Spawning performance and egg quality of wild-caught and first generation southern flounder *Paralichthys lethostigma* broodstock induced with piscine and mammalian GnRH analogs

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ABSTRACT

Spawning performance and egg quality were compared in wild-caught and F1 generation southern flounder broodstock following induction with mammalian mLHRHa or piscine sGnRHa at different doses. Forty F1 and 33 wild vitellogenic females (mean oocyte diameter ≥ 0.385 mm) were implanted with an 80% cholesterol-20% cellulose pellet at doses (μg/kg bw) of 0 (control), 5 (low), 25 (mid) and 50 (high) mLHRHAs, or with similar doses of sGnRHa. Ovulated females (N = 5–8 per treatment) were strip-spawned and eggs were fertilized in vitro with sperm from two males. Egg quality was categorized according to fertilization and hatching success: 75–100% (high), 50–75% (medium-high), 25–50% (medium-low), and 0–25% (low). Egg production (number of eggs/kg female bw × 10^3) was highest at the low dose of mLHRHAs in F1 females (146.4) and sGnRHa in wild females (128) and was lowest at the intermediate dose sGnRHa in F1 females (61.9) and the high dose sGnRHa in wild females (40.6). Number of fertilized eggs produced per kg bw (× 10^3) was highest at the low dose mLHRHAs in F1 females (113.5) and sGnRHa in wild females (95.4) and lowest at the high dose sGnRHa in F1 females (18.7) and sGnRHa in wild females (7.8). Number of yolk sac larvae produced per kg bw (× 10^3) was highest at the low dose of mLHRHAs in F1 females (74) and sGnRHa in wild females (73.5) and was lowest at the high dose of sGnRHa in F1 females (2.0) and in wild females (1.2). Number of larvae per kg bw (× 10^3) surviving to 96 h post-hatching was highest at the low dose mLHRHAs in F1 females (67.5) and sGnRHa in wild females (66.5) and was lowest at the high dose sGnRHa in F1 (72) and sGnRHa in wild females (60.6). In F1 females, larval production was significantly greater (P < .01) at lower doses and greater (P < .01) for mLHRHAs than for sGnRHa at all doses. For wild females, larval production was greater at lower doses of sGnRHa, with the inverse trend observed for mLHRHAs (hormone dose × type interaction, P < .01). Taurine concentrations (nmol/mg dw) were higher (P < .05) in ovulated eggs of high quality (27.4) than in eggs of med-low (14.8) and low (17.5) quality, supporting an important role in broodstock nutrition and in early development. The results help standardize GnRHa therapies for spawning of wild and F1 southern flounder.

1. Introduction

1.1. Southern Flounder

The southern flounder *Paralichthys lethostigma* is a high-value recreationally and commercially harvested flatfish found in estuarine and shelf waters of the Atlantic and Gulf coasts of the U.S. from North Carolina to Mexico (Gilbert, 1986) and is threatened by overfishing (NC DFM, 2018). Southern flounder inhabit estuarine waters, coastal bays, sounds, and lagoons from spring through fall (Dalhberg, 1972). In the late fall and winter adults migrate offshore to spawn when temperatures begin to decline (Ginsberg, 1952). Southern flounder are euryhaline and have the ability to grow well in both fresh and salt water (Daniels et al., 1996; Lasswell et al., 1977); hence, they have significant potential as a commercial aquaculture industry in coastal and inland regions of the South Eastern U.S. With a growing demand for southern flounder, combined with the pressure of commercial and recreational fisheries on wild stocks, research on aquaculture of this species will be important to meet future demand while conserving wild populations.
1.2. Hormone-induced spawning

In captivity, many marine finfish females undergo vitellogenesis, but do not readily undergo final oocyte maturation (FOM), ovulation and spawning (Zohar, 1988). A popular method of hormone-induced spawning in fish involves the application of exogenous gonadotropin releasing hormone (GnRH) to stimulate the release of endogenous gonadotropic hormone (GtH) from the pituitary gland, resulting in FOM, ovulation and spawning (Donaldson, 1981; Peter and Yu, 1997; Zohar and Mylonas, 2001). The action of GtH on FOM is mediated by the stimulation of steroidogenesis in granulosa cells of the ovarian follicle, which secrete the maturation-inducing steroids (MIS) responsible for triggering the resumption of meiosis in the oocyte (FOM) (Nagahama, 1995; Nagahama and Yamashita, 2008).

Two types of synthetic gonadotropin releasing hormone (GnRH) analogs have been used for induced spawning, including mammalian luteinizing hormone releasing hormone analog (mLHRHa), and salmon gonadotropin releasing hormone analog (sGnRHa). These synthetic neurohormone peptides have similar amino acid sequences to the native hormones, with molecular differences usually characterized by a single amino acid substitution at position 6 and an ethylamide (NEI) substitution at position 10 (Zohar and Mylonas, 2001). Mammalian mLHRHa and piscine sGnRHa have different amino acids in positions 6, 7, and 8 of the peptide chain (Zohar and Mylonas, 2001), and their amino acid sequences are Glu-His-Trp-Ser-Tyr-D-Ala-Leu-Arg-Pro-NH2 and Glu-His-Trp-Ser-Tyr-D-Arg-Trp-Leu-Pro-NH2 (Zohar et al., 2017). These differences in amino acid sequence may cause different rates of degradation in the pituitary, kidney and liver, and hence, may elicit different biological activities in fish (Zohar et al., 1989). To date, no studies have compared the efficacies of mammalian mLHRHa and piscine sGnRHa on reproductive performance in the southern flounder or in wild-caught and cultured F1 broodstock.

1.3. Hormone type and dose

The southern flounder is a fractional spawner that shows “group-synchronous” ovarian development in which successive batches of vitellogenic oocytes are recruited into FOM and ovulation during the spawning season (Wallace and Selman, 1981; Berlinsky et al., 1996). Mammalian mLHRHa applied in sustained-release delivery systems at doses of 20 to 1000 μg/kg body weight has been found to be an effective method for inducing ovulation and producing good quality eggs in a number of multiple-clutch, group synchronous spawners, including the black sea bass Centrurrpis striata (Watanabe et al., 2003; Berlinsky et al., 2005; Denson et al., 2007), summer flounder Paralichthys dentatus (Berlinsky et al., 1997; Watanabe et al., 1998), and Senegal sole Solea senegalensis (Auguleiro et al., 2006). Sustained release mLHRHa or sGnRHa delivery systems are believed to be effective with fractional spawners, because they produce long-term elevations in plasma GtH, which in turn stimulates steroidogenesis and completion of vitellogenesis and multiple cycles of FOM, ovulation and spawning, while allowing the smaller vitellogenic oocytes to continue vitellogenic growth. Hormone administration via a single pellet implant also alleviates the stress associated with multiple injection methods.

1.4. Free amino acids in relation to egg quality

Availability of high quality eggs is a key constraint to expansion of the finfish aquaculture industry. Factors that are hypothesized to determine egg quality and production of viable fry include: genetics, broodstock nutrition, water quality, initial physiological processes in the egg (Kjørsvik et al., 1990, 2003), and the biochemical composition of eggs. Lipids are important nutrients for embryonic development in fish (Tocher and Sargent, 1984; Sargent et al., 1989; Sargent, 1995), serving as sources of metabolic energy and of components of biological membranes and affecting larval stress resistance. However, previous studies with southern flounder in our laboratory have not been able to find meaningful differences in total lipids (4.4% wet wt.), DHA (26.7%), EPA (3.3%) and ARA (1.9%) and DHA/EPA ratio (8.3) between low and high quality embryos (Woolridge, 2005).

Amino acids also play important roles in developing marine teleost oocytes and larvae. It is known that the protein synthesis of yolk proteins during oocyte hydration generates an organic osmolyte pool of free amino acids (FAAs) that are used for water influx into the oocyte that aids in achieving buoyancy (Finn and Fyhn, 2010). The process of oocyte hydration can affect egg viability and embryo survival in marine teleosts and therefore has important implications for the control of reproduction and hatchery production of cultivated species (Kjørsvik et al., 1990). The release of FAAs from yolk proteins also contributes to the amino acid supply required for aerobic energy production in developing embryos and larvae (Fyhn and Serigstad, 1987; Rennestad et al., 1992a,b; Sivaloganathan et al., 1998; Finn and Fyhn, 2010) and are major substrates for the synthesis of proteins in fish larvae (Rennestad and Fyhn, 1993). Establishing baseline FAA profiles in marine fish eggs of different quality will further our understanding of their role in the FOM process and aid in the establishment of biochemical correlates of egg quality to improve broodstock husbandry practices for more reliable production of healthy fry (Kjørsvik et al., 2003).

