INTRODUCTION

The immune system of *Xenopus laevis* develops rapidly in the early larval stages following fertilization. The ontogeny of the larval immune system of *X. laevis* is first observed four days after fertilization when recombination-activating gene (RAG) transcripts appear (Du Pasquier *et al*., 2000). The ability to produce antigen-specific B cells and T cells is dependent on RAG encoded enzymes. At about day five, pre-B cells are present in the liver and lymphocytes are found in the thymus. By day twelve (stage 49), antibodies are present in the serum and the spleen is recognizable (Du Pasquier *et al*., 89). An antibody-mediated response can occur at this stage (Hsu & Du Pasquier, 1984a), and these antibodies are classified as isotype IgM. Class switching from IgM to IgY does not occur until day fifteen. By this time, proteins encoded by the genes of the major histocompatibility complex (MHC) are expressed (DuPasquier and Flajnik, 1990) and MHC-disparate allografts can be rejected (Horton, 1969). A diverse set of B cells are present in the liver and spleen after two weeks (Mussmann *et al*., 1998). Following immunization with the antigen 2,4-dinitrophenol (DNP), anti-DNP larval antibodies were detected, but with lower affinity than antibodies produced by adult B cells (Hsu & Du Pasquier, 1984b). This observation may reflect an immunological advantage for larvae, when a limited antibody repertoire could potentially respond to a variety of antigens with, albeit, low affinity (Du Pasquier *et al*., 2000). Although the larval immune system develops rapidly, there is a ~twelve day window where the larva is not immunocompetent. This leads to the question of whether or not antibodies are transferred from mother to eggs in *X. laevis*.

The maternal transfer of antibodies has been demonstrated in several species of vertebrates. Two isotypes, 7S IgM (monomer) and new antigen receptor (IgNAR), were detected in the yolk of nurse sharks, *Ginglymostoma cirratum* (Haines *et al*., 2005). The process has been demonstrated in several teleosts, including channel catfish (*Punctatus ictalurus*) (Hayman & Lobb, 2003), tilapia (*Oreochromis aureus*) (Mor & Avtalion, 1990), guppy (*Peocilia reticulata*) (Takahashi & Kawahara, 1987), char (*Salvelinus leucomaenis*) (Kawahara *et al*., 1993), and Atlantic salmon (*Salmo salar*) (Lillehaug *et al*., 1996). Maternal transfer of antibodies has been well characterized in chickens (Kowalcyk *et al*., 1985). Prior to ovulation, antibodies from the hen's blood are transferred to the egg yolk by IgY receptors on the ovarian follicle. During embryonic development, antibodies are transferred from the egg yolk to the embryonic circulation via yolk sac IgY receptors. In a separate process, IgM and IgA antibodies are transferred to the chicken egg white, and then later to the embryonic digestive tract. In humans, passive immunity is conferred directly to the fetal circulation. During this process, antibodies reach the fetal circulation by trans-placental movement, which is mediated by a receptor/transport molecule known as hFcRII (Stuart *et al*., 1989). Therefore, it seems likely that this process has been conserved through the gnathostome evolutionary lineage. To date, however, it has not been demonstrated in amphibians.

In the present study, sexually mature, adult female *X. laevis* were injected with the hapten-carrier complex, trinitrophenylated bovine serum albumin (TNP-BSA), in order to elicit an anti-hapten antibody response. A hapten-carrier complex was used because the hapten molecule, TNP, has a single antigenic determinant, but it must be attached to a carrier protein to provoke the production of antibodies. If maternal transfer of antibodies

occurs in *X. laevis*, then anti-TNP antibodies will be present in the eggs. When assaying for the presence of anti-TNP antibodies by enzyme-linked immunosorbant assay (ELISA), a different carrier molecule, ovalbumin, was used in order to only detect antibodies binding to TNP.

This study demonstrated the presence of anti-TNP antibodies in the eggs of *X. laevis*. These antibodies, which must have been transferred from mother to egg, were detected by ELISA using a polyclonal mouse anti-*Xenopus laevis* antibody reagent.

