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Reproductive Biology of the Southern Dwarf Siren, *Pseudobranchus axanthus*, in Southern Florida

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
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University of South Florida

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ABSTRACT

The salamander family Sirenidae is composed of two extant genera, *Siren* and *Pseudobranchus*, each with two known species. Several questions regarding Sirenidae life history persist, and much of the available life history data for *Pseudobranchus* are attributed to studies of *P. axanthus* in northern Florida. Information on the reproductive biology of *P. axanthus* is limited, and historical references often suggest contradictory results. This study was undertaken to clarify information and expand on the limited data regarding *P. axanthus* reproductive biology, specifically for southern Florida populations. The study population was most likely the *P. a. belli* subspecies. *P. axanthus* in southern Florida exhibited year round, continuous reproduction with oviposition documented in nine months. Potential clutch size was positively correlated to female size. The largest observed potential clutch was a female with 58 pre-ovulatory oocytes. The largest observed bout was a female with 15 oviductal eggs. Female *P. axanthus* in southern Florida oviposited eggs singly and at total densities of 3-4 eggs/m^2. Developmental time to egg hatching lasted about 30 days, larvae hatched at 16mm TL, and minimum size at female sexual maturity was 115mm TL and 72mm SVL. The reproductive biology of *P. axanthus* was distinctly contrasting to other members of the family Sirenidae, *S. intermedia* and *S. lacertina*, that occupy the same habitat at the same study location.

INTRODUCTION

The salamander family Sirenidae is composed of two extant genera, *Siren* and *Pseudobranchus*, each with two known species: *S. lacertina*, *S. intermedia*, *P. axanthus*, and *P. striatus*. Its members exhibit a unique combination of primitive, derived, and paedomorphic characters, are permanently aquatic, and inhabit mud bottoms and the roots of dense vegetation in a variety of permanent and semi-permanent aquatic habitats in the southeastern United States and northern Mexico (Martof, 1974; Duellman and Trueb, 1986; Petranka, 1998). Several questions regarding Sirenidae life history persist despite its ecological importance (Fauth and Resetarits, 1991) and potential abundance in appropriate habitat (Petranka, 1998; AmphibiaWeb, 2012). This lack of basic life history data is largely attributed to sirenid habitat characteristics and the difficulties that muddy substrate, dense vegetation, and tannic water present for natural observations. Further, the evidence concerning certain documented aspects of sirenid life history are contradictory and confusing (Godley, 1983).

Life history data provide valuable evidence for reconstructing a group's phylogeny (Dunn, 1923; Noble, 1927; Sever et al., 1996), and the family-level phylogenetic relationships of salamanders is an enduring question (Larson and Dimmick, 1993; Larson et al., 2003; Zhang and Wake, 2009). This question largely persists because of the potential polyphyletic origin of paedomorphosis (Adler, 2003; Larson et al., 2003), internal fertilization (Frost et al., 2006), and sperm storage (Sever, 1994), as well as the difficulty of resolving conflicting morphological and molecular data

(Duellman and Trueb, 1986; Larson and Dimmick, 1993; Wiens et al., 2005; Frost et al., 2006; Zhang and Wake, 2009). The phylogenetic relationship of Sirenidae to other salamanders has been problematic since the original description of *P. striatus* (Le Conte, 1824) and *S. intermedia* (Le Conte, 1828), and Sirenidae's placement among other salamander families has undergone several revisions (Cope, 1889; Goin and Goin, 1962; Duellman and Trueb, 1986; Wiens et al., 2005; Frost et al., 2006; Zhang and Wake, 2009). Several researchers have placed Sirenidae in its own order, Trachystomata (Cope, 1889; Goin and Goin, 1962; Cochran and Goin, 1970), because sirenids possess a unique combination of morphological characteristics (e.g., pelvic girdle and hind limbs are absent, premaxillary teeth are absent and replaced by horny beaks) (Duellman and Trueb, 1986). The family Sirenidae is currently designated to its own suborder, Sirenoidea (Duellman and Trueb, 1986; Larson et al., 2003). Zhang and Wake's (2009) study of salamander mitochondrial genomes reestablished Sirenidae as the basal branch and sister group to all other salamanders, suggesting a divergence date of approximately 183 million years ago (MYA). Reproductive biology provides several useful characters for phylogenetic comparison (Dunn, 1923; Noble, 1927), but remains poorly understood for Sirenidae (Petranka, 1998; Larson et al., 2003; Moler, 2005).

Most peer-reviewed studies regarding Sirenidae have focused on the genus *Siren*; consequently, life history data for *Siren* are better known than for the genus *Pseudobranchus* (Petranka, 1998; Moler, 2005; AmphibiaWeb, 2012). Sirenidae monophyly is supported by shared unique morphological characteristics and molecular data (Duellman and Trueb, 1986; Larson, 1991; Zhang and Wake, 2009), but extensive divergence between the genera *Pseudobranchus* and *Siren* is documented (Liu et al., 2004; 2006). In addition, Zhang and Wake (2009) proposed divergence of the two genera at approximately 65.5 MYA. Sirenidae life history generalizations based solely on data from the genus *Siren* should be avoided because of the extensive divergence of the two genera.

According to three recent and extensive accounts on salamander life history, *Pseudobranchus* spp. home range, territory, longevity, diseases, clutch size, age/size at reproductive maturity, and courtship behavior are unknown (Petranka, 1998; Moler, 2005; AmphibiaWeb, 2012). However, some information regarding reproductive biology (i.e., reproductive cycle, clutch size, developmental time to egg hatching, and age/size at reproductive maturity) is reported in peer-reviewed publications, unpublished theses and dissertations, historic field guides, and husbandry articles. Most of these resources include little to no data to substantiate the information, and in many cases, the information between resources is contradictory.

STUDY SPECIES. — The genus *Pseudobranchus* was originally considered to consist of a single species, *P. striatus*, with five subspecies. Two species of *Pseudobranchus*, *P. axanthus* and *P. striatus*, are currently recognized, and are distinguishable by karyological differences, allozyme differences, and habitat type (Moler and Kezer, 1993; Liu et al., 2004; 2006). *P. striatus* is found in cypress ponds in the acidic pine flatwoods of northern Florida, Georgia, and South Carolina (Moler and Kezer, 1993). *P. axanthus* is precintive to peninsular Florida and inhabits open marshes and prairie ponds, with an affinity for water hyacinth communities (Moler and Kezer, 1993). *P. striatus* has never been collected from water hyacinth communities, despite extensive efforts (Moler and Kezer, 1993). Further, water hyacinth has become the primary habitat for *P. axanthus* throughout peninsular Florida, and it is rarely collected in other substrates (Carr, 1940; Netting and Goin, 1942; Ultsch, 1971; Carr, 1994). Moler and Kezer (1993) documented significant sympatry of *P. striatus* and *P. axanthus* in northern Florida.

Two subspecies of *P. axanthus*, *P. a. axanthus* and *P. a. belli* are currently recognized (Moler and Kezer, 1993; Liu et al., 2006). *P. a. axanthus* occupies northern peninsular Florida, and *P. a. belli* occupies southern peninsular Florida. The dividing latitude is approximately located along the northern edge of Lake Okeechobee (Moler and Kezer, 1993; Petranka, 1998). The northern and southern phylogeographic units were confirmed by analyzing *Pseudobranchus* spp. mitochondrial DNA (mtDNA) sequences (Liu et al., 2006).

Historical references refer to the currently recognized *P. axanthus* as *P. striatus*, *P. striatus axanthus*, or *P. striatus belli* because of the recent identification of *P. axanthus* as a separate species. The majority of the genus *Pseudobranchus* life history data (and most of the accounts regarding reproductive biology) are attributed to researchers at the University of Florida (UF) and the specimens they studied from nearby water hyacinth communities in Alachua County (northern), Florida (Carr, 1940; 1994; Goin, 1941; 1943; 1947; Netting and Goin, 1942; Freeman, 1963; Ultsch, 1971). The Alachua County specimens described by Netting and Goin (1942) and studied by UF researchers have subsequently become the type specimens for *P. a. axanthus* (Moler and Kezer, 1993). The reproductive biology of *P. axanthus* in southern Florida has not been investigated.

REPRODUCTIVE CYCLE. — Several accounts of *Pseudobranchus* spp. reproductive cycle exist, but the data are limited and the results are inconsistent. Noble (1930) documented oviposition in March and April in a laboratory for *P. striatus* from Lakeland, Georgia. Pfaff and Vause (2002) reported an oviposition period of mid-June through August for captive *P. striatus* in South Carolina. Carr (1940) and Goin (1941) reported that oviposition occurs in spring months for *P. axanthus* in northern Florida. Additional research from northern Florida documents *P. axanthus* oviposition in February, March, and November (Goin, 1947) and adult *P. axanthus* females with mature ovarian oocytes from February, March, April, and October (Goin, 1941; Netting and Goin, 1942). Goin did not attempt collection in June, July, or August, but did attempt collection for all other months (Netting and Goin, 1942; Goin, 1943). Freeman (1963) collected *P. axanthus* eggs in every month during wet years and asserted late September and early October as the heaviest egg laying period (collection year unknown) in northern Florida. In a central Florida study, Bancroft et al. (1983) suggested a long reproductive season with some individuals in reproductive condition all year. The *P. axanthus* reproductive season has also been proposed as winter and early spring (Ashton and Ashton, 1988) and November through March (Petranka, 1998). The reproductive cycle for *P. axanthus* in southern Florida has not been investigated.

CLUTCH SIZE. — Here, a clutch is defined as the total number of eggs deposited per ovulation event (Altig and McDiarmid, 2007). *Pseudobranchus* spp. lay eggs singly and far apart (Noble, 1930; Noble and Richards, 1932; Goin, 1941; 1947) which has precluded the direct observation of clutch size in natural settings (Goin, 1947). Noble (1930) documented *P. striatus* females from Georgia ovipositing one to ten eggs in a laboratory. Ashton and Ashton (1988) suggested that females may lay up to a dozen eggs. Goin (1941) estimated that *P. axanthus* clutch size can reach 40-50 eggs and later proposed that clutch size can reach 100 eggs (Goin and Goin, 1962; 1971) based on ovarian oocyte counts in females from northern Florida. Clutches of up to five eggs have also been reported in northern Florida (Freeman, 1963). Clutch was not specifically defined in the previous studies. Clutch size for *P. axanthus* in southern Florida has not been studied.

DEVELOPMENTAL TIME TO EGG HATCHING. — Information concerning the genus *Pseudobranchus* developmental time to egg hatching is limited. Godley (1978) suggested an egg development time of 21 – 28 days. An egg hatching time of 24 days is documented for *P. axanthus* reared in captivity (Kowalski, 2004).

AGE OR SIZE AT SEXUAL MATURITY. — *Pseudobranchus striatus* in southern Georgia reach sexual maturity within one year (Moler, 2008), and Ashton and Ashton (1988) suggested that it takes up to two years for *Pseudobranchus* spp. to reach sexual maturity. Mature *P. axanthus* in northern Florida usually exceed 115mm total length (TL) or 70mm snout-vent length (SVL) (Netting and Goin, 1942). *P. axanthus* in southern Florida are smaller than northern Florida specimens (Netting and Goin, 1942; Schwartz, 1952; Freeman, 1959), but there are no data for size at maturity for *P. axanthus* in southern Florida.

This study was undertaken to clarify contradictory information and expand on the limited data regarding *P. axanthus* reproductive biology, specifically for southern Florida populations. Aspects of *S. intermedia* reproductive biology varies geographically (Petranka, 1998), including reproductive cycle, clutch size, and embryo/larval development (Noble and Marshall, 1932; Cagle and Smith, 1939; Davis and Knapp, 1953; Collette and Gehlbach, 1961; Gehlbach and Kennedy, 1978; Godley, 1983; Trauth et al., 1990; Raymond, 1991). Information from this study may also help to identify any geographic variation in *P. axanthus* reproductive biology.

I quantitatively address southern Florida *P. axanthus* reproductive biology, including: 1) reproductive cycle, 2) clutch size, 3) developmental time to egg hatching, and 4) size at maturity. The results are compared to the reproductive biology of *S. intermedia* in southern Florida to evaluate the variation in life history strategies of sirenid salamanders.

MATERIALS AND METHODS

COLLECTION SITE. — *Pseudobranchus axanthus* were collected by J. S. Godley and associates at Rainey Slough, Glades County, Florida (Figure 1). The Rainey Slough collection site is located at the County Road 731 bridge (26°59’03.66”N, 81°29’30.80”W) in Section 23, Township 40S, and Range 28E. Rainey Slough is a linear system of freshwater marshes and ephemeral wet prairies that drain into Fisheating Creek and eventually into Lake Okeechobee (Godley, 1980). Rainey Slough is bordered by agricultural land, pine flatwoods, and oak hammock (Figure 2) and is representative of a typical south Florida slough ecological community (USDA, 1989). The system is bisected north to south by canals that border either side of County Road 731 and are the result of excavation for road creation (Figure 3). Each canal is hydrologically connected to the slough system on one side, and at times, the canals provide the only standing water in Rainey Slough (Godley, 1980). The canals are covered by the non-native water hyacinth (*Eichhornia crassipes*) which forms a uniform, floating layer of dense, interlocking vegetation (Godley, 1980). An excellent description of Florida water hyacinth communities is provided by Carr (1994).

Glades County, Florida experiences mild winters and distinct wet (June – September) and dry (October – May) seasons (Figure 4). Approximately 60% of the annual rainfall occurs during the four-month wet season (Weatherbase, 2012).

Historical climate data (i.e., temperature and rainfall) were acquired from www.weatherbase.com and represent 81 years of data (1930-2011) collected from

Labelle, Glades County, Florida (Weatherbase, 2012). Labelle, Florida is approximately 25km south of the Rainey Slough collection site. Rainfall data for collection years were acquired from the South Florida Water Management District (SFWMD) for Palmdale, Glades County, Florida (SFWMD, 2012). Palmdale, Florida is approximately 18.5km east of the Rainey Slough collection site.