1.5. Objectives

Based on available studies using wild-caught broodstock, the recommended dose for induced spawning of southern flounder is 50 to 100 μg/kg bw administered in a 95% cholesterol- 5% cellulose sustained-release delivery system (Berlinsky et al., 1996; Smith et al., 1999; Watanabe and Carroll, 2001; Watanabe et al., 2001, 2006). However, at the University of North Carolina Wilmington (UNCW), these doses have resulted in overstimulation of first generation (F1) southern flounder broodstock, sometimes resulting in fish death. This phenomenon is characterized by excessive ovarian hydration and swelling due to simultaneous maturation and hydration of a large proportion of oocytes as reported in other fractional spawning marine finfish (Prentice and Colura, 1989; Fornies et al., 2001). To date, no studies have compared the effects of mammalian mLHRHa and piscine sGnRHa at different doses on ovulation and egg quality in the southern flounder. Furthermore, no studies have compared the efficacy of mLHRHa or sGnRHa treatment in wild-caught and F1 broodstock. The purpose of this study was to standardize mLHRHa and sGnRHa induction therapies for spawning wild-caught and F1 generation southern flounder females to support the growing demand for high quality eggs and larvae for commercial scale production. Specific objectives were to compare the effects of mLHRHa and sGnRHa applied at different doses on spawning performance (egg production and quality) in both wild-caught and F1 southern flounder and to determine free amino acid (FAA) profiles of newly spawned eggs as potential biochemical correlates of quality.

2. Materials and methods

2.1. Experimental animals

F1 and wild-caught adult southern flounder were held in three separate controlled-environment recirculation aquaculture systems (RAS) (two tanks per system), at the University of North Carolina Wilmington Center for Marine Science (UNCW CMS) Aquaculture Facility (Wrightsville Beach, NC). Approximately 60 F1 and 60 F1 were held in the experimental system during each of three consecutive spawning seasons, with approximately 50% of the F1 females and 80% of the wild females replaced each year. The F1 southern flounder broodstock were spawned and raised to maturity in captivity at UNCW CMS and at North
Carolina State University and ranged from 2 to 3 years in age. Wild-caught fish were collected off Carolina Beach, NC and in the Atlantic Intracoastal Waterway near Wrightsville Beach, NC by hook and line and were acclimated to captivity for at least 1 year. Newly-captured fish were quarantined in a controlled-environment tank (2.46-m dia., 1-m depth, 4.76-m³ volume) supplied with recirculating seawater (32–35 g/L) at a density of ≤ 7 fish/m³ (5 kg/m³) and under temperature conditions similar to their origin. Fish were treated with formalin (30 ppm) to kill protozoans and monogenetic trematodes and with conditions similar to their origin. Fish were treated with formalin of two, each maintained on a diet of approximately 15% daily. The brood tanks were arranged in three groups with recirculating seawater (32°C) at a density of 4.76-m³ volume) supplied with recirculating seawater (32°C) at a density of approximately 15% daily. The brood tanks were arranged in three groups of two, each maintained on a diet of approximately 15% of total body weight daily. The brood tanks were held at 14 h D and 10 h L and 17 °C for approximately 2 months. Each group of two tanks was supported by a sand fractionator, ultraviolet sterilizer, and a moving bed biofilter containing kelp. Water quality parameters, including temperature, dissolved oxygen, pH, and salinity were measured daily, while ammonia, nitrite, and nitrate were measured weekly.

Wild-caught broodstock could not be weaned to commercial pelleted diets, but gradually accepted de-frozen Atlantic silversides. The brood tanks were arranged in three groups of two, each maintained on a different artificial photothermal regime, which enabled seasonal and aseasonal spawning from October through late May (Watanabe et al., 2006). Timer-controlled fluorescent light fixtures provided a surface light intensity of approximately 240 lux, and temperature was controlled by a heat pump. During the spawning season tanks were held at 14 h D and 10 h L and 17 °C for approximately 2 months. Each group of two tanks was supported by a sand filter, foam fractionator, ultraviolet sterilizer, and a moving bed biofilter containing kelp. Water quality parameters, including temperature, dissolved oxygen, pH, and salinity were measured daily, while ammonia, nitrite, and nitrate were measured weekly.

2.2. Experimental system

Adult broodstock were held in six outdoor controlled-environment fiberglass tanks (2.46-m dia., 1-m depth, 4.76-m³ volume) supplied with recirculating seawater (32–35 g/L). Water was exchanged at approximately 15% daily. The brood tanks were arranged in three groups of two, each maintained on a different artificial photothermal regime, which enabled seasonal and aseasonal spawning from October through late May (Watanabe et al., 2006). Timer-controlled fluorescent light fixtures provided a surface light intensity of approximately 240 lux, and temperature was controlled by a heat pump. During the spawning season tanks were held at 14 h D and 10 h L and 17 °C for approximately 2 months. Each group of two tanks was supported by a sand filter, foam fractionator, ultraviolet sterilizer, and a moving bed biofilter containing kelp. Water quality parameters, including temperature, dissolved oxygen, pH, and salinity were measured daily, while ammonia, nitrite, and nitrate were measured weekly.

Wild-caught broodstock could not be weaned to commercial pelleted diets, but gradually accepted de-frozen Atlantic silversides. Menidia menidia during the quarantine period and were maintained on this diet until the spawning trials were conducted. Although F1 fish were raised on commercial pellets, they readily accepted Atlantic silversides. Hence, to standardize diet among wild-caught and F1 broodstock, F1 fish were transferred to the brood tanks and weaned to silversides during the months of April to June, at least 6 months before the induced spawning trials were conducted in winter. Both F1 and wild-caught broodstock were fed silversides daily Monday thru Saturday to apparent satiation (Watanabe et al., 2001, 2006).

2.3. Experimental design

To evaluate the effects of hormone type, dose, and female origin (wild vs F1) on spawning performance and egg quality, hormone induced spawning trials were conducted on groups of 2–4 post-vitellogenic stage wild-caught or F1 females over the course of three consecutive spawning seasons. To minimize non-treatment effects on spawning performance related to inter-seasonal variation, fish age/size, spawning history, or acclimation history of fish, wild-caught and F1 females in each spawning trial were randomly assigned a prescribed hormone type (mammalian mLHRH or piscine sGnRHα) and a dose (0 μg/kg bw at the UNC-W CMS Aquaculture Research Laboratory (Wrightsville Beach, NC) (Sherwood et al., 1988a, 1988b). To select females for induced spawning trials, the “light table method” (George Nardi, Great Bay Aquaculture, NH) and a mean oocyte diameter (N = 100–150 eggs) of at least 0.385 mm (Bellinsky et al., 1996; Smith et al., 1999; Watanabe et al., 2001) were used to assess stage of gonadal development. Females were anesthetized (2.5 ppm 2-phenoxyethanol) and placed on a clear plexiglass table illuminated from underneath (1000-W bulb), and development was determined from the shape, size, and darkness of the silhouette of the ovary. Females were rated from 0 to 4, depending on the stage of ovarian development: 0 (no development); 1 (early), ovaries slightly darkened, but not extending to the caudal fin; 2 (moderate), ovaries darker and extending nearly to the caudal fin; 3 (full), ovaries fully darkened, extending to the caudal fin, and swelling near the oviduct; and 4 (ovulatory stage), similar to stage 3, but clearing near the oviduct indicating that ovulation had commenced (Watanabe et al., 2006). Females in stage 4 were not eligible for the spawning trial because they had initiated FOM without hormone induction.

Females at stage 3 were further examined by ovarian biopsy. A polyethylene cannula (1.57 mm o.d. × 1.14 mm i.d.) was inserted into the oviduct and a sample of oocytes was withdrawn and placed in 10% formalin in seawater to preserve the sample until analysis. The oocytes were then photographed with a digital camera fitted onto a dissection microscope and the mean oocyte diameter (MOD) (excluding primary and early yolk vesicle stage oocytes) was determined using image analysis software (IMAGEI, National Institute of Health, USA). Females that met the ovarian developmental criteria (stage 3, MOD ≥ 0.385 mm, N = 100–150 oocytes) (Watanabe et al., 2001, 2006) were randomly assigned a hormone treatment and dose and then implanted intramuscularly with a single pellet.

Approximately 48 h post-implantation (hpi), implanted females were anesthetized and checked for ovulation (i.e., stage 4) on the light table. If clearing near the oviduct was evident and ovarian biopsy revealed > 75% hydrated oocytes, females were judged suitable for strip spawning. Partially or non-ovulated females that were premature for strip spawning were rechecked for ovulation within 54–66 hpi, or within 70–78 hpi, respectively, depending on the progress of ovarian maturation as judged from both ovarian biopsy samples and ovarian morphology using the light table method. This approach avoided excessive handling stress (and gonadal atresia) caused by frequent checking of ovulatory status by biopsy, or overripening of ovulated eggs due to delayed stripping.

Males were anesthetized and rinsed with fresh water around the genital pore to prevent sperm activation, and milk was collected with a glass pipette as gentle abdominal pressure was applied near the pelvic fin. Sperm motility was determined by placing one drop of milk on a glass slide, activating with a drop of seawater, and placing a cover slip over the sample. Sperm was observed immediately under a compound microscope and motility was scored on a scale of 0–4 (0 = no movement and 4 = vigorous movement). Milk with highly motile sperm (rating of 3 or 4) was then stored on ice in a 5 mL test tube until used for artificial fertilization.