MATERIALS AND METHODS

Animals

Five female *X. laevis* (Xenopus Express Inc., Brooksville, FL), the African clawed frog, were used in this study. Two female Balb/cJ mice were used to produce antibodies against *X. laevis* antibodies. These animals were maintained according to the guidelines set forth by the Wake Forest University Animal Care and Use Committee.

Production of mouse anti- X. laevis immunoglobulins

X. laevis serum immunoglobulins were purified using a Protein A Antibody Purification Kit (Sigma, St. Louis, MO). Briefly, 1 ml of *X. laevis* serum was diluted with 1 ml Binding Buffer (obtained from kit) and was slowly passed through the 1 ml Protein A column three times over the course of 3 hours. After washing the column with Binding Buffer, purified immunoglobulins were eluted with 5 ml Elution Buffer (obtained from kit). The eluate was then passed through the Desalting Column. Eluted immunoglobulins should include isotypes IgY and IgM (Hsu & Du Pasquier, 1984c). The eluted immunoglobulins were then concentrated in a 100 kilodalton (kDa) cut-off Microsep centrifugal filter device (Pall, East Hills, NY) by centrifugation at 1,000 x *g* for 30 minutes at room temperature. The protein concentration of the purified immunoglobulins solution was adjusted to 200 µg/ml as determined by the Bradford assay (Bio-Rad, Hercules, CA). Two Balb/cJ mice were injected intraperitoneally with 50 µg purified *X. laevis* immunoglobulins in 0.25 ml PBS buffer. Two subsequent injections were performed on days 7 and 14 using the same concentration and volume. On day 21, blood samples were taken from anesthetized mice via the retro-orbital plexus. The blood was allowed to clot for 30 minutes at room temperature and for 24 hours at 4°C. The

serum was collected after centrifugation for 5 minutes at 2,000 x *g*, aliquoted, and stored at -20°C until use. The mouse serum was tested for anti-*X. laevis* immunoglobulins reactivity by Western blot analysis (Fig. 1). Briefly, two dilutions (1:100 and 1:200) of the mouse serum were incubated with a Western blot of 1:200 *Xenopus laevis* serum. For complete methods of SDS-PAGE and Western blot, see *Western blot analysis of egg extract* below.

Preparation of X. laevis egg extract

Pre-immunization blood samples were taken by cardiac puncture from all five *X. laevis*. The sera were collected from the blood samples by the same process described above for the isolation of mouse serum. All five frogs were injected with trinitrophenylated bovine serum albumin (TNP-BSA, Biosearch Technologies, Novato, CA) diluted to 1.0 mg/ml in phosphate buffer saline (PBS, 11.5 g/L Na_2HPO_4, 2 g/L KH_2PO_4, 80 g/L M NaCl, 2 g/L mM KCl, pH 7.4). TNP-BSA was prepared for injection by emulsifying 1 mg/ml TNP-BSA in an equal volume of Freund's complete adjuvant (Sigma, St. Louis, MO). Each frog was injected subcutaneously into the dorsal lymph sac with 50 μg TNP-BSA in 100 μl of PBS/adjuvant. Anesthesia was induced for this procedure (and all other injections/blood samples) by immersion in 0.05% MS-222 (Western Chemical, Ferndale, WA) buffered with 0.05% sodium bicarbonate for 15 minutes. The frogs later received three biweekly immunizations of 50 μg TNP-BSA emulsified in Freund's incomplete adjuvant over the course of six weeks. Prior to all injections, a blood sample was taken by cardiac puncture and the sera were collected as described above. At week eight, the frogs were moved into individual aquaria equipped with a plastic mesh (1 cm x 1 cm pore size), false-bottom surface. The frogs were induced to lay eggs by injection of 250 IU human chorionic gonadotropin (hCG, Sigma, St. Louis, MO) in 0.25 ml sterile 0.9%

sodium chloride solution into the dorsal lymph sac (Etheridge and Richter, 1978). A subsequent 500 IU hCG injection in 0.5 ml sterile 0.9% sodium chloride solution was given 8-12 hours later. Eggs were collected and washed twice with cold PBS. Excess PBS was removed and the washed eggs were stored at -20°C until use. The egg extract isolation began by diluting 0.5 ml eggs 1:10 in cold PBS with 1mM phenylmethylsulphonyl fluoride, 1% Triton X-100, 1 mM EDTA, and 1:100 dilution of a protease inhibitor cocktail (Sigma, St. Louis, MO). The eggs were then disrupted by repeated passage through a 20 gauge needle using a 3 ml syringe. After centrifugation at 5,000 x *g* for 10 minutes, a syringe equipped with a 26 gauge needle was used to collect the supernatant fluid while avoiding the top layer of lipids. The egg extracts were stored at 4°C until use.