COLLECTION SPECIFICS. — The hyacinth-choked canals at Rainey Slough were sampled 20 times from November 1974 through October 1976, and an additional three times from April 1977 through May 1979 (Godley, 1980). Collection occurred at least once in each calendar month. The hyacinth community was sampled by dredging portions of the floating hyacinth mat and sifting through the plant material for entrapped fauna. A hyacinth sieve similar to a Goin dredge (Goin, 1942), as described by Godley (1982), was used for collection (Godley, 1980). The hyacinth sieve was capable of collecting $0.56m^2$ of hyacinth per dredge (Godley, 1980).

The Rainey Slough *P. axanthus* collection was deposited at the Smithsonian Institution National Museum of Natural History (NMNH) and totals 503 specimens, including 17 eggs, 12 larvae, and 474 juveniles and adults (USNM 289096-289569). I examined 100 specimens for this project, representing a stratified random sample. Accession numbers for all examined specimens are provided in Appendix 1. Specimens were selected based on size and sex (sex was distinguishable if the specimen had previously been dissected). I attempted to examine at least three sexually mature females and two males per collection month. I chose additional specimens along a size gradient to determine the minimum size at sexual maturity. The SVL of the entire Rainey Slough *P. axanthus* collection and the SVL of all examined specimens are shown in Figure 5.

SUBSPECIES IDENTIFICATION. — The Rainey Slough collection consists of *P. axanthus* according to the collection habitat type and range (Moler and Kezer, 1993; Liu et al., 2006), and are most likely a member of Liu et al.'s (2006) southern phylogeographic group that correspond to *P. a. belli*. However, Moler and Kezer (1993) collected *P. axanthus* from Glades County, Florida that did not adhere to Schwartz's (1952) type specimen description of *P. a. belli*, and they suggested a potential zone of interbreeding between the subspecies. As a result, I collected morphological data for the Rainey Slough specimens to determine the appropriate subspecies. The southern phylogeographic unit in Liu et al.'s (2006) study consisted of specimens from Sarasota and Glades County, and the Glades County collection site was approximately 15km from Rainey Slough.

Pseudobranchus axanthus belli can be distinguished from *P. a. axanthus* by costal groove count (29-33 vs. 34-37), color (brown vs. gray), stripe pattern (distinct, wide vs. pale, indistinct), and head shape (narrow, pointed vs. bluntly rounded) (Netting and Goin, 1942; Schwartz, 1952; Cochran and Goin, 1970; Martof, 1972) (Table 1). For each examined specimen, I collected data on color pattern (i.e., light, dark, or intermediate), stripe pattern (i.e., distinct/wide stripes, indistinct/pale stripes, or other), and head shape (i.e., tapered/pointed snout, bluntly rounded snout, or other). Costal grooves and vertebrae counts will be examined in a subsequent study.

QUANTIFICATION OF REPRODUCTIVE BIOLOGY. — I collected a suite of general and gonadal morphometric data to quantify aspects of *P. axanthus* reproductive biology. Preservation causes specimen shrinkage (Simmons, 2002) and can potentially distort the relationships among morphometric data (Lee, 1982). However, all Rainey Slough specimens were preserved in the same fashion more than 30 years ago, and it is expected that morphometric comparisons within the collection are appropriate. I measured TL and SVL to the nearest 1mm with a standard metric ruler and measured blotted dry mass to the nearest 0.01g with a digital scale. Prior to recording mass, I removed each specimen from its storage container (immersed in 70% ethanol), allowed it to drip for three seconds, and blotted dry with a disposable towel for five seconds. Specimen identification tags did not skew mass comparisons because most specimens had identical field and USNM identification tags. A few specimens had larger type locality tags that skewed mass measurements; these specimens were not used in analyses with mass as a variable.

I dissected each specimen (if not previously dissected) to ventrally expose the pleuroperitoneal cavity. Dissections were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of South Florida (USF), IACUC protocol number T3808. I recorded all gonad measurements to the nearest 0.01mm with digital calipers. Male and juvenile gonad length and multiple widths (i.e., anterior end, middle, and posterior end) were measured *in situ*. Testes size measurements were collected to determine if testes sizes were uniform between months and seasons.

Females with pigmented, vitellogenic ovarian oocytes were considered sexually mature. I documented ovary location (left vs. right) and position (anterior vs. posterior) prior to removing the ovaries. Previous reports have suggested the right testis is always anterior (Willet, 1965), but no data were presented for the position of female ovaries. Once removed, I measured ovary length and maximum width to the nearest 0.01mm with digital calipers and mass to the nearest 0.01g with a digital scale.

For each ovary, I performed oocyte counts and assigned oocytes to one of five size classes. Oocyte descriptions for each of the five size classes corresponded to the salamander follicular maturation stages described by Uribe (2003a; 2009) (Table 2). Uribe's (2003a; 2009) follicular maturation stage classes are largely described by oocyte nuclear development, and the size classes I utilized were based on oocyte size, pigmentation, and development of the animal and vegetal poles. Figure 6 shows an example ovary with the size classes and size (diameter in mm) labeled for representative oocytes. Counts were obtained for oocytes that were visible from the exterior of the ovary. The larger, pigmented oocytes (size classes 3-5) were always visible, regardless of position within the ovary. Size classes 1 and 2 were small enough to be embedded within the ovary and not always visible from the exterior. Oocyte counts for size classes 1 and 2 may be underrepresented.

I considered oviposited eggs and females with oviductal eggs as documented evidence of *P. axanthus* oviposition for the collection month. Females with oviductal eggs represent documented oviposition for the collection date because the amount of time between oocyte ovulation and egg oviposition is typically only a few hours in oviparous amphibians (Rugh, 1951). Leopard frog (*Lithobates pipiens*) eggs take a total of four to six hours to reach the ovisac after ovulation: two hours from ovulation to entering the ostium of the oviduct and two to four more hours to pass through the oviduct (Rugh, 1951). Eggs are rarely retained in the ovisac for more than one day (Rugh, 1951).

I used the presence of pre-ovulatory oocytes, oviductal eggs, and oviposited eggs in addition to oocyte counts and oviductal egg counts to compare female reproductive condition between months and seasons (i.e., wet vs. dry seasons and six-month seasons).

Godley (1983) identified a six-month reproductive season for *Siren intermedia* at Rainey Slough (October – March). Consequently, I used six-month seasons of April – September and October – March to investigate seasonal changes in reproductive condition in *P. axanthus*, and to compare female reproductive condition between members of Sirenidae. I also used oocyte counts to determine potential clutch size and the presence of pigmented, vitellogenic oocytes to determine the minimum size at female maturity.

Cloacal morphology was examined for each specimen as a potential sexually dimorphic character. Netting and Goin (1942) reported that reproductively active females may exhibit swelling around their vent. In contrast, Willet (1965) reported that male and female cloacas are similar and not useful for externally identifying sex.

I obtained a standard suite of photos for all examined specimens including photos of the dorsum, left side, venter, opened pleuroperitoneal cavity (prior to ovary removal), testes and undifferentiated gonads *in situ*, and removed ovaries. All photos are to be retained at the NMNH as part of the Rainey Slough *P. axanthus* collection.

DEVELOPMENTAL TIME TO EGG HATCHING. — Godley and associates conducted a laboratory experiment in 1976 on *P. axanthus* developmental time to hatching. *P. axanthus* eggs were collected at Rainey Slough on 7-8 February 1976 (*n* = 38) and 14-15 February 1976 (*n* = 61) and transported to USF. Eggs (*n* = 99) were maintained in the lab in glass dishes at 23°C ± 1°C under ambient laboratory light (Godley, 1980). Over the course of egg hatching, water was replaced in the glass dishes 3-4 times with well water. Eggs were counted, the number of hatchlings recorded, and larvae were removed from the glass dishes daily, until all eggs hatched.

STATISTICAL ANALYSES. — I calculated all statistics with STATISTICA 10 (StatSoft, 2011). All means are presented as the mean ± one standard deviation. An alpha level of 0.05 was used to determine significance for all statistics. A Bonferroni adjusted alpha level was used for tests with multiple comparisons. I used Shapiro-Wilk tests to evaluate data normality.

Morphological characters were evaluated (count and percentage data) to determine Rainey Slough *P. axanthus* subspecies. General morphometric data (SVL and mass) were used to test for correlation between size variables. Body mass was not normally distributed; consequently, I used non-parametric tests for analyses. I used Spearman's rank correlation coefficient to determine if SVL and mass were significantly correlated ($\alpha = 0.05$) and regression analyses to determine the regression equation. The sex of specimens with swollen, prominent cloacas were recorded to determine if cloaca morphology is a useful character to externally identify sex.

I used a chi-square (X^2) goodness-of-fit test to determine the frequency of ovary location and position ($\alpha = 0.05$). Ovary sizes were not normally distributed, so I used Mann-Whitney U tests to determine if ovary sizes and number of ovarian oocytes differ by ovary location and position (Bonferroni adjusted $\alpha = 0.01$).

I used an analysis of covariance (ANCOVA) to compare the mean number of oocytes per female between months and seasons (Bonferroni adjusted $\alpha = 0.003$) to determine *P. axanthus* reproductive cycle. SVL and $\log_{10}$ transformed mass were used as covariates. Mass was $\log_{10}$ transformed to establish linearity for analyses.

General morphometric data (SVL and mass) were used to test for correlation between size variables and correlation with oocyte counts. Mass and oocyte counts were not normally distributed; therefore, I used non-parametric tests for analyses. I used Spearman's rank correlation coefficient to determine if the variables were significantly correlated and regression analyses to determine the regression equation. I tested correlation between SVL and number of oocytes per size class (Bonferroni adjusted $\alpha = 0.01$), mass and number of oocytes per size class (Bonferroni adjusted $\alpha = 0.01$), SVL and potential clutch size ($\alpha = 0.05$), and mass and potential clutch size ($\alpha = 0.05$).

Maximum number of days to egg hatching and cumulative percentage of hatched eggs were used to determine developmental time to egg hatching. A scatter plot of TL and SVL of mature females was used to determine minimum size at female maturity.

RESULTS

IDENTIFICATION OF MALES, FEMALES, AND JUVENILES. — Of the 100 specimens examined from the Rainey Slough *Pseudobranchus axanthus* collection, 53 were sexually mature females with at least one or more pigmented, vitellogenic oocytes. Six specimens were clearly juveniles with immature, undifferentiated gonadal tissue, and seven specimens lacked gonads because of previous removal for histological examination. Twelve specimens appeared to match Ogielska and Bartmańska's (2009a) description of mature males with testes in active spermatogenesis (i.e., gonads appeared thick, spongy, and bulbous). The remaining 22 specimens had visually indistinguishable gonads. Their gonadal tissue had a flat and flaccid texture, distinctly different from the spongy and bulbous tissue of the active testes. The size (length and width) of the visually indistinguishable gonads ranged from smaller to larger than the active testes. The visually indistinguishable gonads generally agreed with the morphology of developing anuran gonads (Ogielska, 2009; Ogielska and Bartmańska, 2009a; 2009b), and it is likely that at least some of the specimens were juveniles with developing testes or ovaries. However, some of the flat and flaccid gonads were observed in *P. axanthus* with a relatively large size (SVL) that was comparable to or larger than individuals that were clearly mature females and males. Results and discussion only address female data because of the uncertainty in properly identifying males and juveniles at this time.

SUBSPECIES IDENTIFICATION. — Original specimen color was not distinguishable because of preservation (Simmons, 2002), and therefore, was not a useful character for subspecies identification of the specimens. However, the shade (e.g., dark, light) remained noticeable, and the majority of the examined specimens ($n = 47$, 88.7%) exhibited a dark base coloration. Stripe pattern adhered to the *P. a. belli* characteristic of distinct, wide stripes ($n = 53$, 100%) (Figure 7).

Head shape was variable in the examined specimens (Figure 8). The Rainey Slough collection contained head shapes that are reported as identification characters for all known *Pseudobranchus* species and subspecies: *P. axanthus belli* – long, narrow, tapering, and pointed (Netting and Goin, 1942; Schwartz, 1952; Martof, 1972), *P. axanthus axanthus* – truncate and bluntly rounded (Netting and Goin, 1942; Martof, 1972), *P. striatus striatus* – pointed and acute outline (Netting and Goin, 1942), *P. striatus lustriculous* – abruptly blunt and truncate (Neil, 1951; Martof, 1972), and *P. striatus spheniscus* – narrow and wedge shaped (Goin and Crenshaw, 1949; Martof, 1972). In addition, a broad, rounded, and truncate head shape was observed that has not been previously described.

GENERAL MORPHOLOGY AND MORPHOMETRICS. — Female SVL and mass are exponentially correlated ($y = 0.1821e^{0.0308x}$, $r = 0.8499$, $P < 0.001$). Mass measurements were $\log_{10}$ transformed to establish a linear relationship with SVL ($y = 0.0134x - 0.7396$, $r = 0.9238$, $P < 0.001$). Transformed mass data were utilized for subsequent analyses.

A prominent, swollen cloaca was observed in both sexually mature females and males. Most (six of eight) females with oviductal eggs had a swollen cloaca, and on 20 June 1976, two females, each with a red and swollen cloaca, were collected along with four eggs.

OVARY MORPHOLOGY AND MORPHOMETRICS. — Ovary size (length, width, and mass) was highly variable among females and was determined by the number of large, yolk-filled, vitellogenic oocytes (Figures 9A-D). *P. axanthus* ovaries were asymmetrically placed in the pleuroperitoneal cavity with one anterior to the other. No pattern exists between ovary location (left vs. right) and position (anterior vs. posterior); the left ovary was not positioned anterior more frequently than posterior ($X^2 = 2.083$, $P <$ 0.05).

