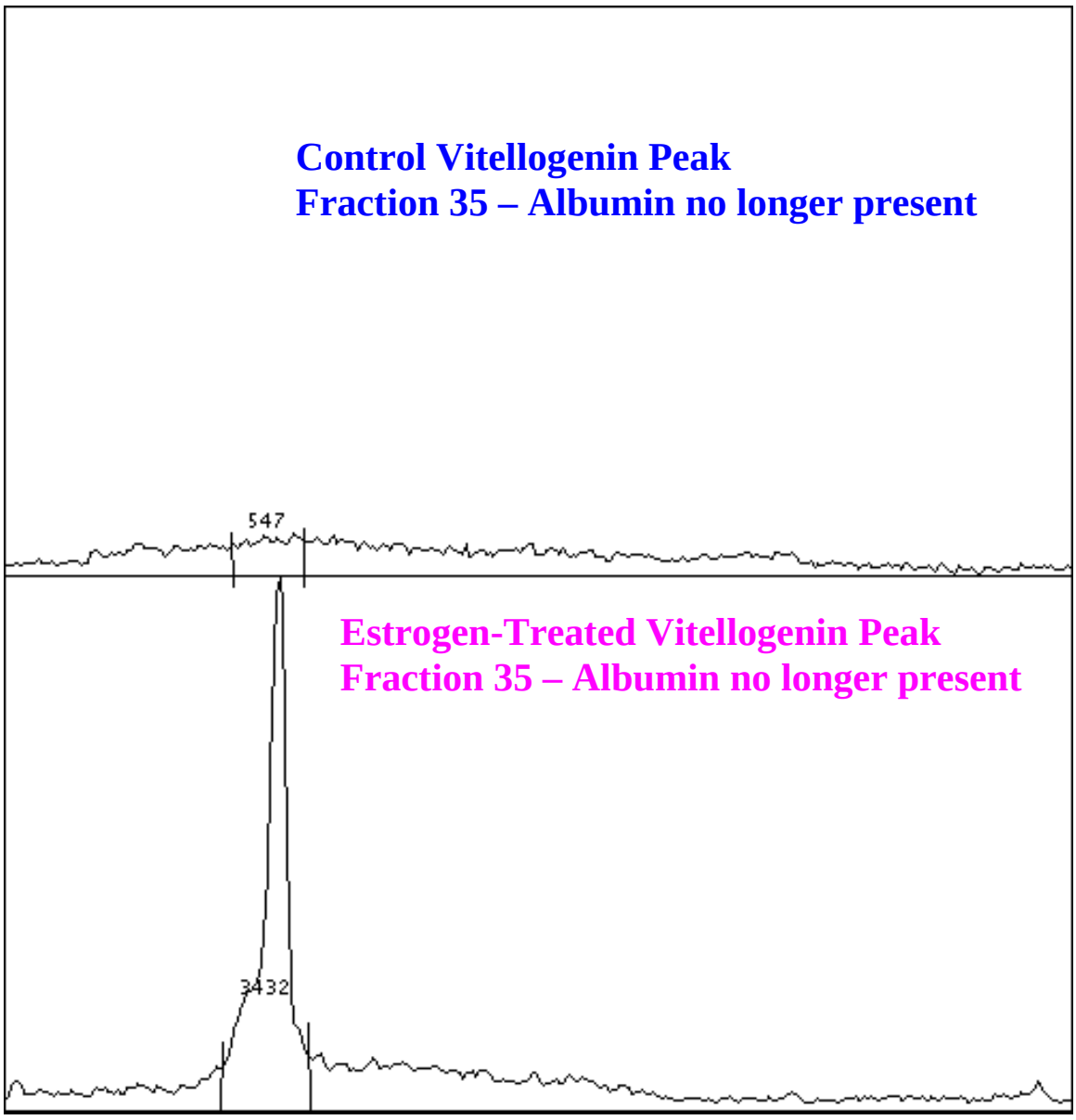
Appendix 8: *An example of the Densitometry Plots*
(This is of Figure 8)




Control Albumin Peak
Fraction 29
2142
Estrogen-Treated Albumin Peak
Fraction 29
848


Control Albumin Peak
Fraction 30
2372
Estrogen-Treated Albumin Peak
Fraction 30 – Still no Vitellogenin present
558


Control Vitellogenin Peak
Fraction 34 – Albumin no longer present
673
Estrogen-Treated Vitellogenin Peak
Fraction 34 – Albumin no longer present
4240


Control Vitellogenin Peak
Fraction 35 – Albumin no longer present
547
Estrogen-Treated Vitellogenin Peak
Fraction 35 – Albumin no longer present
3432