Determination of specific antibody titer in eggs by ELISA

The antigens used in the ELISA were trinitrophenylated ovalbumin (TNP-OVA, Biosearch Technologies, Novato, CA) and ovalbumin (OVA, Sigma, St. Louis, MO). TNP-OVA and OVA were separately diluted to 10 ug/ml in 100 µl carbonate buffer, pH 9.6 (1.59 g/L Na_2CO_3, 2.39 g/L $NaHCO_3$), and were then coated on to Falcon U-bottom 96-well plates (Becton Dickinson, Franklin Lakes, NJ) over night at 4°C. The wells were then blocked with 200 µl of 3% non-fat dry milk (NFDM) in PBS for 4 hours at room temperature. The wells were washed two times with 200 µl PBS containing 0.05% Tween-20 (PBST). The sera were diluted in PBS with 1% NFDM and 50 µl were added per well. Each dilution of egg extract or serum was performed in triplicate. The incubation period (and all others) was carried out at room temperature for 1 hour. Each incubation period was separated by three washes with 200 µl/well PBST. The detector

antibody reagent, mouse anti-*X. laevis* immunoglobulins (described above), was diluted to 1:200 in PBS with 1% NFDM. The indicator antibody reagent was rabbit anti-mouse IgG conjugated to horseradish peroxidase (Sigma, St. Louis, MO) diluted 1:5000 in PBS with 1% NFDM. The substrate solution was prepared by adding 10 µl of 30% H_2O_2 to 10 ml of 100mM anhydrous citric acid, pH 4.35 with 0.6 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)(Sigma, St. Louis, MO). Following development in substrate solution (50 µl/well) for 1 hour at room temperature, absorbance was measured at 405 nm with a Multiskan plate reader (Fisher Scientific, Pittsburgh, PA). There were two sets of negative control wells on each plate, which (1) did not receive the detector antibody or (2) did not receive egg extract or serum. The absorbance values in these wells were averaged and subtracted from the experimental absorbance values on the same plate.

Western blot analysis of egg extract

Proteins in the egg extract and 1:100 dilution of *X. laevis* serum were separated by SDS-PAGE. Samples were prepared by adding 4 µl of 6x sample buffer (60% glycerol, 300 mM Tris pH 6.8, 12 mM EDTA, 12% SDS, 864 mM 2-mecaptoethanol, 0.05% bromophenol blue) to 20 µl of egg extract or diluted serum and heating for 5 minutes at 100°C. Samples were added to wells at 10 µl/well and run on a 0.75 mm gel slab at 100 V using a Mini-Protean 3 Cell apparatus (Bio-Rad, Hercules, CA). The gel slab consisted of a 4% polyacrylamide stacking gel, pH 6.8, and a 10% polyacrylamide running gel, pH 8.8. Pre-stained kaleidoscope molecular weight standards (Bio-Rad) were also run on the gel in a separate lane. Separated proteins were transferred on to a nitrocellulose membrane by electroblotting at 25 V overnight at 4°C. The nitrocellulose

blot was blocked with PBS containing 3% NFDM overnight at 4°C. After washing the blot two times with 20 ml PBST for 5 minutes, the blot was incubated with 20 ml mouse anti-*Xenopus laevis* immunoglobulins diluted to 1:1000 in PBS with 1% NFDM. The incubation was carried out on a rocker platform at room temperature for 4 hours. The blot was washed 4 times with 50 ml PBST for 5 minutes. The indicator antibody was 20 ml of a 1:5000 dilution of rabbit anti-mouse IgG conjugated to horseradish peroxidase (Sigma, St. Louis, MO), which was added and incubated on a rocker platform at room temperature for 1 hour. Following another round of washes, 24 ml substrate solution was added to allow visualization of antibody complexes. The substrate was prepared by combining 4 ml of 3 mg/ml 4-chloro-1-naphthol in absolute methanol, 12 µl 30% H_2O_2, and 20 ml PBS.