No difference exists in *P. axanthus* ovary size (i.e., length, maximum width, or mass) or number of ovarian oocytes between the left and right ovary (Table 3). Respective mean sizes and oocyte counts for the left and right ovaries are: length (22.8 ± 6.55 and 22.5 ± 6.81), maximum width (3.2 ± 1.06 and 3.3 ± 1.17) mass (0.09 ± 0.07 and 0.09 ± 0.08), number of vitellogenic oocytes (32.6 ± 22.22 and 32.0 ± 23.78), and total number of oocytes (370.9 ± 189.26 and 370.1 ± 197.64).

No difference exists in *P. axanthus* ovary size (i.e., length, maximum width, or mass) or number of ovarian oocytes between the anterior and posterior ovary (Table 3). Respective mean sizes and oocyte counts for the anterior and posterior ovaries are: length (24.4 ± 7.24 and 20.9 ± 5.42), maximum width (3.2 ± 1.13 and 3.4 ± 1.12) mass (0.09 ± 0.08 and 0.08 ± 0.07), number of vitellogenic oocytes (34.0 ± 25.33 and 31.1 ± 21.94), and total number of oocytes (370.5 ± 196.29 and 376.8 ± 198.81).

All mature *P. axanthus* females contained ovarian oocytes in various stages of development, and almost all females had all five size classes of oocytes represented simultaneously. Mean oocyte counts per female are 625.3 ± 343.65 size class 1, 52.2 ± 32.91 size class 2, 28.1 ± 21.51 size class 3, 20.6 ± 15.82 size class 4, 15.9 ± 13.60 size class 5 (Table 4). The total number of oocytes for each size class is significantly correlated to female SVL and mass (Table 5).

FEMALE REPRODUCTIVE CYCLE. — *Pseudobranchus axanthus* eggs were collected at Rainey Slough in January 1975 and February, March, June, July, August, and October 1976. Eggs were collected in both the wet and dry seasons and in both of the six-month seasons. Egg density per collection event ranged from 0.89-6.89 eggs/m^2, with a mean egg density of 3.58 ± 1.81 eggs/m^2 (Table 6).

Pseudobranchus axanthus with pre-ovulatory oocytes were collected in all months (and seasons). Females examined from July, August, September, and October 1975 and May 1978 contained oviductal eggs (Figure 10). *P. axanthus* with oviductal eggs were collected in both the wet and dry seasons and in both of the six-month seasons (Figure 11). Oviductal eggs were approximately the same size or slightly larger than the pre-ovulatory (size class 5) oocytes.

The results of the ANCOVA analyses reveal that the number of size class 1 oocytes per *P. axanthus* females are significantly different between months for both SVL and mass covariates. No difference exists in the number of size class 2-5 oocytes per female between months for both SVL and mass covariates (Tables 7A and 7B and Figure 12).

No difference exists in the number of size class 1-5 oocytes per female between wet and dry seasons for both SVL and mass covariates In addition, no difference exists in the number of size class 1-5 oocytes per female between six-month seasons for both SVL and mass covariates (Tables 7A and 7B). The ANCOVA results are not statistically significant, but *P. axanthus* at Rainey Slough are trending toward fewer pre-ovulatory oocytes in the dry season compared to the wet season and fewer pre-ovulatory oocytes from October – March compared to April – September (Figure 13).

CLUTCH SIZE. — The total number of pre-ovulatory oocytes was considered to be the potential clutch size for each female. The mean number of pre-ovulatory oocytes for the 53 females is 15.9 ± 13.60 (Table 8). For the eight females with oviductal eggs, the mean potential clutch size (sum of pre-ovulatory oocytes and oviductal eggs) is 30.1 ± 13.27 (Table 8). However, this assumed that the female was collected while beginning to oviposit her full complement of pre-ovulatory eggs, as there was no way to know how many eggs were oviposited prior to collection. Because the number of oocytes is positively correlated with size (i.e., SVL and mass), *P. axanthus* potential clutch size increased as female size increased (Figures 14A and 14B).

Mean relative clutch mass (the sum of each female's left and right ovary mass divided by the total mass of the specimen) is 0.045 ± 0.0261, or 4.5%. The maximum relative clutch mass recorded was 10.1% (Table 8).

DEVELOPMENTAL TIME TO EGG HATCHING. — Eggs collected on 7-8 February 1976 ($n = 38$) hatched between 9 and 30 days post-collection. The rate of egg hatching was stable over the 22 days, with four eggs as the maximum number that hatched in one day (Figure 15A). One egg did not hatch. Eggs collected on 14-15 February 1976 ($n = 61$) hatched between 8 and 25 days post-collection. The majority of egg hatching (83.3%) occurred between 16-23 days post-collection (Figure 15B). One egg did not hatch. Larvae were approximately 16mm TL at hatching.

FEMALE SIZE AT SEXUAL MATURITY. — The minimum observed size of a female with pigmented, vitellogenic oocytes was 125mm TL and 72mm SVL (USNM 289151).

USNM 289151 had 11 pigmented, yolk-rich ovarian oocytes, but none were pre-ovulatory (size class 5). However, USNM 289125 was 127mm TL and 72mm SVL and had 34 pigmented, yolk-rich ovarian oocytes, 13 of which were pre-ovulatory. Sexually mature females that did not contain pre-ovulatory oocytes ranged in size from 72mm to 95mm SVL. The smallest TL for a mature female was 115mm and the smallest SVL for a mature female was 72mm (Figure 16). Twenty-four specimens were dissected with a SVL smaller than 72mm (ranging from 38mm to 71mm), and none had mature ovaries.

DISCUSSION

IDENTIFICATION OF MALES, FEMALES, AND JUVENILES. — Davis and Knapp (1953) documented similar difficulty in visually distinguishing immature and mature male *Siren intermedia*. The most likely explanations for *Pseudobranchus axanthus* with visually indistinguishable (flat and flaccid gonads) are: 1) *P. axanthus* mature at variable sizes, 2) the large specimens with visually indistinguishable gonads were developing females, and females mature at a larger size than males, and/or 3) the *P. axanthus* population concurrently contained mature, reproductively active males with mature, reproductively inactive males. Changes in salamander testis size and shape associated with different stages of spermatogenesis is well documented (Uribe, 2003b), and Hanlin (1975) documented this phenomenon in *Siren lacertina*. However, it seems unlikely that any adult sized males would be reproductively inactive when females appear to be in reproductive condition year round. These questions will be addressed by histological examination of the presumed active testes and visually indistinguishable gonads in future work.

SUBSPECIES IDENTIFICATION. — The distinct, wide stripe pattern exhibited in the Rainey Slough *P. axanthus* population clearly agrees with Schwartz's (1952) type description for *P. a. belli*, which Schwartz described as a "striking character". In addition, the Rainey Slough collection site is approximately 15km from the southern *P. a. belli* phylogeographic unit identified with mtDNA sequences by Liu et al. (2006).

The head shape of the Rainey Slough specimens was different than the *P. a. belli* type description, and in fact, none of the *Pseudobranchus* subspecies type descriptions document the variable head shape that was observed in the Rainey Slough specimens. All type descriptions clearly identify a unique and presumably consistent head shape (Netting and Goin, 1942; Goin and Crenshaw, 1949; Neil, 1951; Schwartz, 1952; Martof 1972). Further, Netting and Goin (1942) reported uniform head shape in 297 specimens from Alachua County, Florida (the *P. a. axanthus* paratypes).

P. axanthus head shape was more variable than previously reported, and is probably a poor morphological character to use for subspecies identification. Moler and Kezer (1993) suggested that areas in Glades County could represent intergrades of the two *P. axanthus* subspecies, but this does not appear to be supported by Liu et al.'s (2006) results. It is uncertain how valuable costal groove counts may be for subspecies identification because of the variation documented in this character (Netting and Goin, 1942; Schwartz, 1952; Davis and Knapp, 1953). The available data indicate that the Rainey Slough *P. axanthus* population is most likely *P. a. belli*.

GENERAL MORPHOLOGY AND MORPHOMETRICS. — Cloaca morphology results were consistent with Willet (1965) that cloaca morphology is not a definitive character to externally determine sex. In most cases, a *P. axanthus* with a prominent, swollen cloaca was a gravid female, in agreement with *P. axanthus* from northern Florida (Netting and Goin, 1942), but males were also observed with this character.

OVARY MORPHOLOGY AND MORPHOMETRICS. — *P. axanthus* ovaries were asymmetrically placed in the pleuroperitoneal cavity, but were symmetrical in size (i.e., length, maximum width, and mass) and number of oocytes. In addition, there was no pattern observed for which ovary (left or right) was positioned anterior, contrary to previous reports for *Pseudobranchus* testes (Willet, 1965).

FEMALE REPRODUCTIVE CYCLE. — The data associated with egg collection, oviductal eggs, and oocyte development support year-round (continuous) reproduction for *P. axanthus* at Rainey Slough. Continuous reproduction agrees with Freeman's (1963) observations for *P. axanthus* in northern Florida and with Bancroft et al.'s (1983) suggestion. Even though oviposition (i.e., egg collection or females with oviductal eggs) was not documented in every month (i.e., April, November, and December), oviposition was documented in enough months to strongly suggest that *P. axanthus* are at least capable of continuous reproduction. *P. axanthus* oocyte development was asynchronous, as most females had all size classes of oocytes concurrently, and pre-ovulatory oocytes were present year-round. The Rainey Slough population had females in vitellogenesis throughout the year (i.e., females were continuously maturing and yolking oocytes).

The obvious question is why was oviposition not documented in April, November, and December? The absence of oviposition data in April was attributed to sample size, as the Rainey Slough collection contained only one mature female. But, is the contiguous block of November-December meaningful? One potential explanation is that *P. axanthus* are capable of continuous reproduction, but only breed when stimulated by an external environmental cue (e.g., rainfall). Because all of the unsubstantiated months were within the south Florida dry season, there could have been a lack of stimulus to breed, as rainfall is the primary factor that initiates amphibian breeding activity (Duellman and Trueb, 1986). Kowalski (2004) provided details on breeding *P. axanthus* in captivity, and noted that introducing large amounts of distilled or deionized water to the tank (simulating a decrease in water hardness due to rainfall) will stimulate courtship and breeding. In addition, I reviewed rainfall data for all dates when females with oviductal eggs were collected. Females with oviductal eggs were presumably collected while actively ovulating or were collected within a few hours of ovulation (Rugh, 1951). Therefore, the environmental data for the day of or for the day preceding collection provided valuable information on any environmental cues that may have stimulated the females to ovulate. In all cases, females with oviductal eggs were collected on a day with rainfall or on a day following rainfall. In four of the five instances, the rainfall event was preceded by several days without rain. In addition, these collection days yielded multiple females with oviductal eggs.

These data suggest that oviposition may be stimulated by rainfall. However, the egg collection events from February 1976, which yielded the greatest number of eggs collected during the study, were not associated with any rainfall events. The 7-8 and 14-15 February 1976 egg collection events were preceded by 24 and 31 days without rainfall, respectively (SFWMD, 2012).

I suggest that *P. axanthus* breeds year-round, but not necessarily synchronously, as long as conditions are appropriate (i.e., surface water is present). Synchronous reproduction may be stimulated by external cues (i.e., rainfall) explaining the correlation between females with oviductal eggs on or after days with rain and oviposition after the introduction of deionized water for captive individuals.

Egg collection data verified the historic accounts that *P. axanthus* oviposit eggs singly and far apart (Noble, 1930; Noble and Richards, 1932; Netting and Goin, 1942).

P. axanthus at Rainey Slough oviposited eggs at a total mean density of approximately 3-4 eggs/m^2.

CLUTCH SIZE. — Historical reports of clutch size vary from less than a dozen eggs (Noble, 1930; Freeman, 1963; Ashton and Ashton, 1988) to 50-100 eggs (Goin, 1941; Goin and Goin, 1962; 1971). It appears that all reports may be correct to a certain extent. A wide range of pre-ovulatory oocytes were observed for Rainey Slough *P. axanthus* females, and potential clutch size ranged from 0 to 58 eggs. The largest female within the collection (*n* = 503) was examined, and exhibited a potential clutch of 56 pre-ovulatory oocytes. Because potential clutch size is positively correlated to female size, and the largest female was examined, 60 eggs is probably an appropriate estimate for maximum potential clutch size for *P. axanthus* at Rainey Slough.

It is not known if the total number of pre-ovulatory oocytes is an accurate projection of clutch size. The pre-ovulatory oocyte data clearly show potential for large clutches, in agreement with Goin (1941) and Goin and Goin (1962; 1971). However, the egg collection data and egg hatching data indicate that a few eggs are oviposited at a time, in agreement with Noble (1930), Freeman (1963), and Ashton and Ashton (1988).

Sever et al. (1996) reported on the oviductal anatomy of *Siren intermedia* and commented that the ovisac (posterior portion of the oviduct) cannot hold more than 10-11 eggs. The oviductal anatomy of *P. axanthus* agrees with Sever et al.'s (1996) description of *S. intermedia*, and two *P. axanthus* females were examined with eggs in their ovisac, one with eleven and one with four (Figure 17). This may indicate that *P. axanthus* oviposit their clutches in several small bouts (*sensu* Altig and McDiarmid, 2007) with the capacity of the ovisac representing the number of eggs per bout. This process could explain why *P. axanthus* appear to be capable of ovipositing large clutches, but the limited egg collection data suggests oviposition of only a few eggs. At Rainey Slough, the observed maximum potential bout was a female with fifteen oviductal eggs.

DEVELOPMENTAL TIME TO EGG HATCHING. — The egg hatching data are difficult to interpret with regards to oviposition time because of the somewhat contrasting results from the two collection events. If treated separately, the relatively stable egg hatching timeline of the 7-8 February data indicate that *P. axanthus* oviposited their clutch in relatively small bouts, possibly over several days. In contrast, the 14-15 February data had a distinct concentration with 83% egg hatching over about a week's time, possibly suggesting a heavier oviposition period for a few days. The variation in hatching rates between the two samples could have been caused by differences in the number, body size, and reproductive condition of the females that oviposited these eggs, and/or a differential rate in egg predation. Or, perhaps an unapparent environmental stimulus could have caused differences in the timing of oviposition and number of oviposited eggs between the two collection events.