Once milk was collected, selected females were anesthetized and rinsed with freshwater around the genital pore. Beginning from the anterior portion of the ovary, gentle pressure was applied toward the genital pore and ovulated eggs were collected in a 1000 mL glass beaker while avoiding contamination by water, mucus, blood or urine. Milk was added at a ratio of 1 mL: 100 mL of stripped eggs and the eggs were gently mixed by swirling until the suspension of non-activated eggs and sperm appeared homogenous. Seawater (17 °C) was then added at a ratio of 1:1 (volume of seawater: eggs) to initiate artificial fertilization, and the eggs were gently swirled for 3 mins and then transferred into a 2-L separatory cone. Seawater was then added to a total volume of 1 L, and buoyant (viable) eggs were separated from non-buoyant (non-viable) eggs after 10 mins. Non-viable eggs (sinking) and viable eggs...
2.3.2. Evaluation of spawning performance

Approximately 10,000 floating eggs (i.e., 10 mL) from each female were transferred into 2-L plastic incubation cones containing 1.75 L of seawater at 17 °C with gentle aeration and allowed to incubate for 2–3 h. Fertilization success was determined as the percentage of developing embryos in a sample of at least 100 eggs measured at the 4-cell stage from 2 to 3 hours post-fertilization (hpf). To determine hatching success and survival, individual floating eggs were placed into three, 24-cell transparent plexiglas trays (1 egg and 2 mL seawater in each cell) and incubated at 17 °C in a constant temperature incubator. Hatching success (%) was determined 96 hpf based on the number of yolk sac stage larvae produced. Survival (%) to first-feeding stage was measured at 144 hpf and was based on the number of larvae with functional eyes and digestive system and with yolk reserves near exhaustion.

2.3.3. Egg free amino acid analysis

Newly spawned eggs from each female were collected and transferred to 2 mL polypropylene cryogenic vials, flushed with N2 gas (triplicate samples per female) and stored at −80 °C until analysis. Eggs from each female were categorized into four quality groups (high, med-high, med-low, and low) by selecting batches that showed comparable fertilization and hatching success according to the following percentages; 75–100% (high), 50–75% (med-high), 25–50% (med-low), and 0–25% (low). Fertilization success was determined at the 4–8 cell stage approximately 2–3 hpf, and hatching was determined approximately 96 hpf. For these determinations, only normally developing embryos and yolk sac stage larvae were counted. Each egg quality group was represented by samples obtained from four to six replicate female spawners.

2.3.4. Free amino acid extraction

Free amino acids were extracted from egg samples and were quantified by high performance liquid chromatography (HPLC) at MARBIONC (UNCW CMS). Extractions were done separately for each egg sample. One week before amino acid analysis, egg samples were freeze-dried and organized according to egg quality group. The extraction solution, 8% trichloroacetic acid, TCA (Sigma-Aldrich, USA), was spiked with 1 μM/L of a 10 nmol/mL stock solution of Norvaline internal standard to determine the extraction efficiency. Approximately 100 mg of each dry egg sample was placed into triplicate 10 mL glass test tubes for homogenization. Each sample was homogenized in 3 mL 8% TCA extraction solution for 60 s and then rinsed with an additional 3 mL solution to collect the sample from the homogenizer. The sample was homogenized with and rinsed with DI water three times for 30 s. Samples were transferred into 10 mL plastic centrifuge tubes with 1 mL of TCA solution and then centrifuged for 30 min at 2000 rpm. Five millilitre of the supernatant was transferred into clean plastic test tubes with a glass Pasteur pipette. The samples were washed three times with ether to remove the TCA before HPLC analysis. Samples were then left overnight in the refrigerator with the caps loosely fit to evaporate remaining ether. The samples were then filtered through 0.2 μm nylon membrane filters, washed with DI water and then acetone. Filtered samples were stored in the refrigerator until HPLC analysis.

2.3.5. HPLC analysis

HPLC analysis was conducted at the UNCW CMS Marine Biotechnology Laboratory. Samples were analyzed on the Agilent 1100 series HPLC comprising a LC binary pump, automated liquid sampler, thermostatic column compartment, column and guard cartridge, and diode array detector. The column selected for the analysis was the Agilent ZORBAX Eclipes Plus C18 and the method used was detailed by Henderson and Brooks (2010).

2.4. Statistics

Quantitative values of egg quality (production, fertilization, hatching, and survival to first-feeding stage success) were expressed as treatment means ± standard error. Prior to analysis, percentage data were arcsine transformed. To meet the assumptions of ANOVA, all data were analyzed using Levene’s homogeneity of variance test prior to the two-way ANOVA. If the data violated the assumptions of the ANOVA, a Kruskal-Wallis test was used to compare the independent effects of hormone type and dose. The effects of hormone type and dose (for both F1 and wild-caught separately) and their interaction on egg production, fertilization success, hatching success, and survival to first-feeding stage were analyzed using two-way ANOVA. When no interactions were observed, hormone type treatments were combined across doses and dose treatments were combined across hormone types. A Tukey-Kramer honest significant difference test was used for multiple comparisons among means. All statistical analyses were performed using JMP 7 and SAS statistical software (SAS version 9.1.3, SAS Institute, Cary, North Carolina USA) and a P-value of ≤0.05 was considered significant.

3. Results

3.1. Hormone-induced spawning: wild-caught females

For wild-caught females, a total of 33 female brooders were implanted with either mLHRHa, or sGnRHa applied at different doses (of 5, 25 or 50 μg/kg = low, medium, or high), comprising 5–7 females per treatment (Table 1). As evidenced by successful ovulation and strip spawning, 100% of the females responded to hormone treatment, except for the mLHRHa 5 μg/kg treatment in which six of seven females (85.7%) responded (Table 1). In contrast, five females were implanted

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<th>Hormone dose (μg/kg)</th>
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Values represent means ± SEM (N = 5–7). Means not followed by the same letter are significantly (P < .05) different.
with a placebo pellet (0 µg/kg), with no response to treatment (data not shown in Table 1).

There were significant (P < .05) differences among treatment groups in mean female body weights, which ranged from 1.72 to 3.32 kg (Table 1). When data on reproductive performance in Table 1 were standardized per unit female weight, actual hormone doses administered were close to those prescribed, ranging from 5.06–5.06, 25.0–25.1, and 50.1–51.3 µg/kg bw at the treatment doses of 5, 25, and 50 µg/kg bw, respectively, and were not significantly (P > .05) different between hormone types (mLHRHa vs. sGnRHa) at each treatment dose (Table 1). The mean latency periods (i.e., time from hormone implantation to first successful strip-spawning) ranged from 49.8 h post-implant (hpi) to 58.8 hpi among treatments, with no significant main (hormone type or dose), or interactive (hormone dose × hormone type) effects (Table 1).

3.1.1. Egg production: wild-caught females

In wild-caught females, no significant main or interactive effects on total eggs produced (range = 40.6–128 × 10³ eggs per kg female bw) (Table 2), or percent buoyant eggs (66.0–96.1%) were observed (Table 2). There were no significant main effects on fertilization success (% buoyant eggs), with an apparent interaction (P = .059) between these effects. For females treated with mLHRHa, fertilization success (% buoyant eggs) was highest at the mid dose of 25 µg/kg bw (82.6%), intermediate at the high dose of 50 µg/kg bw (79.2%) and lowest at the low dose of 5 µg/kg bw (67.3%). For females treated with sGnRHa, a different pattern was seen, where number of fertilized eggs decreased at higher doses from a maximum at the low dose of 5 µg/kg bw body wt. (67.3) to a minimum at the high dose of 50 µg/kg bw (23.9%) (Table 2, Fig. 1a).

When fertilization success was expressed as a percentage of overall (floating + sinking) eggs, there were no significant effects of hormone type (P > .05) or dose (0.05 < P < .10), but a significant interaction (P < .05) between these effects was observed (Table 2, Fig. 1a). For females treated with mLHRHa, overall fertilization success was highest at the mid dose of 25 µg/kg bw (79.2%), intermediate at the high dose of 50 µg/kg (45.8%) and lowest at the low dose of 5 µg/kg bw (24.4%). For females treated with sGnRHa, a different pattern was evident, where overall fertilization success decreased at higher doses from a maximum at the low dose of 5 µg/kg bw body wt. (57.7%) to a minimum at the high dose of 50 µg/kg bw (23.9%) (Table 2, Fig. 1a).

There was a significant interaction (P < .05) between hormone type and dose on number of fertilized egg produced per kg female bw, while there were no significant effects of hormone type (P > .05) or dose (0.05 < P < .10) (Table 2, Fig. 1a). For females treated with mLHRHa, number of fertilized eggs per kg bw (×10³) was highest at the mid dose of 25 µg/kg bw (92.2), intermediate at the high dose of 50 µg/kg (45.8) and lowest at the low dose of 5 µg/kg bw (18.2). For females treated with sGnRHa, a different pattern was evident, where number of fertilized eggs per kg bw (×10³) decreased at higher doses from a maximum at the low dose of 5 µg/kg bw (95.4) to a minimum at the high dose of 50 µg/kg bw (7.8) (Table 2, Fig. 1a).

Fig. 1. Fertilization success (% overall) for wild-caught (a) and F1 (b) female southern flounder. P. lethostigma broodstock implanted with pelleted mLHRHa or sGnRHa at doses of 5, 25, and 50 µg/kg. Values represent means ± SEM (N = 5–7).