Statistical analysis

Student's t-tests were performed to determine statistical significance ($p < 0.05$) between the mean absorbance values from the wells coated with TNP-OVA and mean absorbance values from wells coated with OVA for each egg extract and diluted serum.

RESULTS

ELISA to determine presence of anti-TNP specific antibodies in serum and egg extract

An ELISA was performed to determine whether serum from female frogs that were immunized with TNP-BSA contained specific antibodies against TNP. The serum from immunized frogs contained antibodies with reactivity to BSA, TNP-BSA, and TNP-OVA, but not to OVA. The reactivity of antibodies was detected using the mouse anti-*X. laevis* immunoglobulins reagent. Figure 2 shows the ELISA results for serial dilutions from frog #1. These data are representative of sera from all of the frogs.

The egg extracts, which were prepared from eggs collected from immunized female frogs, contained antibodies with anti-TNP reactivity (Fig. 3). After averaging the absorbance values from 1:10 dilutions of all five egg extracts, there was a significantly higher mean absorbance in TNP-OVA-coated wells, 0.787 +/- 0.082 (mean absorbance +/- SEM), than in OVA-coated wells, 0.306 +/- 0.058 ($p < 0.001$, n=5). When comparing mean absorbance values within egg extracts, there was significant binding with TNP-OVA compared to OVA. For example, the mean absorbance for egg extract from frog #1 (E1) in TNP-OVA-coated wells, 1.089 +/- 0.075 (mean absorbance +/- SD), was significantly higher than in OVA-coated wells, 0.573 +/- 0.028 ($p = 0.012$, n=3) (Fig. 3). All other egg extracts contained significantly higher mean absorbance values in TNP-OVA-coated wells than in OVA-coated wells ($p < 0.05$).

Western blot

A Western blot analysis was performed to determine what the mouse anti-*X. laevis* immunoglobulins reagent recognized in the egg extracts. The mouse anti-*X. laevis* immunoglobulins reagent had specific binding capacity for the heavy chain of the *X.*

laevis immunoglobulins in the sera and egg extracts. Figure 4 shows representative results for the Western blot analysis. The mouse anti-*X. laevis* immunoglobulins recognized the 70 kDa heavy chain in all 3 lanes, representing diluted serum from frog #2 and egg extracts from frogs #2 (E2) and #5 (E5). In the 1:100 serum lane (S2), there was a very strong band at the molecular weight of the heavy chain, and there was also apparent binding with the light chain at 27 kDa.

DISCUSSION

In this study, adult female frogs were immunized with the hapten-carrier complex, TNP-BSA, and it was found that the frogs produced antibodies against BSA and TNP-BSA, as expected. To determine if there were antibodies against the hapten (TNP), the ELISA was repeated with TNP-OVA as the antigen. In this case, antibodies were found to be against the hapten, but not OVA. It was, of course, critical to confirm that immunized adult female frogs were producing these antibodies before assays were performed to determine if antigen-specific antibodies were being transferred to the eggs.

In order to perform the assays, an immune reagent was produced to be used as a detector antibody (i.e., mouse anti-*X. laevis* immunoglobulins). Its reactivity was examined by Western blot analysis. The reagent was used to probe a blot that contained separated *X. laevis* serum proteins. The mouse anti-*X. laevis* immunoglobulins reagent detected the heavy and light chains of *X. laevis* immunoglobulin in serum. This analysis confirmed that the reagent could be used to specifically detect *X. laevis* immunoglobulins.

The eggs from all five frogs were processed to remove membranes and lipids. During this procedure, protease inhibitors were added to preserve protein structure during homogenation. Egg extracts were analyzed by ELISA to determine if hapten-specific antibodies were present. All egg extracts were found to contain antibodies against the hapten (TNP). There was not, however, reactivity when OVA was used as the antigen. The presence of anti-TNP antibodies in the egg extracts demonstrates that these antibodies were transferred from adult female frogs to their eggs.