Maximum hatching dates of 30 and 25 days post-collection suggest that developmental time to hatching for southern Florida *P. axanthus* is approximately one month. Kowalski (2004) reported a development time of 24 days for *P. axanthus* eggs reared in captivity at 22°C. Both results agree with Godley (1978), who suggested a hatching time of three to four weeks for *Pseudobranchus* spp.

Pseudobranchus axanthus eggs were robust, as 97 out of 99 collected eggs successfully hatched. The larvae hatching size of 16mm TL agrees with *P. axanthus* hatching size of 14.5-16mm TL in northern Florida (Goin, 1947).

FEMALE SIZE AT SEXUAL MATURITY. — Southern Florida females exhibited sexual maturation and oogenesis at a minimum size of 115mm TL and 72mm SVL. These results agree with Netting and Goin's (1942) comment that mature *P. axanthus* usually exceed 115mm TL or 70mm SVL in northern Florida. It is not clear if live or preserved measurements were reported by Netting and Goin (1942). Limited live TL and SVL measurements were available for the Rainey Slough *P. axanthus* (n=5) and *S. intermedia* (n=6) collections. Specimen shrinkage averaged 5.7% for the 11 specimens. An estimate for female size at sexual maturity for live *P. axanthus* at Rainey Slough is 121.6mm TL and 76.1mm SVL.

Size at sexually maturity was variable, as seven females larger than 72mm SVL had few pigmented, vitellogenic oocytes and no pre-ovulatory oocytes. The largest female without pre-ovulatory oocytes measured 139mm TL and 95mm SVL, 32% larger than the smallest female with pre-ovulatory oocytes.

VARIATION IN REPRODUCTIVE STRATEGIES OF SIRENID SALAMANDERS. — Three of the four species of the family Sirenidae occupy the water hyacinth community at Rainey Slough, *Siren intermedia* and *Siren lacertina* in addition to *Pseudobranchus axanthus* (Godley, 1983). Godley (1983) described several aspects of the Rainey Slough *S. intermedia* reproductive biology, and the results are distinctly contrasting to *P. axanthus*. Rainey Slough *S. intermedia* have a defined reproductive season in which vitellogenesis begins in October, females have pre-ovulatory oocytes from December through March, and females are spent by April. Females do not exhibit continuous vitellogenesis and do not concurrently contain multiple sizes of vitellogenic oocytes. *S. intermedia* oviposit a large clutch of eggs (nests of 206 and 362 eggs were discovered), and females guard their nests. Larvae exhibit synchronous hatching at about 11.5mm TL. Rainey Slough male *S. intermedia* bite females as a courtship behavior (Godley, 1983). In the 100 *P. axanthus* I examined, none had bite marks from conspecifics. However, male *P. axanthus* will use their head to nudge the female's cloaca as a courtship behavior (Kowalski, 2004). Both *P. axanthus* and *S. intermedia* attach their eggs to submerged aquatic vegetation at Rainey Slough (Godley, 1983).

The reproductive biology for *S. lacertina* at Rainey Slough has not been investigated, but *S. lacertina* larvae were collected at Rainey Slough in March and April 1977 indicating synchronous breeding with *S. intermedia*. Also, *S. lacertina* reproductive season has been defined as February through March for northern Florida (Ultsch, 1973) and Alabama (Hanlin and Mount, 1978), and as approximately January through April for central Florida (Bancroft et al., 1983).

Clutch size data for *Siren* spp. is unclear. Large clutch/nests (> 200 eggs) have been observed in the field (Noble and Marshall, 1932; Gehlbach and Kennedy, 1978; Godley, 1983) and females with several hundred mature, pre-ovulatory oocytes have been observed through dissection (Noble and Marshall, 1932; Cagle and Smith, 1939; Trauth et al., 1990). Hanlin and Mount (1978) documented a female *S. lacertina* with over 1,400 pre-ovulatory oocytes. However, all oviposition observations (in laboratories) document that eggs are deposited singly or in groups of less than ten (Noble and Richards, 1932; Ultsch, 1973). Also, *S. intermedia* ovisac capacity is 10-11 eggs (Sever et al., 1996). This may indicate that relatively few follicles are ovulated at one time and that eggs are laid in small groups (Sever et al., 1996).

Siren intermedia and *Siren lacertina* exhibit the same reproductive strategy of annual reproduction, a defined synchronous reproductive season, associated spermatogenesis and oogenesis, and large clutch sizes (Hanlin and Mount, 1978; Godley, 1983; Sever et al., 1996). This strategy is retained throughout their range even though oviposition time and clutch size show geographic variability (Noble and Marshall, 1932; Cagle and Smith, 1939; Davis and Knapp, 1953; Collette and Gehlbach, 1961; Gehlbach and Kennedy, 1978; Godley, 1983; Trauth et al., 1990; Raymond, 1991).

CONCLUSIONS

Pseudobranchus axanthus Reproductive Strategy. — This study provides some of the first quantitative data to clarify, confirm, and describe several aspects of *Pseudobranchus axanthus* reproductive biology. Southern Florida *P. axanthus* size at maturity and larvae size at hatching was similar to previously reported data from northern Florida, and therefore, these traits do not appear to vary geographically. Previous reports for reproductive cycle and clutch size appeared to be contradictory, but this may have been the result of incomplete data and failure to provide a clear definition for clutch. Reports for reproductive cycle and clutch size of northern populations of *P. axanthus* are congruent with *P. axanthus* from Rainey Slough.

P. axanthus in southern Florida were reproductively active year round and appear capable of two oviposition strategies. I suggest that when standing water is present, *P. axanthus* exhibit continuous reproduction by oviposition of small bouts or clutches of probably ten or fewer eggs. This reproductive strategy is typical of tropical and subtropical amphibians that breed in areas where conditions are appropriate most of the year (Duellman and Trueb, 1986; Patton and Harris, 2010). Continuous oviposition of small clutches allows females to reduce reproductive effort and scatter eggs, bouts, or clutches to enhance egg survivorship (Duellman and Trueb, 1986).

I also suggest that *P. axanthus* reproduce synchronously if stimulated by an environmental cue (i.e., rainfall). Reproduction stimulated by rainfall may result in ovulation of a larger clutch, perhaps up to 60 eggs. This reproductive strategy is typical of amphibians from areas that have seasonal limitations that require explosive breeding, e.g., temperate regions and ephemeral aquatic habitats (Duellman and Trueb, 1986; Patton and Harris, 2010).

A variable reproductive strategy is advantageous because *P. axanthus* inhabit both permanent and ephemeral aquatic habitats. This is not unique among salamanders, as *Batrachoseps attenuatus* demonstrates a variable clutch size and reproductive cycle that maximizes reproductive effort based on seasonal fluctuations and available resources (Maiorana, 1976). Maiorana (1976) concluded that environmental unpredictability can be a major selection pressure resulting in life history flexibility.

Independent of reproductive strategy, female *P. axanthus* in southern Florida oviposited eggs singly at total densities of approximately 3-4 eggs/m^2. Developmental time to egg hatching lasted about 30 days, larvae hatched at 16mm TL, and minimum size at female sexual maturity was 115mm TL and 72mm SVL.

VARIATION IN REPRODUCTIVE STRATEGIES OF SIRENID SALAMANDERS. — The genus *Siren* and *Pseudobranchus axanthus* have contrasting reproductive strategies within the same habitat at Rainey Slough and throughout their ranges. Life history data for *P. striatus* is especially fragmentary (Petranka, 1998; Moler, 2005) and is not included for comparison. *P. axanthus* and *Siren* spp. demonstrate opposing breeding frequency, clutch size, size at larval hatching, and courtship behavior. *P. axanthus* demonstrates greater flexibility in reproductive strategy than *S. intermedia* and *S. lacertina*.

Table 1. Distinguishing characteristics of *Pseudobranchus axanthus axanthus* and *Pseudobranchus axanthus belli* subspecies.

	Costal Groove Count	Color	Stripe Pattern	Head Shape
P. a. axanthus	34-37[1]	dark gray[1, 3]	narrow, pale, indistinct [1, 3, 4]	truncate, bluntly rounded[1, 4]
P. a. belli	29-33[2]	brown with gray venter[2, 4]	distinct, wide[2]	long, narrow, tapering[2,4], pointed[1]

[1] Netting and Goin (1942)

[2] Schwartz (1952)

[3] Cochran and Goin (1970)

[4] Martof (1972)

Table 2. Description of size classes used to group *Pseudobranchus axanthus* ovarian oocytes and the corresponding follicular maturation stage class.

	Oocyte Description[1]	Follicular Maturation Stage[2]
Size Class 1	no pigmentation; distinctly previtellogenic; ≈ 0.25 – 0.5mm	Previtellogenesis, Stage 2
Size Class 2	little to no pigmentation; some showing initial yolk accumulation; ≈ 0.6 – 0.85mm	Vitellogenesis, Stage 3
Size Class 3	distinctly vitellogenic; marked yolk accumulation and melanin pigment deposition; ≈ 0.85 – 1.0mm	Vitellogenesis, Stage 4
Size Class 4	increased darkening due to melanin deposition; does not display distinct animal and vegetal poles; ≈ 1.0 – 1.8mm	Vitellogenesis, Stage 5
Size Class 5	distinct animal and vegetal poles; ≈ 1.8 – 2.25mm	Preovulatory, Stage 6

[1] Sizes (mm) refer to oocyte diameter.

[2] Uribe (2003a; 2009)

Table 3. Comparison of ovary size and number of ovarian oocytes by ovary location (left vs. right) and ovary position (anterior vs. posterior). Analyses performed with a Mann-Whitney U test (*U*).

	Ovary Location		Ovary Position	
	Left vs. Right		Anterior vs. Posterior	
	U	*P*	*U*	*P*
Ovary Length	1,338.0	0.677	815.5	0.014
Ovary Width	1,325.5	0.620	1,002.5	0.275
Ovary Mass	1,380.5	0.882	1,086.0	0.631
Number of Pigmented Oocytes	1,374.0	0.850	1,094.0	0.674
Total Number of Oocytes	1,385.0	0.904	1,129.5	0.872

n = 53 for all variables.

Bonferroni adjusted $\alpha = 0.01$.

Table 4. Descriptive statistics for Rainey Slough *Pseudobranchus axanthus* oocyte counts.

	Mean ± 1 SD	Minimum – Maximum
Oocyte Size Class 1	625.3 ± 343.65	11 – 1,397
Oocyte Size Class 2	52.2 ± 32.91	6 – 164
Oocyte Size Class 3	28.1 ± 21.51	1 – 105
Oocyte Size Class 4	20.6 ± 15.82	0 – 72
Oocyte Size Class 5	15.9 ± 13.60	0 – 56

n = 53 for all size classes.

Statistics calculated using the total oocyte count for each female (sum of oocytes in the left and right ovaries).

Table 5. Correlation of female *Pseudobranchus axanthus* snout-vent length (SVL) and mass to total number of oocytes per size class. Analyses performed with Spearman's rank correlation coefficient (r).

	SVL[1]		Mass[2]	
	r	P	r	P
Oocyte Size Class 1	0.557	< **0.001**	0.605	< **0.001**
Oocyte Size Class 2	0.551	< **0.001**	0.541	< **0.001**
Oocyte Size Class 3	0.485	< **0.001**	0.470	< **0.001**
Oocyte Size Class 4	0.593	< **0.001**	0.611	< **0.001**
Oocyte Size Class 5	0.477	< **0.001**	0.515	< **0.001**

[1] $n = 53$

[2] $n = 51$

Bonferroni adjusted $\alpha = 0.01$. Significant probabilities are indicated in bold.

Table 6. Rainey Slough *Pseudobranchus axanthus* egg density.

Collection Date	Collection Effort[1] (m^2)	Number of Eggs	Density (eggs / m^2)
2 Jan 1975	unknown	1	NA
7 Feb 1976	6.72	37	5.51
8 Feb 1976	7.84	10	1.28
14 Feb 1976	unknown	34	NA
15 Feb 1976	3.92	27	6.89
27 Mar 1976	2.80	12	4.29
28 Mar 1976	1.68	5	2.98
20 Jun 1976	6.16	26	4.22
16 Jul 1976	3.36	3	0.89
17 Jul 1976	6.16	24	3.90
28 Aug 1976	8.96	27	3.01
9-10 Oct 1976	8.96	25	2.79
Mean ± 1 SD			**3.58 ± 1.81**

[1] Collection effort represents the area (m^2) of water hyacinth sampled. Sampling was conducted with a $0.56 m^2$ seine. Mean egg density indicated in bold.

Table 7A. Mean number of oocytes per female *Pseudobranchus axanthus* at Rainey Slough for each month and season.