3.1.2. Hatching success and yolk sac larval production: wild-caught females

In wild-caught females, no significant main or interactive effects of hormone dose or type were observed on hatching success (% buoyant eggs), which ranged from 15.8 to 47.7% among treatments (Table 3). When hatching success was expressed as a percentage of overall eggs produced, there were no significant main effects, while there was a significant (P < .05) interaction between these effects (Table 3, Fig. 3a). For females treated with mLHRHa, overall hatching success (%) was lowest at the low dose of 5 µg/kg body wt. (6.47) and increased at the mid (20.7) and high doses (30.5) (Table 3, Fig. 3a). For females treated with sGnRHa, a clear trend toward lower hatching success (%) overall with increasing dose was evident, from a maximum at the low dose (38.4%) to a minimum at the high dose (2.96) (Table 3, Fig. 3a).

When hatching success was expressed as number of yolk sac larvae produced per kg female bw (×10³), significant (P < .05) hormone dose and interactive effects were evident, while the effects of hormone type were not significant (P > .05). When hatching success was expressed as a percentage of overall eggs produced, there was no significant main effect, while there was a significant (P < .05) interaction between these effects (Table 3, Fig. 3a).
Type were not significant (Table 3, Fig. 3a). For females treated with mLHRHa, the number of yolksac larvae per kg bw was lowest at the low dose of 5 μg/kg (4.56) and increased to a maximum at the high dose of 50 μg/kg (33.6) (Table 3). For females treated with sGnRHa, a clear trend toward lower numbers of yolksac larvae per kg bw with increasing dose was evident, from a maximum at the low dose (34.8) to a minimum at the high dose (1.2) (Table 3, Fig. 4a).

### 3.1.3. Yolksac larval survival to first-feeding: wild-caught females

In wild-caught females, there were no significant main effects on survival (% buoyant eggs) of yolksac larvae to the first feeding stage, while there was an interaction (0.05 < P < 0.10) between these effects (Table 3). For females treated with mLHRHa, survival (% buoyant eggs) to first feeding increased from a minimum of 17.4% at the low dose of 5 μg/kg bw to a maximum of 33.6% at the high dose of 50 μg/kg bw. In contrast, for females treated with sGnRHa, survival to first feeding decreased from a maximum of 43.3% at the low dose to a minimum of 13.1% at the high dose of 50 μg/kg bw (Table 3).

When survival of yolksac larvae to the first-feeding stage was expressed as a percentage of overall eggs, there were no significant main effects, while there was a significant interaction (P < 0.05) between these effects (Table 3, Fig. 5a). For females treated with mLHRHa, survival to first feeding (% overall) was lowest at the low dose of 5 μg/kg bw (4.56) and increased to a maximum at the high dose of 50 μg/kg bw (25.0) (Table 3, Fig. 5a). For females treated with sGnRHa, a clear trend toward lower survival to the first-feeding stage (% overall) with increasing dose was evident, from a maximum at the low dose (34.8) to a minimum at the high dose (0.76) (Table 3, Fig. 5a).

### Table 3

Hatching success of buoyant and overall eggs produced (%), number of yolksac larvae per kg bw, survival to first-feeding stage of buoyant and overall eggs (%), and number of first-feeding larvae per kg bw for wild-caught southern flounder *P. lethostigma* female broodstock implanted with pelleted mLHRHa or sGnRHa pellets at dose rates of 5, 25, and 50 μg/kg.

<table>
<thead>
<tr>
<th>Hormone dose (μg/kg)</th>
<th>mLHRHa</th>
<th>sGnRHa</th>
<th>mLHRHa</th>
<th>sGnRHa</th>
<th>mLHRHa</th>
<th>sGnRHa</th>
<th>Probability levels for 2-way ANOVA&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hatching success (% buoyant)</td>
<td>22.3 ± 9.49</td>
<td>47.7 ± 18.5</td>
<td>35.0 ± 4.40</td>
<td>42.3 ± 11.8</td>
<td>38.6 ± 12.1</td>
<td>15.8 ± 4.79</td>
<td>NS</td>
</tr>
<tr>
<td>Hatching success (% overall)</td>
<td>6.47 ± 3.35</td>
<td>38.4 ± 16.6</td>
<td>27.9 ± 3.74</td>
<td>24.3 ± 8.08</td>
<td>30.5 ± 13.2</td>
<td>2.96 ± 1.48</td>
<td>NS</td>
</tr>
<tr>
<td>No. yolksac larvae per kg bw (× 10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>4.2 ± 2.4</td>
<td>73.5 ± 30.0</td>
<td>33.7 ± 12.6</td>
<td>14.5 ± 13.4</td>
<td>30.7 ± 27.0</td>
<td>1.2 ± 4.0</td>
<td>Dose**</td>
</tr>
<tr>
<td>Survival to first-feeding (% buoyant)</td>
<td>17.4 ± 8.79</td>
<td>43.3 ± 16.7</td>
<td>25.0 ± 4.94</td>
<td>39.9 ± 11.4</td>
<td>33.6 ± 12.6</td>
<td>13.1 ± 5.44</td>
<td>NS</td>
</tr>
<tr>
<td>Survival to first-feeding (% overall)</td>
<td>4.56 ± 2.11</td>
<td>34.8 ± 16.7</td>
<td>25.9 ± 4.94</td>
<td>39.9 ± 11.4</td>
<td>33.6 ± 12.6</td>
<td>13.1 ± 5.44</td>
<td>NS</td>
</tr>
<tr>
<td>No. first-feeding larvae per kg bw (× 10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>2.8 ± 1.4</td>
<td>66.5 ± 30.8</td>
<td>26.8 ± 12.9</td>
<td>13.8 ± 13.8</td>
<td>30.0 ± 26.6</td>
<td>0.68 ± 4.1</td>
<td>Dose**</td>
</tr>
</tbody>
</table>

Values represent means ± SEM (N = 5–7).<sup>a</sup> ⁎⁎ 0.05 < P < 0.10, ⁎⁎⁎ < 0.05, ⁎⁎⁎⁎ < 0.01, NS = not significant.
When yolksac larval survival was expressed as numbers of first-feeding stage larvae produced per kg female bw, significant dose (P < .05) and interactive effects (P < .05) were observed, with no significant effects of hormone type (Table 3, Fig. 6a). For females treated with mLHRHa, number of first-feeding stage larvae per kg bw (× 10³) increased with hormone dose from a minimum at the low dose (2.8) to a maximum at the high dose (30.0) (Table 3, Fig. 6a). Regression analysis of the relationship between wild-caught female body weight and the number of first-feeding stage larvae per kg female bw showed no statistical significance (r² = 0.0248 P = .4063, N = 30).

3.2. Hormone-induced spawning: first generation (F1) females

A total of 40 first-generation (F1) female brooders were implanted with either mammalian mLHRHa, or piscine sGnRHa applied in a fast-release pellet (80% cholesterol, 20% cellulose) at different nominal doses of 5, 25, and 50 µg/kg, comprising 5–8 females per treatment (Table 4). As evidenced by successful ovulation and strip-spawning, all females responded to hormone treatment, except for the mLHRHa 5 µg/kg and sGnRHa 25 µg/kg in which six of seven females (85.7%) responded (Table 4). In contrast, five females were implanted with a placebo pellet (0 µg/kg), with no response (data not shown in Table 4). There were no significant differences among treatment groups in mean female body weights, which ranged from 1.61 to 2.32 kg (Table 4). Actual hormone doses administered were close to those prescribed, ranging from 4.96–5.22, 25.2–25.8, and 50.6–50.6 µg/kg at the treatments doses of 5, 25, or 50 µg/kg, respectively, and were not significantly different between hormone types (mLHRHa and sGnRHa) at each treatment dose (Table 4). The mean latency periods (i.e., time from hormone implantation to first successful strip-spawning) ranged from 52.4 to 66.2 hpi, with no significant main or interactive effects (Table 4).

3.2.1. Egg production: F1 females

In F1 females, there was a significant effect of hormone type (P < .05) on total eggs produced per kg female bw, but with no significant effects of hormone dose or interaction between these effects (Table 5). At all hormone doses, egg production per kg bw was higher for females treated with mLHRHa (mean = 112.1 × 10³) than for females treated with sGnRHa (mean = 72.5 × 10³). There appeared to be an effect of hormone type (0.05 < P < .10) on percent buoyant eggs, with no significant effects of hormone dose or interaction between these effects (Table 5). At all hormone doses, percent buoyant eggs appeared to be higher for females treated with mLHRHa (mean = 80.7%) than for females treated with sGnRHa (mean = 67.9%).

There was a significant effect of hormone type (P < .05) on fertilization success (% buoyant eggs) (Table 5), with no significant effects of hormone dose or interaction between these effects (Table 5). Fertilization success (% buoyant eggs) was higher for females treated with mLHRHa (mean = 64.2%) than sGnRHa (mean = 43.3%) at all doses (Table 5).