Determining the presence of antibodies in the egg extract provides evidence that maternal transfer of immunity has been conserved in the gnathostome lineage. Antibody

deposition in the egg yolk has been demonstrated in representative species at several branch points of the vertebrate lineage including an elasmobrach, several fishes, and birds (citations above). Therefore, it is hypothesized that the anti-TNP antibodies in the egg extract were located in the yolk of the *X. laevis* eggs and not in the jelly coat. While this process appears to be highly conserved, the functional aspect of these antibodies has yet to be demonstrated. Based on the presence of egg yolk antibodies, it would be parsimonious to believe that these antibodies could impart antigen-specific immunity to the developing embryo and larvae. The process of producing antibodies and transferring antibodies to the egg requires energy and resources that could otherwise be used elsewhere. Further studies should be performed, however, to determine if the presence of antibodies conveys immunological protection to the developing embryo and larva.

Over the course of a year in temperate ecosystems, the immune response in amphibians displays significant plasticity (Raffel *et al*., 2006; Maneiro & Carey, 1997). During long-term periods of low temperature, some immune system parameters (e.g. circulating lymphocyte count) decline to a lower level. This is thought to be due to a trade-off between temperature-dependent proliferation rates of typical pathogens and the costs of immunity. The trade-off is necessitated by the combination of changing environmental temperature and the ectothermic physiology of amphibians. Therefore, it is likely that environmental temperature affects the concentration of antibodies transferred from mother to egg. Some other factors that may affect the maternal transfer of antibodies are nutrient availability, UV exposure (Vermeer & Hurks, 1994), and toxic chemical exposure (Sharma & Reddy, 1987).

The presence of antibodies in eggs might also serve a purpose in epidemiological studies of amphibian populations. When surveying for pathogen or disease prevalence, it may be informative to determine what pathogens were encountered in the previous generation by detecting antibodies in eggs that are specific to pathogens of interest. The present study was conducted in the context of a time period when amphibian populations are declining in several parts of the world at alarming rates. While some declines are due to habitat destruction, there is strong evidence for disease-related declines. The amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), has been implicated in causing population declines, and even extirpation, in Latin America (Lips *et al*., 2006) and Australia (Berger *et al*., 1998). Currently, the mechanism that leads to *Bd*-induced mortality in susceptible species is unknown. Therefore, more studies need to be conducted to better understand the amphibian immune system in the context of large-scale amphibian declines.

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Figure 1. Western blot analysis of the specificity of the mouse anti-*Xenopus* immunoglobulins. Lane 1: Molecular weight markers. Lane 2: 1:100 dilution of mouse anti-*Xenopus* immunoglobulins. Lane 3: 1:200 dilution of mouse anti-*Xenopus* immunoglobulins. Molecular weight standards are expressed in kiloDaltons.

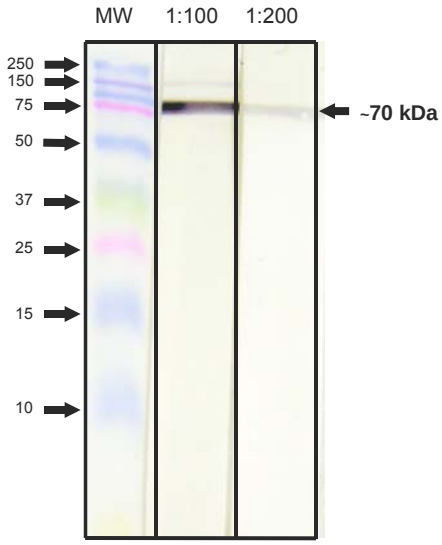

MW
1:100
1:200
250
150
75
50
37
25
15
10
~70 kDa

Figure 2. ELISA results are shown for serial dilutions of serum from frog #1. The dilutions are indicated on the x-axis. For each dilution, the four bars correspond to respective antigens used to coat the wells. The reactivity of 1:1,000 pooled serum from pre-immunized frogs is also shown. Each group was performed in triplicate. The results are representative of those found for all of the remaining frogs. Error bars represent SD.