Collection Category	Oocyte Size Class 1	Oocyte Size Class 2	Oocyte Size Class 3	Oocyte Size Class 4	Oocyte Size Class 5
Jan (*n* = 4)	290.3 ± 142.35	40.5 ± 7.05	20.25 ± 9.74	18.75 ± 25.08	12.5 ± 15.24
Feb (*n* = 3)	1157.3 ± 270.52	73.7 ± 42.16	29.3 ± 39.15	31.0 ± 37.03	19.3 ± 31.77
Mar (*n* = 3)	359.7 ± 131.34	39.0 ± 23.64	9.0 ± 1.00	7.7 ± 8.62	5.7 ± 5.13
Apr (*n* =1)	799.0 ± 0	56.0 ± 0	38.0 ± 0	25.0 ± 0	9.0 ± 0
May (*n* =4)	1171.5 ± 117.90	96.8 ± 15.71	27.3 ± 11.67	32.0 ± 19.82	26.5 ± 16.78
Jun (*n* =2)	732.5 ± 242.54	38.0 ± 31.11	13.0 ± 5.66	6.5 ± 0.71	1.0 ± 1.41
Jul (*n* =11)	596.2 ± 285.73	49.8 ± 34.61	40.8 ± 31.59	21.91 ± 16.75	18.73 ± 15.42
Aug (*n* =5)	836.2 ± 69.03	51.6 ± 11.99	37.2 ± 12.64	19.8 ± 7.26	17.2 ± 7.53
Sep (*n* =5)	678.8 ± 275.78	70.8 ± 57.48	34.2 ± 20.18	30.0 ± 14.70	28.2 ± 9.99
Oct (*n* =3)	595.67 ± 181.27	41.67 ± 9.82	17.7 ± 5.13	15.0 ± 7.00	9.3 ± 2.89
Nov (*n* =7)	469.3 ± 335.54	42.9 ± 34.26	19.0 ± 16.70	16.00 ± 5.42	10.14 ± 5.27
Dec (*n* =5)	243.6 ± 59.41	32.2 ± 17.71	25.4 ± 16.38	18.0 ± 9.92	13.4 ± 7.54
Wet Season (*n* =23)	678.2 ± 252.55	53.7 ± 36.23	36.2 ± 24.88	21.9 ± 14.62	18.9 ± 13.64
Dry Season (*n* =30)	582.9 ± 399.12	51.0 ± 30.71	21.9 ± 16.38	19.7 ± 16.87	13.5 ± 13.32
Apr – Sep (*n* =28)	753.0 ± 290.36	60.0 ± 36.48	35.0 ± 23.02	23.4 ± 15.20	19.6 ± 13.95
Oct – Mar (*n* =25)	480.1 ± 346.48	43.4 ± 26.45	20.4 ± 17.01	17.5 ± 16.23	11.6 ± 12.10

Mean ± 1 SD presented. Wet and dry seasons correspond to south Florida rainfall patterns. April – September and October – March correspond to *Siren intermedia* non-reproductive and reproductive seasons, respectively, at Rainey Slough (Godley, 1983).

Table 7B. ANCOVA results comparing the mean number of oocytes per female between months, wet and dry seasons, and six-month seasons. Results of two ANCOVA analyses are provided, one utilizing female snout-vent length (SVL) as a covariate and one utilizing female $\log_{10}$ transformed mass as a covariate.

		Continuous Predictor			
		SVL Covariate		$\log_{10}$ Mass Covariate	
Categorical Predictor	Dependent Variable	*F*	*P*	*F*	*P*
Month	Oocyte Size Class 1	4.553	**< 0.001**	4.547	**< 0.001**
	Oocyte Size Class 2	0.789	0.650	0.799	0.640
	Oocyte Size Class 3	1.003	0.461	0.968	0.490
	Oocyte Size Class 4	0.456	0.919	0.446	0.925
	Oocyte Size Class 5	0.968	0.490	0.961	0.496
Wet and Dry Season	Oocyte Size Class 1	1.334	0.254	1.136	0.292
	Oocyte Size Class 2	0.090	0.765	0.053	0.818
	Oocyte Size Class 3	8.085	0.006	7.689	0.008
	Oocyte Size Class 4	0.292	0.591	0.201	0.651
	Oocyte Size Class 5	2.381	0.129	2.206	0.144
Six-month Season	Oocyte Size Class 1	6.891	0.011	6.647	0.013
	Oocyte Size Class 2	1.532	0.222	1.476	0.230
	Oocyte Size Class 3	4.271	0.044	4.119	0.048
	Oocyte Size Class 4	0.340	0.562	0.304	0.584
	Oocyte Size Class 5	2.896	0.095	2.799	0.101

Bonferroni adjusted $\alpha = 0.003$. Significant probabilities are indicated in bold.
Wet and dry seasons correspond to south Florida rainfall patterns. Six-month seasons are defined as April – September and October – March which corresponds to *Siren intermedia* non-reproductive and reproductive seasons, respectively, at Rainey Slough (Godley, 1983).

Table 8. *Pseudobranchus axanthus* potential clutch size.

	n[1]	Mean ± 1 SD	Minimum – Maximum
Pre-ovulatory Oocytes	53	15.9 ± 13.60	0 – 56
Oviductal Eggs	8	9.1 ± 4.32	4 – 15
Sum of Pre-ovulatory Oocytes and Oviductal Eggs	8	30.1 ± 13.27	15 – 58
Relative Clutch Mass[2]	51	0.045 ± 0.0261	NA[3] – 0.101

[1] Number of females with appropriate variable.

[2] Relative clutch mass calculated as the sum of each female’s left and right ovary mass divided by the total mass of the female specimen.

[3] Female ovary mass totaled < 0.00 (*n* = 6).

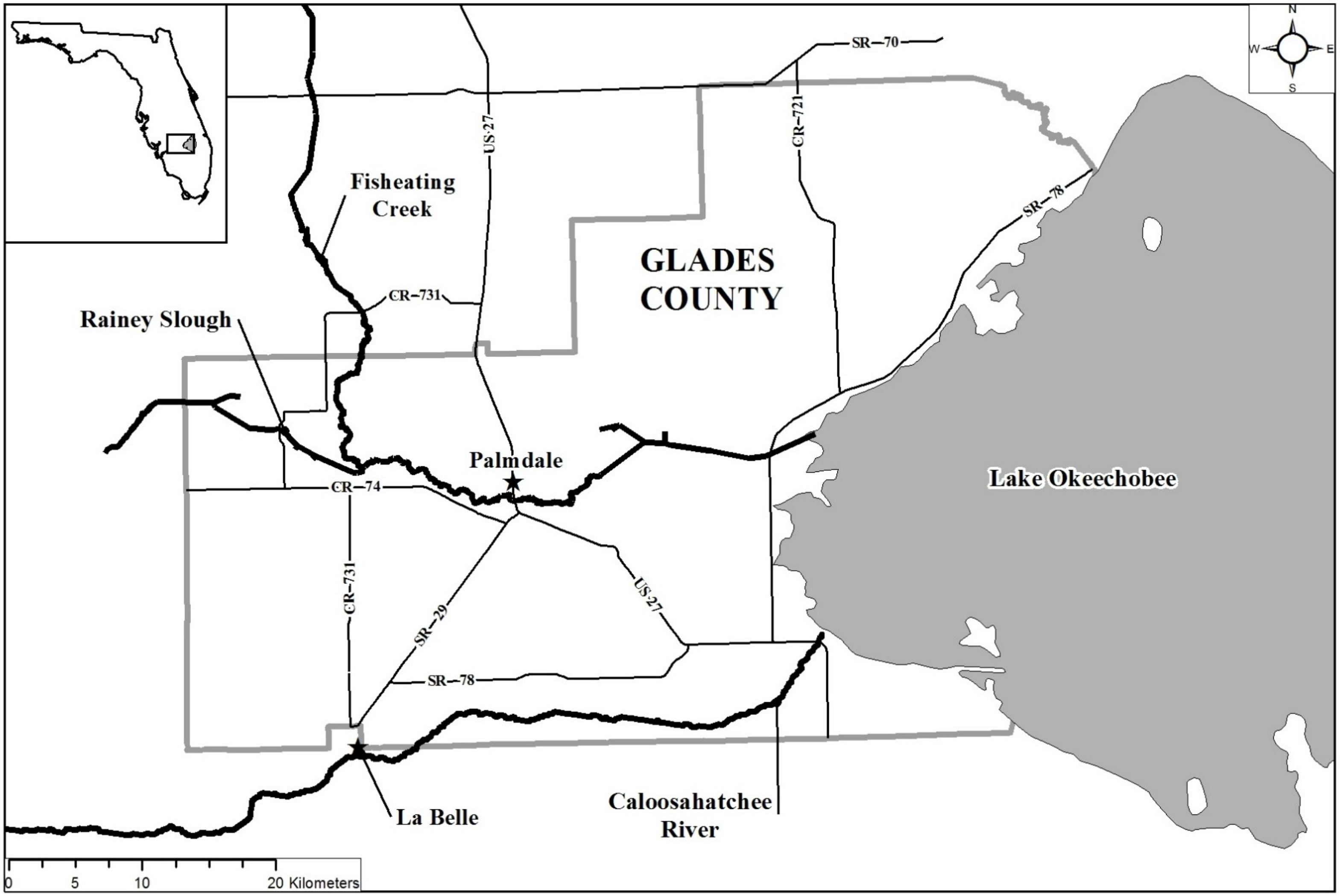

Figure 1. Rainey Slough, Glades County, Florida location map.

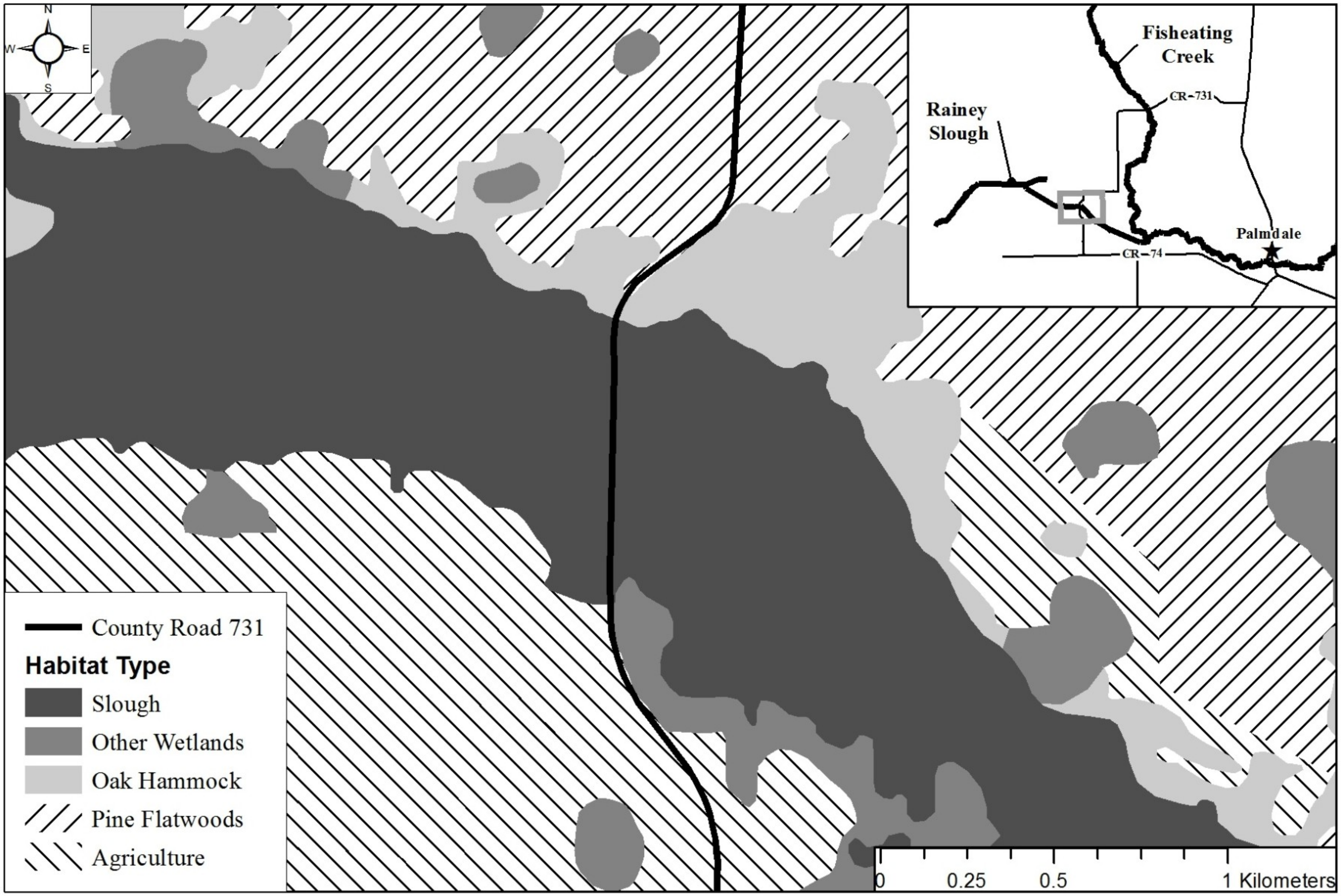

Figure 2. Rainey Slough, Glades County, Florida habitat map.

Figure 3. Aerial photo of Rainey Slough, County Road 731, and excavated canals (Glades County, Florida).

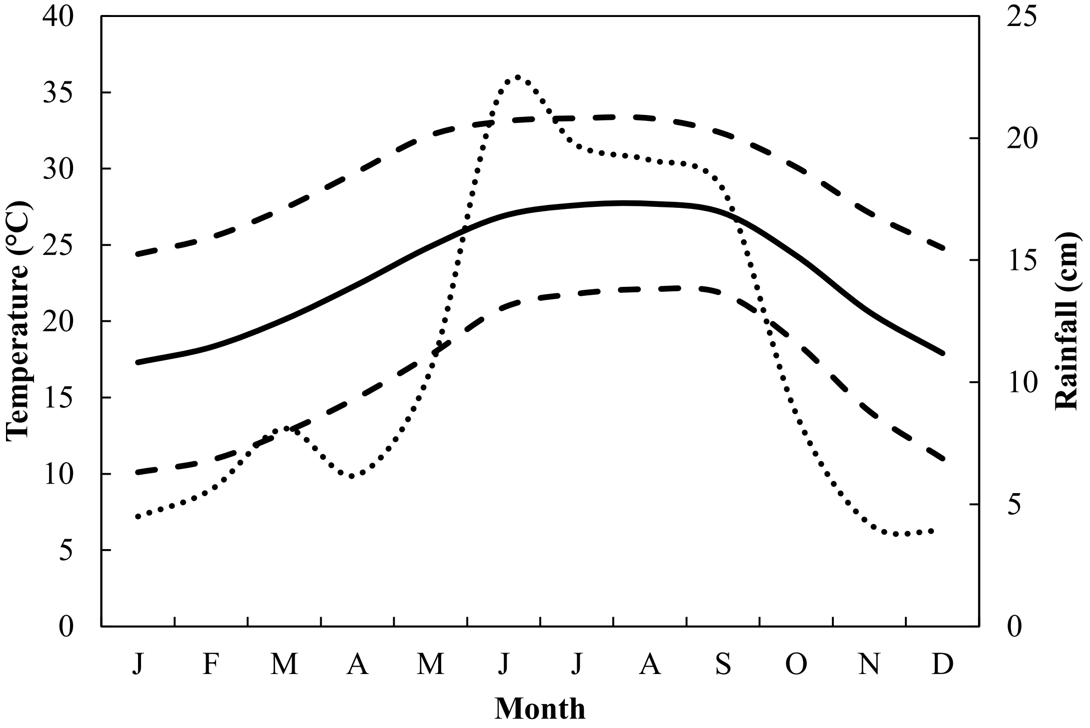

Figure 4. Mean monthly temperature and mean monthly rainfall for Labelle, Glades County, Florida (1930-2011). Solid line represents mean monthly temperature (°C); dashed lines represent mean monthly high and low temperatures (°C); dotted line represents mean rainfall (cm). Labelle, Florida is located approximately 25km south of the Rainey Slough collection site. Climate data acquired from Weatherbase (2012).