When fertilization success was expressed as a percentage of the overall (floating + sinking) eggs, there was also a significant effect of hormone type (P < .05) (Table 5), with no significant effects of hormone dose, or interaction between these effects (Table 5, Fig. 1b). Fertilization success (% overall) was higher for females treated with mLHRHa (mean = 53.6%) than sGnRHa (mean = 33.3%) at all doses (Table 5, Fig. 1b).

There was a significant (P < .05) effect of hormone type and an
Apparent effect of hormone dose (0.05 < P < 0.10) on number of fertilized egg produced per kg female bw, with no interaction between these effects (Table 5, Fig. 2b). Number of fertilized eggs per kg bw (×10³) was higher for females treated with mLHRHa or sGnRHa at doses of 5, 25, and 50 μg/kg. Values represent means ± SEM (N = 5–8). For wild females (a) a significant interaction (hormone dose × type, P < .05) was evident. For F1 females (b), highly significant effects of hormone dose and hormone type (P < .01) were evident.

### 3.2.2. Hatching success and yolksac larval production: F1 females

In F1 females, there was a significant effect of hormone type (P < .05) on hatching success (% buoyant eggs) (Table 6), but with no significant effects of hormone dose or interaction between these effects (Table 6). At all hormone doses, hatching success (% buoyant eggs) was higher for females treated with mLHRHa (mean = 37.9%) than for females treated with sGnRHa (mean = 19.4%) (Table 6).

In F1 females, there appeared to be significant (0.05 < P < 0.10) main effects on hatching success (% overall), with no interaction between these effects (Table 6, Fig. 3b). Hatching success (% overall) was higher for females treated with mLHRHa (mean = 24.9%) than with sGnRHa (mean = 10.7%). For females treated with both mLHRHa and sGnRHa, hatching success (% overall) showed an inverse trend associated with dose; maximum hatching success was observed at the low dose of 5 μg/kg bw (37.9, 20.7) with the minimum hatching success observed at the high dose of 50 μg/kg bw (13.0, 2.62) (Table 6, Fig. 3b).

When hatching success was expressed as the number of yolksac larvae per kg female bw, there was an effect of hormone type (P < .05 < 0.10) and a significant (P < .05) effect of hormone dose, with no interaction between these effects (Table 6, Fig. 4b). For F1 females, mLHRHa appeared to produce greater numbers of yolksac larvae per kg bw than sGnRHa at all doses. For both hormone types, a trend toward decreasing yolksac larval production with increased dose was evident. Yolksac larval production per kg bw (×10³) was maximized at the low dose (74.0, 25.4), intermediate at the mid dose (19.9, 9.45) and decreased to a minimum at the high dose (10.8, 2.0) (Table 6, Fig. 4b).

#### 3.2.3. Yolksac larval survival to first-feeding: F1 females

For F1 females, there was a significant effect of hormone type (P < 0.05), and there appeared to be a significant effect of hormone dose (0.05 < P < 0.10) on yolksac larval survival to first-feeding (% buoyant eggs), with no interaction between these effects (Table 6). At all hormone doses, survival to first-feeding (% buoyant eggs) was higher in females treated with mLHRHa (mean = 32.8%) than for those treated with sGnRHa (mean = 15.6%). For both hormone types, survival to first feeding (% buoyant eggs) appeared to be maximized at the low dose (41.6–30.6), intermediate at the mid dose (29.1–13.0) and decreased to a minimum at the high dose (27.7–3.07) (Table 6).

When survival to first-feeding was expressed as a percentage of overall eggs, there appeared to be significant effects (0.05 < P < 0.10) of hormone type and hormone dose on yolksac larval survival to first-feeding (% overall eggs), with no interaction between these effects (P > .05) (Table 6, Fig. 5b). For F1 females, larval survival to first-feeding (% overall) across all doses appeared to be higher in females treated with mLHRHa (mean = 22.1%) than for those treated with sGnRHa (mean = 9.31%). For both hormone types, survival to first feeding (% overall eggs) appeared to decrease from a maximum at the low dose (33.8–20.2), to intermediate values at the mid dose (20.4–6.20) and to a minimum at the high dose (12.2–2.12) (Table 6, Fig. 5b).

When yolksac larval survival was expressed as numbers of first-feeding stage larvae produced per kg female bw, highly significant

**Table 4**

<table>
<thead>
<tr>
<th>Hormone type:</th>
<th>mLHRHa</th>
<th>sGnRHa</th>
<th>mLHRHa</th>
<th>sGnRHa</th>
<th>mLHRHa</th>
<th>sGnRHa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hormone dose (μg/kg):</td>
<td>5</td>
<td>25</td>
<td>50</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Fish implanted (total)</td>
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<td>7</td>
<td>8</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fish responding (%)</td>
<td>85.7</td>
<td>100</td>
<td>85.7</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Female body wt. (kg)</td>
<td>1.61 ± 0.12</td>
<td>2.10 ± 0.27</td>
<td>1.86 ± 0.10</td>
<td>2.32 ± 0.26</td>
<td>1.80 ± 0.19</td>
<td>1.96 ± 0.18</td>
</tr>
<tr>
<td>Range</td>
<td>(1.24–2.07)</td>
<td>(1.44–2.71)</td>
<td>(1.43–2.29)</td>
<td>(1.71–3.72)</td>
<td>(1.06–2.64)</td>
<td>(1.60–2.59)</td>
</tr>
<tr>
<td>Actual dose (μg/kg):</td>
<td>5.22 ± 0.13</td>
<td>4.96 ± 0.18</td>
<td>25.8 ± 0.37</td>
<td>25.2 ± 0.19</td>
<td>50.6 ± 0.72</td>
<td>50.6 ± 0.64</td>
</tr>
<tr>
<td>Range</td>
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<td>(4.11–5.28)</td>
<td>(24.4-27.5)</td>
<td>(24.5–26.2)</td>
<td>(48.4–54.6)</td>
<td>(49.1–52.6)</td>
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<tr>
<td>Latency period (hpi):</td>
<td>54.3 ± 4.01a</td>
<td>53.3 ± 3.6a</td>
<td>57.0 ± 3.16a</td>
<td>55.3 ± 3.66a</td>
<td>52.4 ± 2.99a</td>
<td>66.2 ± 4.04a</td>
</tr>
<tr>
<td>Range</td>
<td>(46.8–73.4)</td>
<td>(48.1–70.7)</td>
<td>(48.6–72.25)</td>
<td>(49.0–73.1)</td>
<td>(48.5–73.15)</td>
<td>(52.1–73.37)</td>
</tr>
</tbody>
</table>

Values represent means ± SEM (N = 5–8). No significant differences were detected.
3.2.4. Free amino acids

Spawned eggs were grouped according to their quality as measured by fertilization and hatching success (% buoyant eggs): high (75–100%), med-high (50–75%), med-low (25–50%), and low (0–25%) (Table 7). No significant differences in total free amino acid (FAA) concentrations (nmol/mg dry egg weight) among eggs of different quality were observed (Table 7).

There were no significant differences in concentrations (nmol/mg dry egg wt.) among the essential amino acids. Among the essential amino acids, leucine (57.1–60.1) was present in highest concentrations, followed by lysine (45.7–46.8), valine (36.9–44.6), isoleucine (31.5–39.1) and arginine (22.0–28.8). Essential amino acids found in intermediate concentrations included threonine (18.8–21.9), methionine (10.4–13.3), histidine (8.1–12.0) and phenylalanine (7.5–9.4). Tryptophan (1.3–2.3) was found in relatively low concentrations. Among the non-essential amino acids, serine (54.9–60.6) and alanine (47.6–51.2) were present in highest concentrations. Non-essential amino acids found in intermediate concentrations included glycine (12.2–21.9), glutamate (19.9–22.7), asparagine (14.3–16.5), tyrosine (7.1–9.5), and aspartate (8.2–9.8).

The conditionally essential amino acid taurine showed significantly different (P < .05) concentrations (nmol/mg dry egg wt.) among eggs of different quality. Taurine concentration was significantly higher in high quality eggs (27.4) than in med-low (14.8) and low (17.5) quality eggs. Concentration of taurine in the med-high (22.1) group was significantly higher than in med-low quality eggs (Table 7, Fig. 7). Glutamine concentrations ranged from 11.9 to 13.7 nmol/mg dry egg wt., with no significant differences among eggs of different quality.