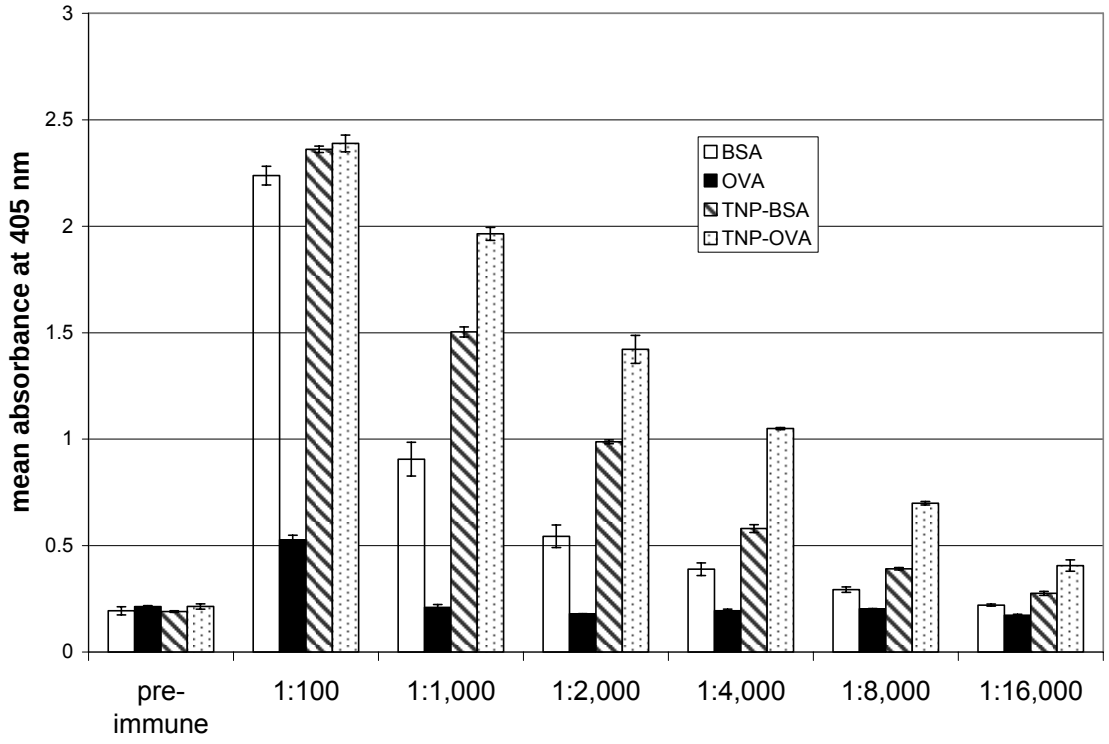

mean absorbance at 405 nm
3
2.5
2
1.5
1
0.5
0
BSA
OVA
TNP-BSA
TNP-OVA
pre-immune
1:100
1:1,000
1:2,000
1:4,000
1:8,000
1:16,000

Figure 3. ELISA results are shown from 1:10 dilutions of all five egg extracts (E1-E5), the mean of all 1:10 dilutions of egg extracts (All E), and 1:1000 dilution of serum from frog #2 (S2). "*" denotes that mean absorbance value is significantly higher in TNP-OVA-coated wells than in OVA-coated wells ($p < 0.05$). For E1-E5 and S2, error bars represent SD; for All E, error bars represent SEM.