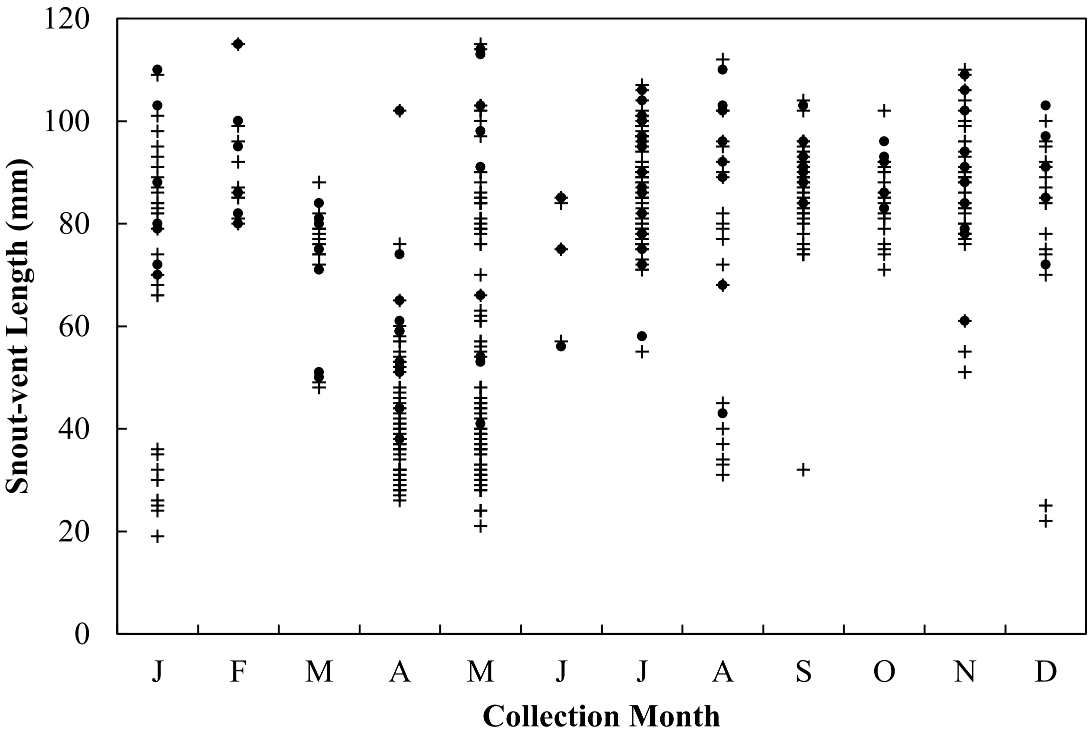

Figure 5. Snout-vent length (mm) and collection month of Rainey Slough *Pseudobranchus axanthus*. Plus signs represent all adult and juvenile *Pseudobranchus axanthus* specimens in the Rainey Slough collection ($n = 474$); closed circles represent all examined specimens ($n = 100$).

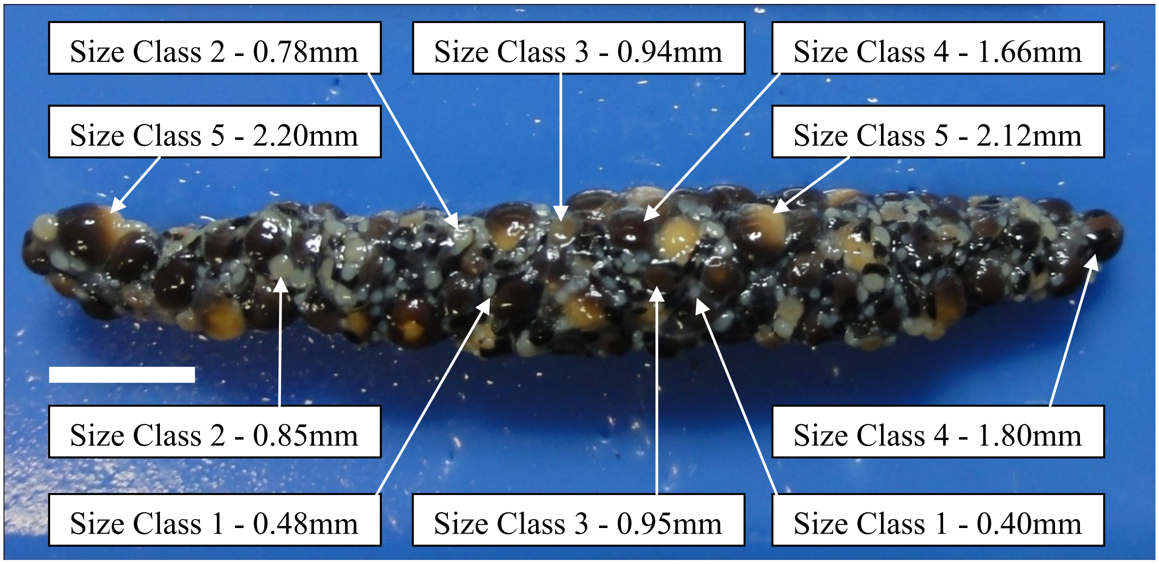

Figure 6. *Pseudobranchus axanthus* ovary with oocyte size classes and diameters. Left ovary from USNM 289386 shown as example. Scale bar = 5mm.

Figure 7. Representative dark coloration and distinct, wide stripes observed in the Rainey Slough *Pseudobranchus axanthus* collection. USNM 289347 shown as example. Scale bar = 20mm.

Figure 8. Variable head shapes observed in the Rainey Slough *Pseudobranchus axanthus* collection. USNM 289256 and USNM 289288 shown as examples. Scale bar = 5mm.

Figure 9A. Variable ovary size and oocyte numbers observed in the Rainey Slough *Pseudobranchus axanthus* collection. Both photos display the opened pleuroperitoneal cavity of USNM 289386. Bottom photo displays ovaries after separation from one another. Scale bars = 10mm.

Figure 9B. Variable ovary size and oocyte numbers observed in the Rainey Slough *Pseudobranchus axanthus* collection. Both photos display the opened pleuroperitoneal cavity of USNM 289218. Scale bars = 10mm.

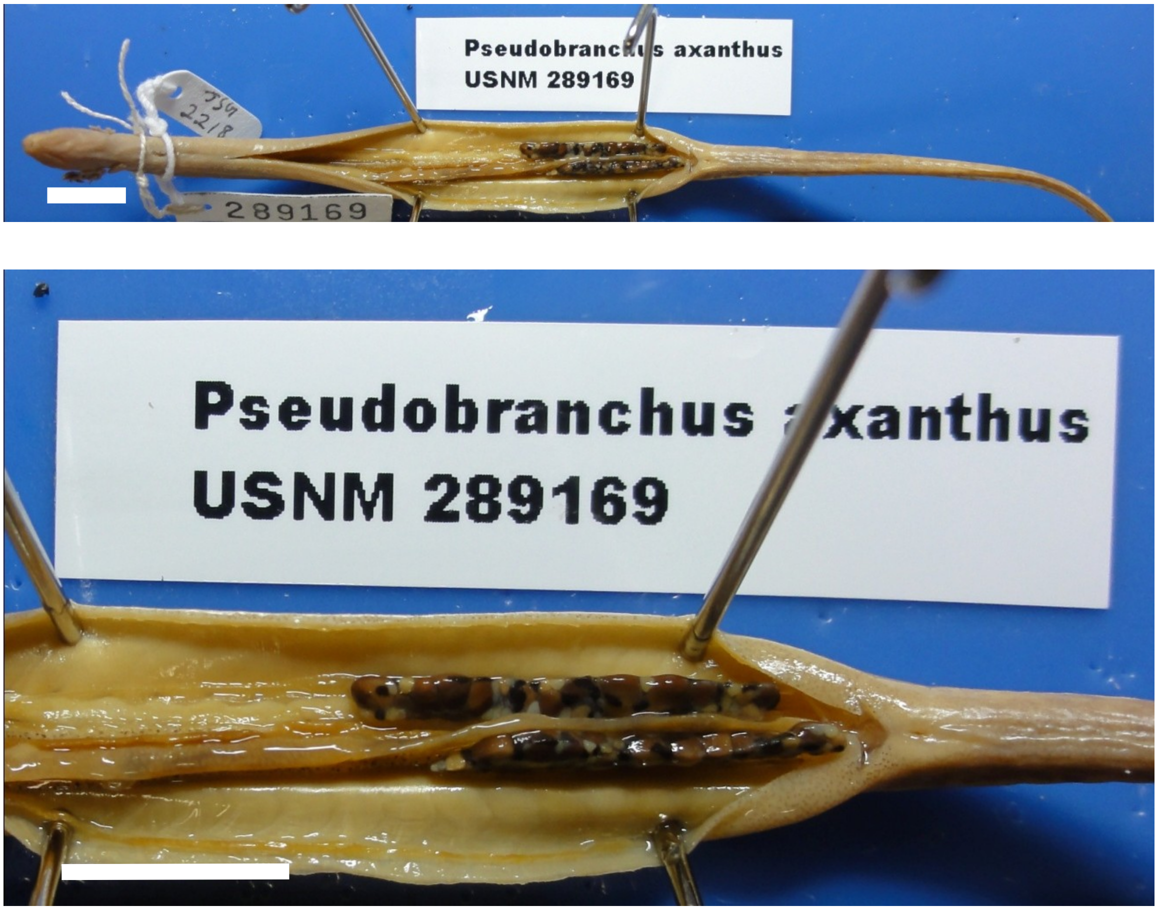

Figure 9C. Variable ovary size and oocyte numbers observed in the Rainey Slough *Pseudobranchus axanthus* collection. Both photos display the opened pleuroperitoneal cavity of USNM 289169. Scale bars = 10mm.

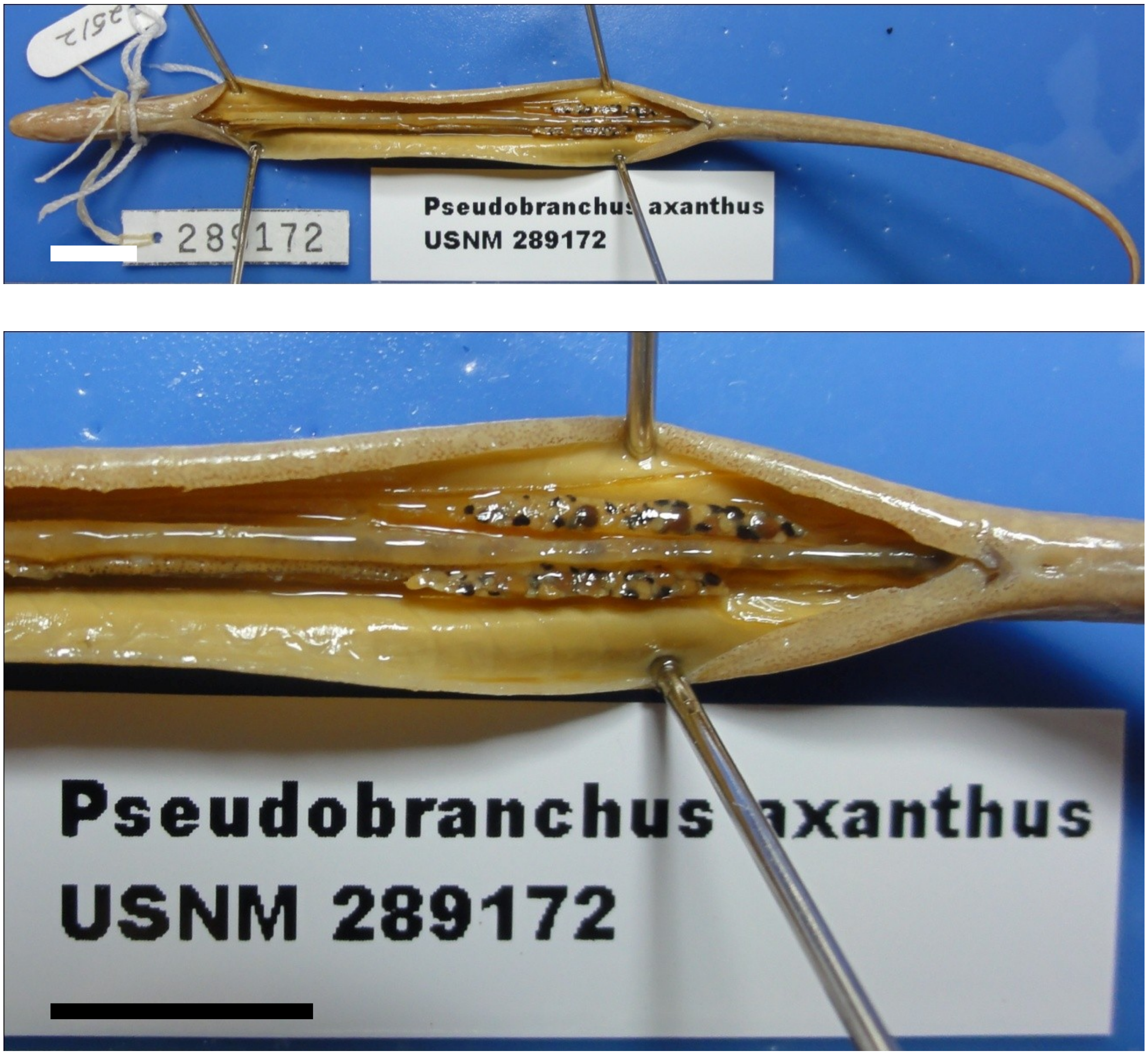

Figure 9D. Variable ovary size and oocyte numbers observed in the Rainey Slough *Pseudobranchus axanthus* collection. Both photos display the opened pleuroperitoneal cavity of USNM 289172. Scale bars = 10mm.