4. Discussion

4.1. Wild-caught females: effects of GnRHα type (mammalian mLHRHa vs piscine sGnRHa) and dose on spawning performance

4.1.1. Ovulatory response, egg production and buoyancy: wild females

In this study, there were no differential effects of mammalian mLHRHa or piscine sGnRHa applied over a wide dose range (5, 25, or 50 μg/kg bw) on ovulatory response (86–100%), response latency (49.8–58.8 hpi) (Table 1), egg production (41–128 × 10^3 per kg bw) or egg buoyancy (63–96%) of post-vitellogenic stage MOD > 385 μm wild-caught southern flounder females under a temperature range of 15.9–16.3 °C (Table 2). These results indicated that mLHRHa and sGnRHa were equally effective in stimulating final oocyte maturation (FOM) and ovulation in vitellogenic stage southern flounder females, yielding large numbers of ovulated eggs during strip spawning with similar rates of buoyancy in seawater. These findings are consistent with previous reports where treatment of vitellogenic stage southern flounder females with GnRHa implants over wide dose ranges of up to 100 μg/kg bw successfully yielded ovulated eggs of varying quality. Berlinsky et al. (1996) reported a 92% ovulatory response in southern flounder females with an initial maximum follicle diameter ≥ 500 μm following implantation with mLHRHa at a dose of 100 μg/kg bw. Implanted females were biopsied at intervals no < 24 h to assess ovulatory status and were strip-spawned from 1 to 3 times on consecutive days, with no difference in the viability of eggs stripped on consecutive days. Under a mean temperature of 17 °C, these workers reported that

Table 5

<table>
<thead>
<tr>
<th>Hormone type:</th>
<th>mLHRHa</th>
<th>sGnRHa</th>
<th>mLHRHa</th>
<th>sGnRHa</th>
<th>mLHRHa</th>
<th>sGnRHa</th>
<th>Probability levels for 2-way ANOVAa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hormone dose (μg/kg):</td>
<td>5</td>
<td>25</td>
<td>50</td>
<td>Type</td>
<td>Dose</td>
<td>Interaction</td>
<td></td>
</tr>
<tr>
<td>No. eggs produced per kg bw (×10^5)</td>
<td>146.4 ± 38.4</td>
<td>76.0 ± 20.2</td>
<td>85.8 ± 13.3</td>
<td>61.9 ± 14.5</td>
<td>104.3 ± 10.9</td>
<td>80.0 ± 14.3</td>
<td>**</td>
</tr>
<tr>
<td>Buoyant eggs (%)</td>
<td>82.3 ± 11.0</td>
<td>67.2 ± 12.7</td>
<td>80.0 ± 4.2</td>
<td>77.5 ± 8.5</td>
<td>79.9 ± 5.0</td>
<td>58.9 ± 12.2</td>
<td>NS</td>
</tr>
<tr>
<td>Fertilization success (%)</td>
<td>70.8 ± 10.1</td>
<td>49.5 ± 12.7</td>
<td>66.1 ± 8.7</td>
<td>45.6 ± 14.8</td>
<td>55.7 ± 6.4</td>
<td>34.8 ± 4.0</td>
<td>**</td>
</tr>
<tr>
<td>Fertilization success (%) overall</td>
<td>63.0 ± 13.3</td>
<td>39.8 ± 12.8</td>
<td>53.3 ± 8.9</td>
<td>38.3 ± 14.6</td>
<td>44.5 ± 6.1</td>
<td>21.9 ± 5.3</td>
<td>**</td>
</tr>
<tr>
<td>No. fertilized eggs per kg bw (×10^5)</td>
<td>113.5 ± 39.6</td>
<td>40.4 ± 20.6</td>
<td>44.2 ± 10.5</td>
<td>26.5 ± 13.5</td>
<td>45.7 ± 3.6</td>
<td>18.7 ± 4.9</td>
<td>**</td>
</tr>
</tbody>
</table>

Values represent means ± SEM (N = 5–7).

(P < .01) effects of hormone type and dose were observed, on with no interactive effects (Table 6, Fig. 6b). In F1 females, yolksac larval production (number per kg bw × 10^3) across all doses was higher in females treated with mLHRHa (mean = 32.2) than for those treated with sGnRHa (mean = 10.8). For both hormone types, yolksac larval production decreased from a maximum at the low dose (67.5–24.9), to intermediate values at the mid dose (18.5–6.66) and to a minimum at the high dose (10.4–0.72) (Table 6, Fig. 6b). Regression analysis of the relationship between F1 female body weight and the number of first-feeding stage larvae per kg bw showed no statistical significance (r^2 = 6.02 × 10^-7, P = .9967, N = 30).

Table 6

<table>
<thead>
<tr>
<th>Hormone type:</th>
<th>mLHRHa</th>
<th>sGnRHa</th>
<th>mLHRHa</th>
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<th>sGnRHa</th>
<th>Probability levels for 2-way ANOVAa</th>
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<tr>
<td>Hormone dose (μg/kg):</td>
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<td>25</td>
<td>50</td>
<td>Type</td>
<td>Dose</td>
<td>Interaction</td>
<td></td>
</tr>
<tr>
<td>Hatching success (%)</td>
<td>48.3 ± 11.5</td>
<td>31.8 ± 11.7</td>
<td>35.2 ± 12.7</td>
<td>16.8 ± 7.72</td>
<td>30.1 ± 7.85</td>
<td>9.50 ± 2.93</td>
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<tr>
<td>Hatching success (%) overall</td>
<td>37.9 ± 11.3</td>
<td>20.7 ± 10.4</td>
<td>23.8 ± 11.4</td>
<td>8.65 ± 6.49</td>
<td>13.0 ± 4.81</td>
<td>2.62 ± 1.15</td>
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</tr>
<tr>
<td>No. yolksac larvae per kg bw (×10^3)</td>
<td>74.0 ± 37.3</td>
<td>25.4 ± 17.7</td>
<td>19.9 ± 7.1</td>
<td>9.5 ± 6.5</td>
<td>10.8 ± 0.57</td>
<td>2.0 ± 0.63</td>
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<tr>
<td>Survival to first-feeding (%)</td>
<td>41.6 ± 12.2</td>
<td>30.6 ± 11.9</td>
<td>29.1 ± 10.8</td>
<td>13.0 ± 5.2</td>
<td>27.7 ± 8.06</td>
<td>3.07 ± 1.02</td>
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</tr>
<tr>
<td>Survival to first-feeding (%) overall</td>
<td>33.8 ± 11.5</td>
<td>20.2 ± 10.5</td>
<td>20.4 ± 11.1</td>
<td>6.20 ± 4.41</td>
<td>12.2 ± 4.85</td>
<td>1.52 ± 0.03</td>
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<tr>
<td>No. first-feeding larvae per kg bw (×10^3)</td>
<td>67.5 ± 33.7</td>
<td>24.9 ± 17.8</td>
<td>18.5 ± 6.9</td>
<td>6.7 ± 4.6</td>
<td>10.4 ± 0.184</td>
<td>0.72 ± 0.38</td>
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</table>

Values represent means ± SEM (N = 5–8).

* P < .05, ** P < .10, *** P < .01, NS = not significant.

Table 7
Free amino acid (FAA) profiles (nmol/mg dry egg) (mean ± SEM, \(N = 4–6\) per group) of southern flounder \(P. lethostigma\) eggs of different quality as measured by fertilization and hatching success (% buoyant eggs): high (75–100%), medium-high (50–75%), medium-low (25–50%), and low (0–25%).

<table>
<thead>
<tr>
<th>FAA concentration (nmol/mg dry egg)</th>
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<th>Med-high</th>
<th>Med-low</th>
<th>Low</th>
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<tr>
<td>Mean ± SEM</td>
<td>75–100% fert</td>
<td>50–75% fert</td>
<td>25–50% fert</td>
<td>0–25% fert</td>
</tr>
<tr>
<td><strong>Essential AA</strong></td>
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<tr>
<td>Leucine</td>
<td>60.1 ± 6.53</td>
<td>62.1 ± 5.47</td>
<td>57.4 ± 5.47</td>
<td>57.1 ± 3.85</td>
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<tr>
<td>Lysolecithin</td>
<td>46.4 ± 3.92</td>
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<td>45.9 ± 4.15</td>
<td>45.7 ± 2.03</td>
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<tr>
<td>Valine</td>
<td>36.9 ± 6.85</td>
<td>41.3 ± 4.89</td>
<td>44.6 ± 5.89</td>
<td>41.1 ± 5.34</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>31.5 ± 5.31</td>
<td>36.6 ± 2.69</td>
<td>39.1 ± 6.40</td>
<td>33.0 ± 4.14</td>
</tr>
<tr>
<td>Arginine</td>
<td>28.8 ± 3.60</td>
<td>25.3 ± 3.34</td>
<td>22.0 ± 1.85</td>
<td>24.4 ± 1.08</td>
</tr>
<tr>
<td>Threonine</td>
<td>21.9 ± 4.01</td>
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<td>21.9 ± 3.71</td>
<td>18.8 ± 3.74</td>
</tr>
<tr>
<td>Methionine</td>
<td>13.3 ± 2.01</td>
<td>11.1 ± 1.31</td>
<td>10.4 ± 1.55</td>
<td>11.2 ± 1.36</td>
</tr>
<tr>
<td>Histidine</td>
<td>12.0 ± 3.01</td>
<td>10.7 ± 2.47</td>
<td>8.8 ± 2.34</td>
<td>8.1 ± 2.56</td>
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<tr>
<td>Phenylalanine</td>
<td>9.2 ± 2.52</td>
<td>8.8 ± 2.29</td>
<td>7.5 ± 2.49</td>
<td>9.4 ± 2.16</td>
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<td>Tryptophan</td>
<td>2.3 ± 0.74</td>
<td>1.3 ± 0.45</td>
<td>1.5 ± 0.62</td>
<td>1.5 ± 0.62</td>
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<tr>
<td><strong>Nonessential AA</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Serine</td>
<td>60.6 ± 7.21</td>
<td>57.7 ± 6.08</td>
<td>54.9 ± 8.67</td>
<td>56.1 ± 6.15</td>
</tr>
<tr>
<td>Alanine</td>
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<td>49.6 ± 4.93</td>
<td>51.2 ± 7.38</td>
<td>47.6 ± 5.60</td>
</tr>
<tr>
<td>Glycine</td>
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<td>21.1 ± 5.65</td>
<td>13.9 ± 1.73</td>
<td>12.2 ± 0.81</td>
</tr>
<tr>
<td>Glutamate</td>
<td>21.6 ± 5.03</td>
<td>20.9 ± 3.44</td>
<td>22.7 ± 4.44</td>
<td>19.9 ± 5.48</td>
</tr>
<tr>
<td>Asparagine</td>
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<td>15.9 ± 3.27</td>
<td>16.4 ± 4.33</td>
<td>14.3 ± 4.48</td>
</tr>
<tr>
<td>Tyrosine</td>
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<td>7.1 ± 2.76</td>
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<tr>
<td>Aspartate</td>
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<td>9.8 ± 3.07</td>
<td>8.8 ± 2.88</td>
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<tr>
<td><strong>Conditionally essential AA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taurine**</td>
<td>27.4 ± 6.26a</td>
<td>22.1 ± 5.38ab</td>
<td>14.8 ± 1.05c</td>
<td>17.5 ± 2.0bc</td>
</tr>
<tr>
<td>Glutamine</td>
<td>13.7 ± 2.48</td>
<td>12.7 ± 1.53</td>
<td>12.2 ± 2.65</td>
<td>11.9 ± 1.71</td>
</tr>
<tr>
<td>Total</td>
<td>489.4</td>
<td>482.7</td>
<td>462.0</td>
<td>446.6</td>
</tr>
</tbody>
</table>