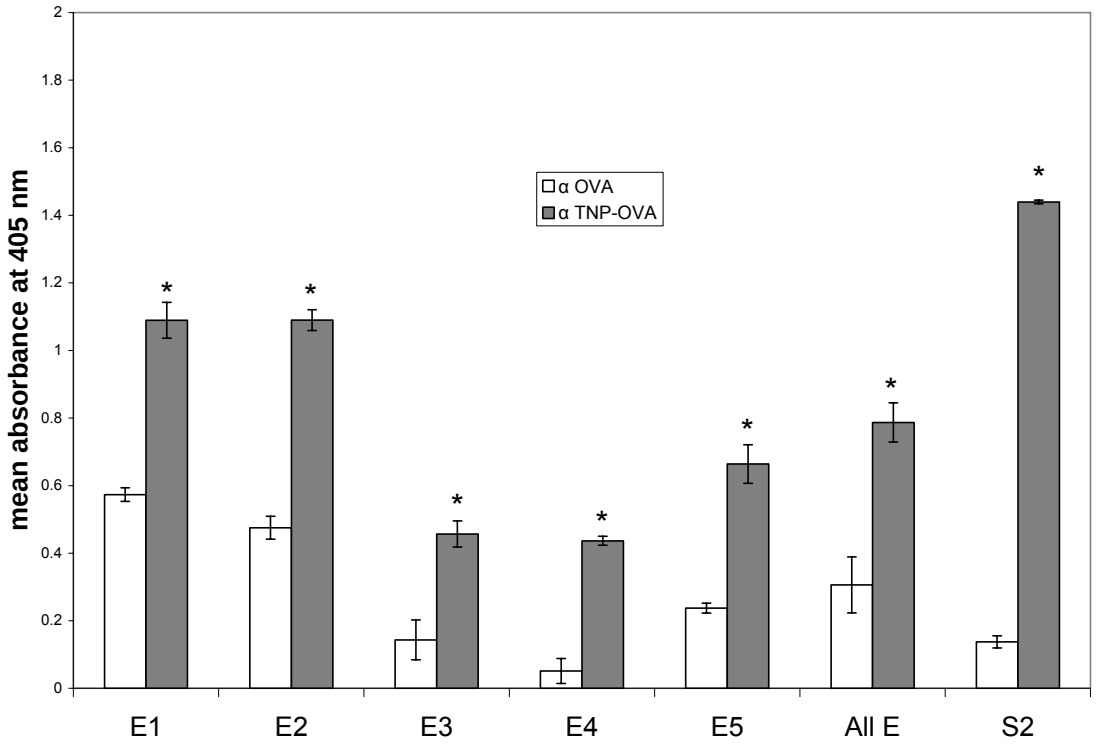
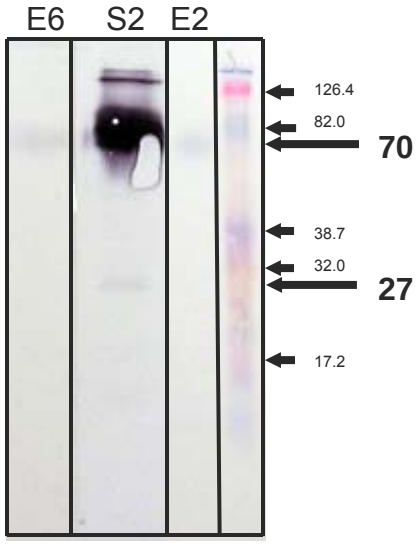

mean absorbance at 405 nm
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
α OVA
α TNP-OVA
*
E1
E2
E3
E4
E5
All E
S2

Figure 4. Western blot results are shown for 1:10 dilution of egg extract 5 (E5), 1:100 serum 2 (S2), and 1:10 dilution of egg extract 2 (E2). Molecular weight standards are expressed in kiloDaltons.


E6
S2
E2
126.4
82.0
70
38.7
32.0
27
17.2

Thomas J. Poorten

WORK EXPERIENCE:

Wake Forest University, 2006-2008
Teacher Assistantship

- Comparative Physiology Lab (BIO 112L), 3 semesters
- Developmental Biology Lab (BIO 360), 1 semester

Aqualutions LLC, Winston-Salem, North Carolina
Research and Development

EDUCATION:

Wake Forest University, Winston-Salem, North Carolina
M.S., Biology (May 2008)
GPA: 3.77

B.S., Biology (May 2006)
Overall GPA: 3.373/4.000 Dean's List (7/8 semesters)
GPA in Major: 3.476/4.000
Beta Beta Beta – Biological Honor Society

School for Field Studies: Summer 2004, Sustaining Tropical Ecosystems in Costa Rica