Figure 10. *Pseudobranchus axanthus* with oviductal eggs. Both photos display the opened pleuroperitoneal cavity of USNM 289218. Left oviduct is visible in the top photo; right oviduct is visible in the bottom photo. Ovaries were removed prior to taking photos. Various stages of egg jelly deposition are visible from the anterior (left) to posterior (right) regions of the oviduct. Scale bars = 10mm.

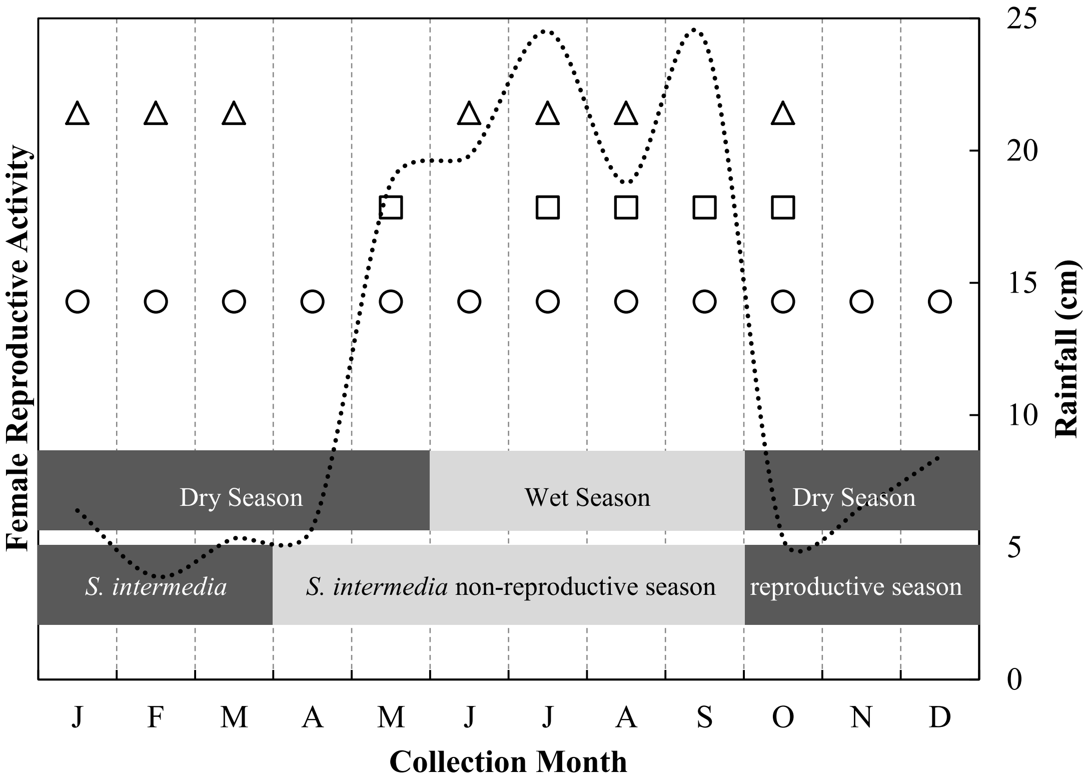

Figure 11. Female *Pseudobranchus axanthus* reproductive activity and Rainey Slough mean rainfall (cm) per month. Female reproductive activity is presented as observed females with pre-ovulatory oocytes (open circles), observed females with oviductal eggs (open squares), and observed oviposited eggs (open triangles). Dashed line represents mean rainfall (cm) for the collection timeframe (1974-1979). Rainfall data presented for Palmdale, Florida, approximately 18.5km east of the Rainey Slough collection location (SFWMD, 2012). Top dark gray bar represents the south Florida dry season, and top light gray bar represents the south Florida wet season. Bottom dark gray bar represents the *Siren intermedia* reproductive season at Rainey Slough, and bottom light gray bar represents the *Siren intermedia* non-reproductive season at Rainey Slough (Godley, 1983).

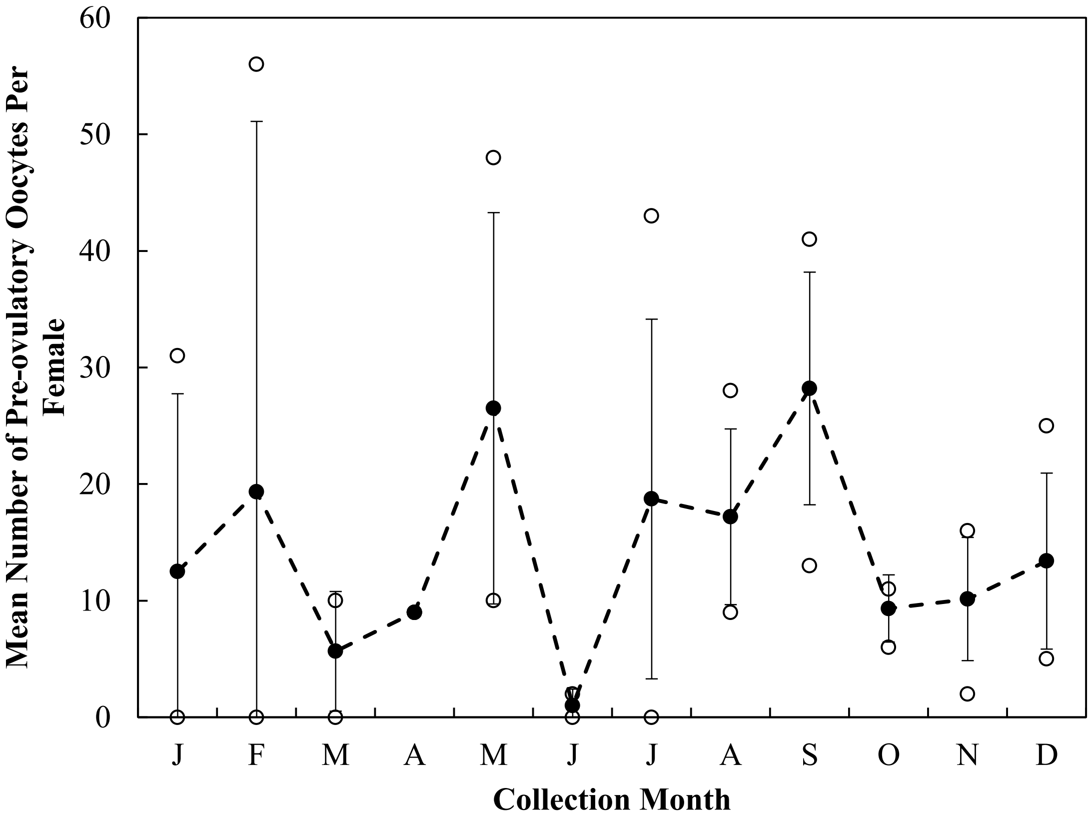

Figure 12. Mean number of pre-ovulatory oocytes per *Pseudobranchus axanthus* females in each collection month. Closed circles represent mean number of oocytes, error bars show ± one standard deviation, and open circles represent maximum and minimum recorded oocyte numbers.

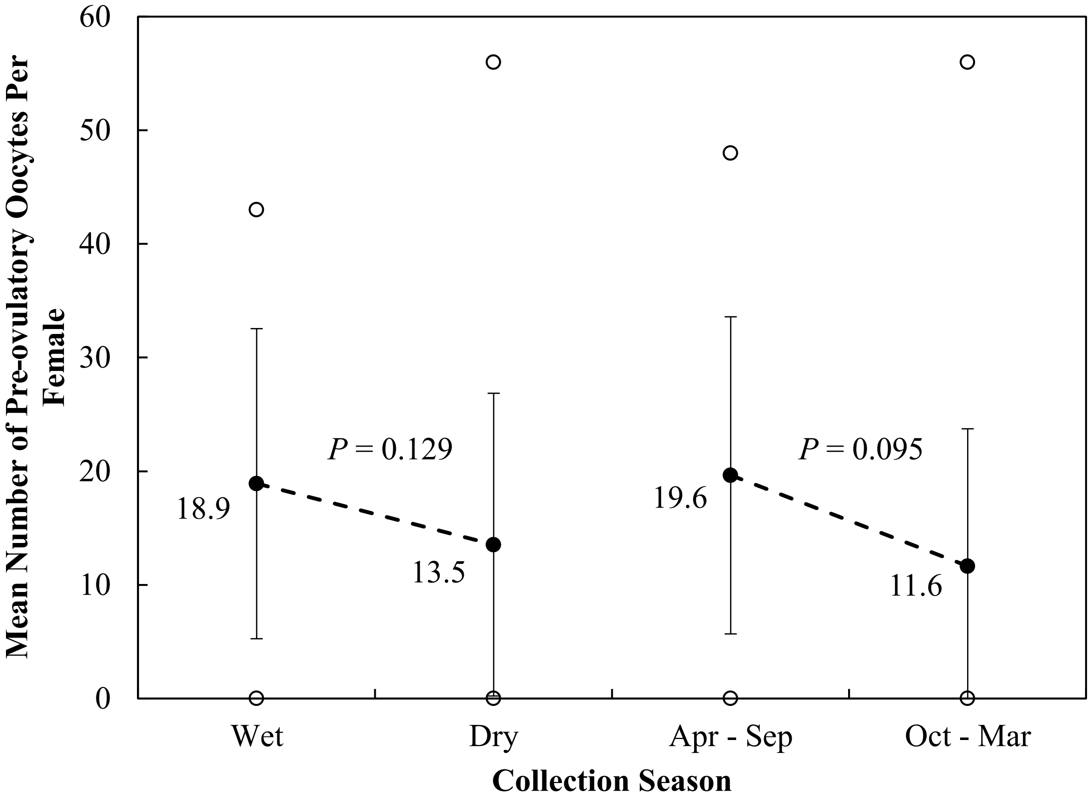

Figure 13. Mean number of pre-ovulatory oocytes per *Pseudobranchus axanthus* females in each collection season. Closed circles represent mean number of oocytes, error bars show ± one standard deviation, and open circles represent maximum and minimum recorded oocyte numbers. Wet and dry seasons correspond to south Florida rainfall patterns. April – September and October – March seasons correspond to *Siren intermedia* non-reproductive and reproductive seasons, respectively, at Rainey Slough as reported by Godley (1983). Probability (*P*) provided for the results of an ANCOVA analysis comparing the wet and dry season means and April – September and October – March means with female snout-vent length as a covariate.

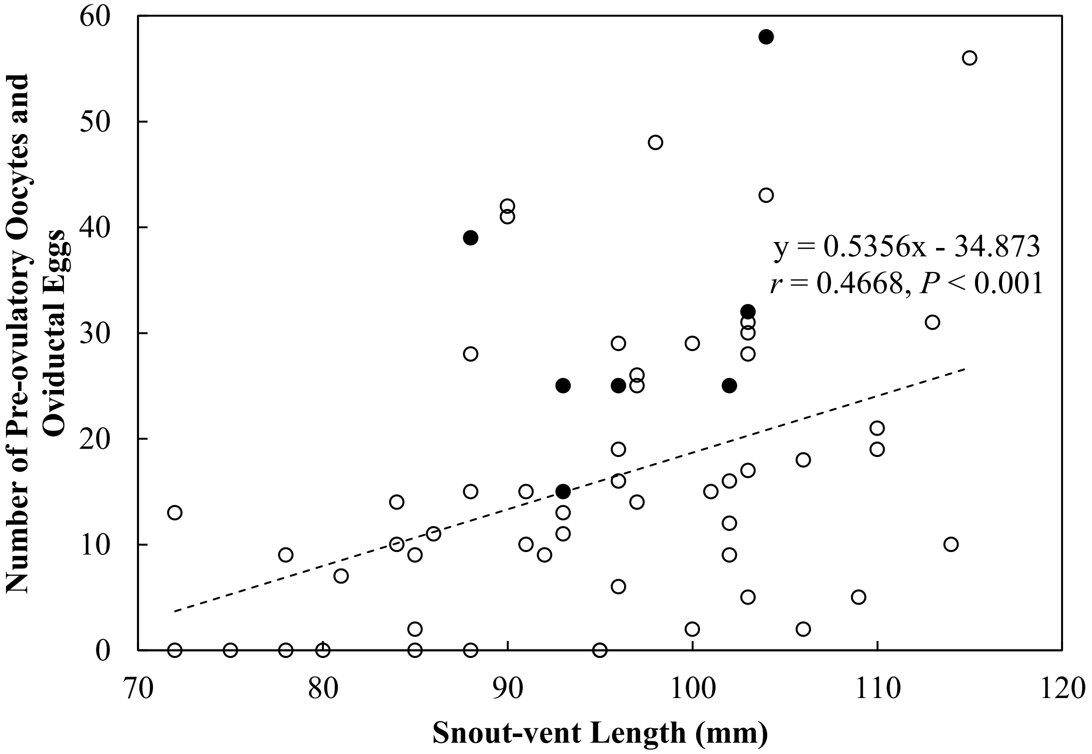

Figure 14A. *Pseudobranchus axanthus* snout-vent length and potential clutch size. The total number of pre-ovulatory oocytes (open circles) or the total number of pre-ovulatory oocytes plus the total number of oviductal eggs (closed circles) were considered to be each female's next potential clutch. Line equation, Spearman's *r* statistic, and probability (*P*) are provided for the correlation between the female snout-vent length (mm) and total number of pre-ovulatory oocytes.