Groups not sharing the same letter are significantly different.

* \(0.05 < P < .10\).

** \(P \leq .05\).

Fig. 7. Taurine concentrations (nmol/mg dry egg weight, mean ± SEM, \(N = 4–6\)) in ovaulated southern flounder eggs of different quality as measured by fertilization and hatching success (% buoyant eggs): high (75–100%), medium-high (50–75%), medium-low (25–50%), and low (0–25%). Groups not sharing the same letter are significantly different.

females ovaulated within 65–90 hpi with an average egg buoyancy rate of 65% (range = 26–87%), results very similar to those in the present study in which latency periods and egg buoyancy (%) ranged from 48.3–77.3 hpi and 62.5–96.1% in wild females (Table 1) and from 46.8–73.4 hpi and 58.9–82.3% in F1 females (Table 4).

Watanabe et al. (2001, 2006) observed a similar response in southern flounder females (mean oocyte diameter ≥ 385 μm) following implantation with mLHRHa at doses of 50 and 100 μg/kg bw under 16–18°C. These workers likewise observed a strong ovulatory response (85–92%), a response latency of approximately 48 hpi, egg production of 26–74 × 10³ per kg bw, and average egg buoyancy rate in seawater ranging from 26.5–91.5% overall (Watanabe et al., 2001, 2006).

Similar findings have also been reported in other Paralichthid flounder species. In the congenic summer flounder \(P. dentatus\) treatment of vitellogenic stage females (mean oocyte diameter = 258–456 μm) with mLHRHa implants at a high dose of 100 μg/kg bw under 17°C produced a 100% ovulatory response, a response latency of 60–132 hpi, egg production of 78.9 × 10³ per kg bw, and a buoyancy rate that varied from 0 to 94.3% (Watanabe et al., 1998). Except for a longer response latency, possibly related to smaller than optimum initial MOD and to the use of volitional spawning rather than strip-spawning, overall egg production and buoyancy were comparable to those obtained with southern flounder in the present study. In another study with the summer flounder (Berlinsky et al., 1997), only females with maximum follicle diameters > 585 μm responded (80%) to mLHRHa implants at 100 μg/kg bw at a temperature of 16°C, although egg production and buoyancy were not reported. In the Brazilian flounder \(P. orbignyanus\) vitellogenic stage wild-caught females (MOD = 484 ± 15 μm) implanted with 100 μg/kg bw mLHRHa under 24–25°C showed a shorter response latency of 36 ± 5 hpi, with an egg production of 64 × 10³ per kg bw, although egg buoyancy was not reported (Sampaio et al., 2008). Except for shorter response latency in Brazilian flounder, likely due to the higher temperature regime, all of these results show similar responsibility among Paralichthid flounder species to GnRHa implants across a wide dose range of 5–100 μg/kg bw with respect to ovulation, egg production and egg buoyancy. Similar to the present findings with southern flounder, no differences in efficacy between mLHRHa and sGnRHa were found in gilthead seabream Sparus aurata (Zohar et al., 1989) or Atlantic cod Gadus morhua (Garber et al., 2009) on spawning performance measured in terms of ovulatory response, egg production, and egg buoyancy (% floating eggs).

4.1.2. Fertilization success, hatching success and yolksac larval survival to the first-feeding stage as indices of egg quality: wild females

Many studies have assessed quality of marine fish eggs by...
determining the percentage of buoyant eggs in seawater, but few have attempted to verify egg quality by monitoring the fertilization success of the eggs, the survival of the embryos to hatching, and then the survival of the newly hatched yolk sac stage larvae until endogenous yolk reserves are exhausted (i.e., first-feeding stage). In this study, although mLHRHa and sGnRHa at different doses ranging from 5 to 50 μg/kg produced no differences in ovulatory response, or in production of buoyant eggs in wild females, clear effects of hormone type and dose became evident as the development of spawned eggs progressed through fertilization, hatching, and the first-feeding larval stage. Strikingly different dose-response relationships between mLHRHa and sGnRHa (i.e., hormone type × dose interaction) were first observed at the fertilization stage and became more pronounced as embryos developed to the hatching stage and as yolk sac larvae developed to the first-feeding stage.

4.1.2.1. mLHRHa: wild females. For wild-caught females treated with mLHRHa, fertilization success peaked at the intermediate dose (25 μg/kg, 79.3% overall fertilization success) and was lower at high (50 μg/kg, 40.5% overall) or low (5 μg/kg, 24.4% overall) doses (Table 2). As embryonic development progressed, however, a trend toward higher hatchling success and yolk sac larval survival to the first-feeding stage with increasing mLHRHa dose was seen (Table 3), and production of yolk sac larvae and first-feeding stage larvae was maximized at the highest dose of 50 μg/kg bw, which produced a hatching rate of 38.6% buoyant (30.5% overall) (Table 3) and a mean of 30.0 × 10³ first-feeding larvae per kg bw (Table 3). Watanabe et al. (2001) reported that treatment of wild-caught post-vitellogenic stage southern flounder females with mLHRHa at a much higher dose of 100 μg/kg bw resulted in poor overall fertilization (17%) and hatching success (26%), but later found that mLHRHa at a lower dose of 50 μg/kg bw produced higher overall fertilization (37.7%) and hatching success (28.7%) (Watanabe et al., 2006). Smith et al. (1999) likewise observed a low fertilization success of 32% for southern flounder females implanted with mLHRHa at a high dose of 100 μg/kg bw. These results suggest that a dose of 100 μg/kg bw mLHRHa is excessive for southern flounder, stimulating high rates of ovulation and egg production, but with low fertilization success and survival to the first-feeding stage (i.e., poor egg quality).

4.1.2.2. sGnRHa: wild females. In contrast to mLHRHa, treatment of wild-caught southern flounder females with piscine sGnRHa in the present study resulted in an inverse dose-response relationship, with fertilization and hatching success and yolk sac larval survival trending downward as sGnRHa dose was increased, and production of first-feeding stage larvae was maximized at the low dose of 5 μg/kg bw (Tables 2, 3; Figs. 1–6a) which resulted in an average of 66.5 × 10³ first-feeding larvae per kg bw (Table 3; Fig. 6a). Distinctive dose-response relationships between mLHRHa and sGnRHa on spawning performance have not been previously reported during induced spawning of flounder.

In this study, egg buoyancy and fertilization success were not reliable indices of egg quality, which was more accurately assessed as development progressed to the first-feeding stage. Likewise, in Atlantic cod Gadus morhua egg production per kg bw and percent fertilization were similar in LHRHa- and sGnRHa-treated and naturally spawning females, but the number of hatched larvae per kg bw (i.e., egg quality) was higher in naturally spawning females (Garber et al., 2009).