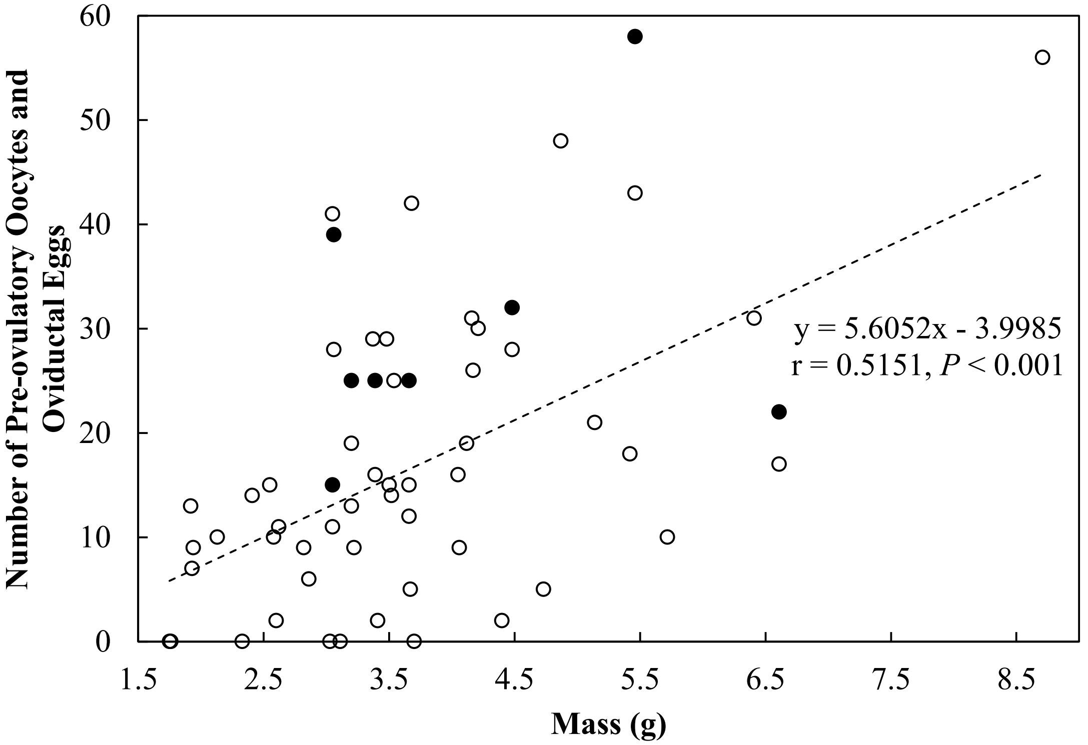

Figure 14B. *Pseudobranchus axanthus* mass and potential clutch size. The total number of pre-ovulatory oocytes (open circles) or the total number of pre-ovulatory oocytes plus the total number of oviductal eggs (closed circles) were considered to be each female's next potential clutch. Line equation, Spearman's *r* statistic, and probability (*P*) are provided for the correlation between the female mass (g) and total number of pre-ovulatory oocytes.

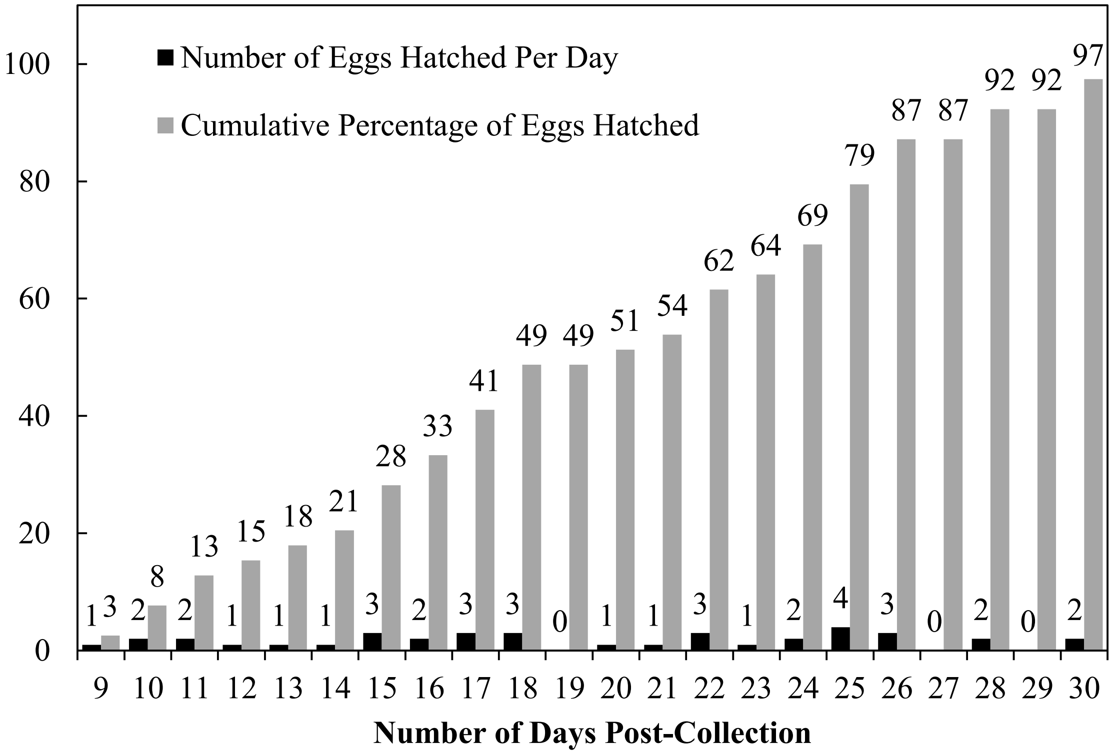

Figure 15A. Hatching timeline for *Pseudobranchus axanthus* eggs collected on 7-8 February 1976 at Rainey Slough.

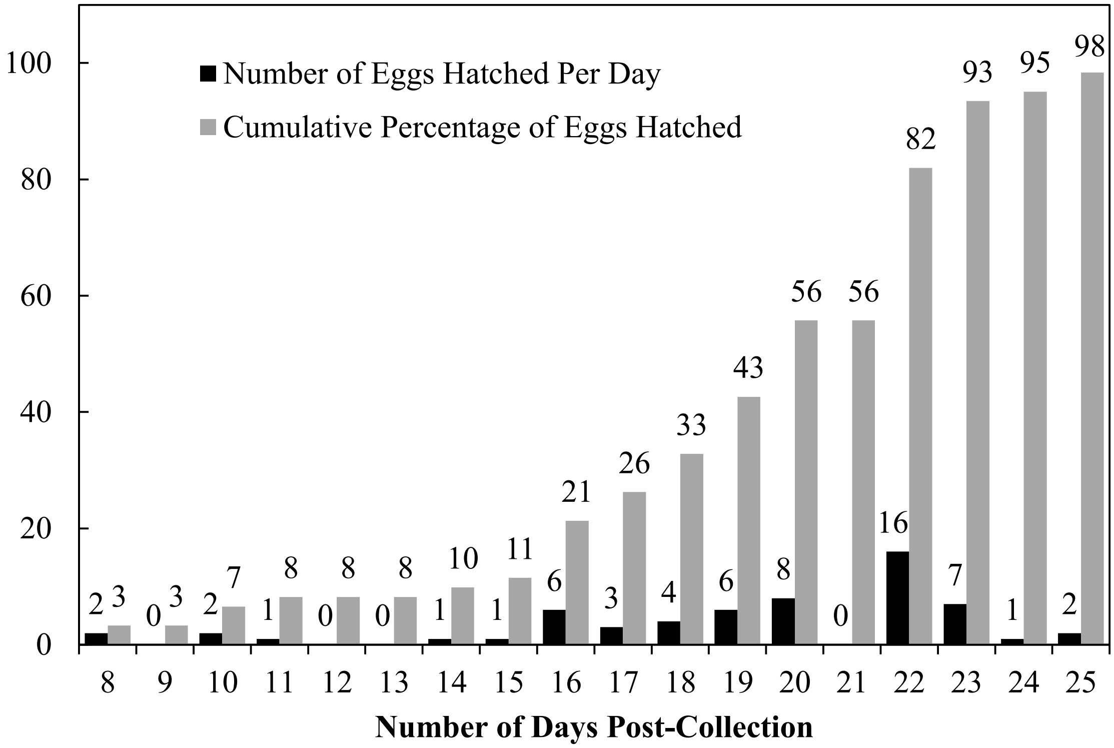

Figure 15B. Hatching timeline for *Pseudobranchus axanthus* eggs collected on 14-15 February 1976 at Rainey Slough.

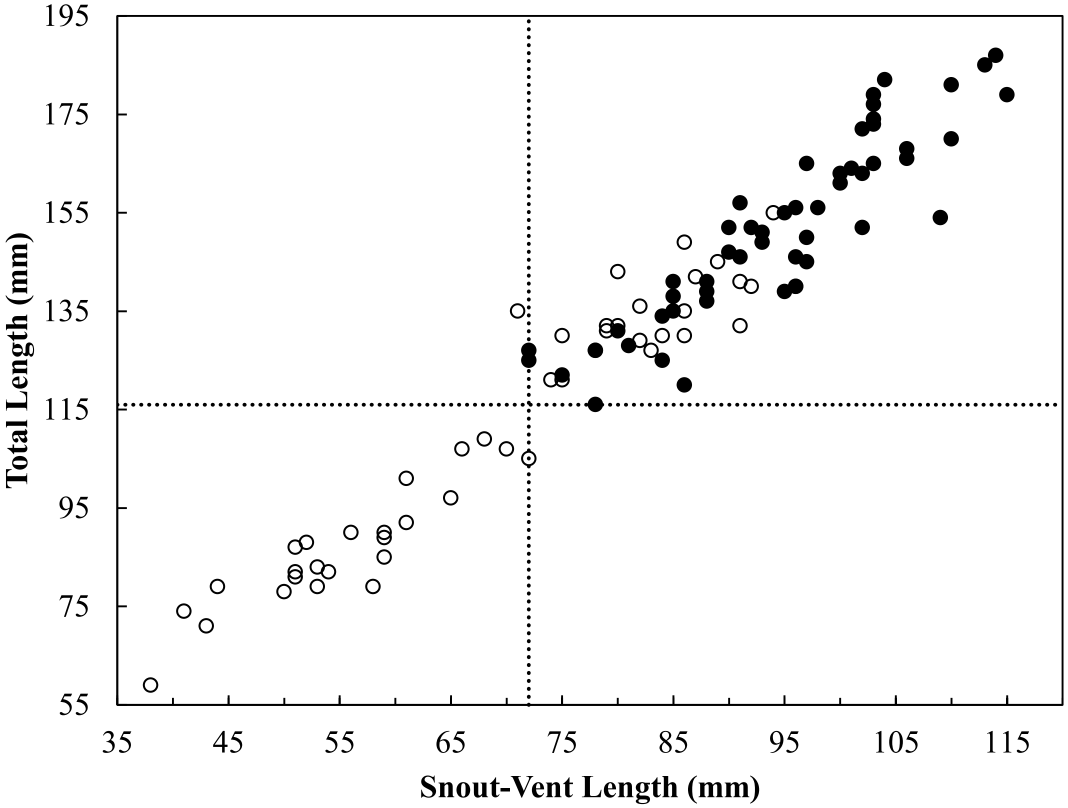

Figure 16. Female size at sexual maturity. Scatter plot includes snout-vent length (mm) and total length (mm) of all examined *Pseudobranchus axanthus* from Rainey Slough (n = 100). Open circles represent males, juveniles, and specimens with visually indistinguishable gonads; closed circles represent sexually mature females. Dashed lines represent the smallest observed snout-vent length and smallest observed total length for mature females. Females with pigmented, vitellogenic oocytes were considered sexually mature.

Figure 17. *Pseudobranchus axanthus* with four ovisac eggs. Both photos display the opened pleuroperitoneal cavity of USNM 289295. Ovaries removed for second photo. Scale bars = 10mm.

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APPENDIX 1

NATIONAL MUSEUM OF NATURAL HISTORY LOAN INFORMATION. — The Division of Amphibians and Reptiles at the Smithsonian Institution National Museum of Natural History loaned a portion of their *Pseudobranchus axanthus* collection (USNM 289096 – 289569 and USNM 290650 – 290741) for this project. I examined 100 of the loaned specimens: USNM 289096, 289098, 289099, 289100, 289102, 289104, 289105, 289107, 289109, 289113, 289118, 289120, 289125, 289127, 289129, 289134, 289137, 289143, 289151, 289152, 289154, 289158, 289163, 289164, 289168, 289169, 289172, 289174, 289175, 289177, 289180, 289181, 289182, 289183, 289184, 289185, 289186, 289187, 289188, 289190, 289206, 289207, 289210, 289212, 289213, 289218, 289223, 289225, 289228, 289232, 289233, 289243, 289246, 289248, 289259, 289267, 289281, 289290, 289295, 289296, 289297, 289301, 289309, 289325, 289337, 289338, 289340, 289341, 289347, 289352, 289360, 289370, 289378, 289381, 289383, 289386, 289387, 289388, 289389, 289390, 289394, 289398, 289399, 289400, 289401, 289408, 289409, 289441, 289442, 289445, 289453, 289469, 289475, 289477, 289494, 289496, 289548, 289560, 289562, and 289567. All examined specimens were collected at Rainey Slough, Glades County, Florida.