4.2. F1-females: effects of GnRHa type (mammalian mLHRHa vs piscine sGnRHa) and dose on spawning performance

4.2.1. Ovulatory response, egg production and buoyancy (F1 females)

In F1 southern flounder females, there were no significant effects of hormone type on ovulatory response (87.6–100%), response latency (52.4–66.2 h) (Table 4), results similar to wild-caught females. Unlike wild-caught females, however, significant effects of hormone type on total egg production, egg buoyancy, and fertilization success were observed (Table 5, Figs. 1b). In general, higher egg production, buoyancy and fertilization success were obtained with mLHRHa than with sGnRHa at all three doses. As development progressed, however, significant effects of both hormone type and dose became evident. Hatching success and yolk sac larval survival were generally higher with mLHRHa than with sGnRHa, and clear trends toward lower embryo viability at higher doses were observed for both types of hormones, so that maximum production of first-feeding stage larvae was obtained at the low dose of 5 μg/kg bw mLHRHa (Table 4, Figs. 6b). Since sGnRHa is a piscine-type analog, this hormone would be expected to have greater potency than mammalian mLHRHa. However, in this study, production of first-feeding stage larvae was higher in response to mLHRHa than to sGnRHa at equivalent doses. While counterintuitive, the results obtained with wild-caught females may be related to the limited dose range tested, which may not have allowed the full dose-response relationships between these types of hormones to be clearly delineated. Hence, it is possible that even higher spawning performance in F1 females could be attained at sGnRHa doses lower than 5 μg/kg bw. Based on maximum numbers of first-feeding stage larvae produced (Fig. 6b), a low dose of 5 μg/kg bw is recommended for both sGnRHa and mLHRHa for induced spawning of F1 females.

4.3. Comparison of wild-caught and F1 females

In this study, differences in spawning performance and egg quality between wild and F1 broodstock in response to mLHRHa and sGnRHa induction were apparent from their distinctive dose-response relationships. In wild females treated with mLHRHa, maximum larval production (number per kg bw × 10³) (30.0) was obtained at the high dose (Fig. 6a). In contrast, maximum larval production of wild females treated with sGnRHa (66.5) was obtained at the low dose (Table 3, Fig. 6a). For F1 females, on the other hand, maximum larval production was obtained at the low dose of both mLHRHa (67.5) and sGnRHa (24.9) (Fig. 6b). These results demonstrate that, depending on the origin (wild vs F1) of the female spawner, both the type and dose of GnRHa are critical to optimizing spawning performance. In wild-caught and F1 southern flounder females, we recommend sGnRHa and mLHRHa, respectively, at a low dose of 5 μg/kg bw. The results also showed that F1 southern flounder breeders can be treated with GnRHa therapies to produce eggs and larvae comparable in numbers and quality to wild breeders, although lower doses of both mLHRHa and sGnRHa within the range of 5–50 μg/kg bw maximized spawning performance in F1 fish.

In this study, the lower doses of exogenous mLHRHa and sGnRHa required to induce ovulation and spawning of F1 hatchery-reared southern flounder females were likely related to lower stress levels and greater responsibility to these gonadotropin releasing hormone analogues than in wild females. It is known that stress can inhibit reproduction in fish (Campbell et al., 1992) and is associated with elevations of plasma cortisol (Donaldson, 1981; Barton and Iwami, 1991) and depression of plasma testosterone and 17β-estradiol levels (Sumpter et al., 1987). Hatchery-reared rainbow trout Salmo gairdneri showed significantly lower cortisol production and glucose levels and more stable plasma chloride levels in response to stress than wild fish (Woodward and Strange, 1987). Stress and resultant cortisol production has also been shown to affect gamete, embryo and larval quality as well as the neuroendocrine axis by reduction in levels of sex steroids and vitellogenin in plasma, and decreasing fertilization, hatching and larval survival in Atlantic salmon Salmo salar and rainbow trout Oncorhynchus mykiss (Carragher et al., 1989; Campbell et al., 1992; Contreras-Sanchez et al., 1998; Pankhurst and Van Der Kraak, 2000; Schreck et al., 2001; Eriksen et al., 2006). Hence, it is likely that blood cortisol levels were higher in wild than in F1 southern flounder, inhibiting GH secretion (Pankhurst and Van Der Kraak, 2000) and requiring a higher dose of mLHRHa to induce successful spawning.
4.4. Ovarian overstimulation

Previous work at UNCW has shown that induced spawning of southern flounder with relatively high doses of 50–100 μg/kg LHRHa administered in a 95% cholesterol–5% cellulose (slow-release) delivery system (Berlinsky et al., 1996; Smith et al., 1999; Watanabe and Carroll, 2001; Watanabe et al., 2006) sometimes caused ovarian overstimulation, characterized by excessive ovarian hydration and swelling, particularly in F1 females. F1 females may be more sensitive to hormone overstimulation than wild-caught females, which are less adapted to captive conditions and are under greater stress. In the present study, one F1 female exhibited ovarian overstimulation in response to the high dose of sGnRHa (50 μg/kg), whereas no overstimulation was observed in F1 females treated at the intermediate (25 μg/kg) or low dose (5 μg/kg) mLHRHa or sGnRHa. The data suggest that overstimulation in F1 southern flounder should not be a problem when the recommended low dose of 5 μg/kg mLHRHa or sGnRHa is used.

4.5. Free amino acids in relation to egg quality

In the present study, the most abundant FAA found in newly ovulated eggs of southern flounder were leucine, lysine, valine, isoleucine, and arginine (EAA); serine, alanine, and glycine (non-EAA); and taurine (conditionally EAA) (Table 7). These FAA were also found to be most abundant in newly ovulated eggs of other flatfish species such as Atlantic halibut Hippoglossus hippoglossus, turbot Scophthalmus maximus (Rønnestad et al., 1992a, 1992b), plaice Pleuronectes platessa, lemon sole Microstomous kitt (Thorsen and Fyhn, 1996), and Barfin flounder Verasper moseri (Ohkubo and Matusubara, 2002), but also other types of marine finfish such as Asian sea bass Lates calcarifer (Nocillado et al., 2000) and red snapper Lutjanus campechanus (Hastey et al., 2009). Although the total amount of FAA in marine fish eggs varies among species, relative concentrations are similar and are related to proteolysis of a common yolk lipoprotein vitellogenin (Vtg) during FOM and oocyte hydration in pelagic eggs (Wallace and Selman, 1985; Craik and Harvey, 1987; Thorsen and Fyhn, 1996). The pool of FAA within the oocyte creates the osmotic gradient that causes osmotic water influx during oocyte hydration (Greeley Jr et al., 1986; Thorsen and Fyhn, 1996; Finn and Fyhn, 2010) and provides an energetic substrate during embryonic development (Fyhn and Serigstad, 1987; Rønnestad et al., 1992a, 1992b; Sivaloganathan et al., 1998; Finn and Fyhn, 2010).

In the present study, total FAA concentrations in newly ovulated southern flounder eggs appeared to decrease in eggs of lower quality (Table 7), with greater taurine concentration in eggs of high quality than in eggs of medium or low quality. In ovulated eggs of marine finfish, taurine is not derived from the hydrolysis of vitellogenin, but is taken up from the extracellular fluid during final oocyte maturation (Thorsen and Fyhn, 1996). In flatfish, including Atlantic halibut, turbot, and lemon sole, taurine concentrations remained relatively unchanged during embryonic and yolk-sac larval stages, suggesting that taurine is incorporated into the FAA pool of the egg before spawning (Rønnestad and Fyhn, 1993) and is conserved, rather than catabolized during the embryonic and yolk sac larval stages. In the present study, significantly higher taurine concentrations found in newly-spawned southern flounder eggs of higher quality (i.e., higher fertilization and hatching success) suggests that taurine may be a critical component during final oocyte maturation and hydration as well as during the early embryonic stages. Dietary taurine supplementation to broodstock may improve egg quality in fish as reported in longfin yellowtail Seriola quinquemaculata (Matsunari et al., 2006) and in Nile tilapia Oreochromis niloticus broodstock (Al-Feky et al., 2016). Additional studies are needed to determine if taurine is conserved during the yolk sac larval stages in southern flounder and to elucidate its physiological role.

4.6. Summary and conclusions

Results of this study showed that spawning performance and egg quality of wild and F1 southern flounder in response to mLHRHa and sGnRHa induction at three dose levels (5, 25 and 50 μg/kg bw = low, mid and high) was accurately measured in terms of the numbers of first-feeding stage larvae produced per kg female bw. For wild females, a significant interaction between hormone dose and type was evident, with sGnRHa producing the best larval production at the low dose of 5 μg/kg bw, whereas mLHRHa produced the best larval production at the high dose of 50 μg/kg bw. This interaction likely reflected the limited dose range tested, which was at the high end of the dose response range for sGnRHa, but in the optimal or low range for mLHRH. This is also consistent with the assumption that piscine sGnRHa is more potent in wild southern flounder females than mammalian mLHRHa. Conversely, mammalian mLHRHa requires higher doses to be effective. For F1 females, on the other hand, no interactive effects between hormone type and dose were observed. This is in accord with the ovarian overstimulation observed in an F1 female treated with the high dose of 50 μg/kg bw of sGnRHa and that spawning performance of F1 fish was improved at low dose of 5 μg/kg bw. This further suggests that even lower doses of GnRHa may improve spawning performance in both wild and F1 fish and that overstimulation was prevented with the lower doses.

Eggs of high quality contained significantly higher concentrations of the free amino acid taurine than in eggs of medium to low quality. The role of taurine during embryonic development and dietary taurine supplementation to broodstock diets to improve egg quality require further study. These findings will help standardize GnHRa induction therapies to improve the reliability of spawning and the quality of eggs produced by wild-caught and F1 southern flounder broodstock to support large-scale hatchery production.

Acknowledgements

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