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INDUCED SPAWNING OF CAPTIVE TRIPLETAIL LOBOTES SURINAMENSIS

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INDUCED SPAWNING OF CAPTIVE TRIPLETAIL *LOBOTES SURINAMENSIS*

by

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ABSTRACT

The Tripletail, *Lobotes surinamensis*, is an emerging candidate species for U.S. marine aquaculture. This work aimed to address two bottlenecks for hatchery production of the species by developing a hormonal induction protocol to obtain fertile spawns from captive brooders and a method for sex identification of candidate brooders.

Single pairs selected among a captive-held broodstock conditioned under a natural photothermal cycle were induced with one of five treatments (n = 5 or 6 replicates per treatment). Control (no hormone) and hCG (1,100 $IU.kg^{-1}$ for females, 550 $IU.kg^{-1}$ for males) pairs did not spawn. Pairs treated with GnRHa slow-release implants (75 $mg.kg^{-1}$ for females, 55 $mg.kg^{-1}$ for males) produced 1 to 2 spawns and, on average 695,899 eggs but fertility was very low (0.58%). Administration of Domperidone at 5 or 10 $mg.kg^{-1}$ in conjunction with GnRHa implants improved all metrics with best results obtained in the 10 $mg.kg^{-1}$ treatment-group (65.3% fertility, 2.83 spawns following induction, over 1.5 M eggs per mating pair, and larval survival through 4 dph averaging 33.3%). Treatment with GnRHa and Domperidone a week after an initial hCG injection did not improve these results.

Plasma levels of 11-Ketotestosterone and Estradiol 17β were assayed in males and females during the spawning season using a competitive ELISA assay. Sex identification was most effective using 11-Ketotestosterone with a 1.19% error rate in cross validation of the training set. Males with low levels of this hormone in the test dataset were mis-identified with a higher rate (7.83%) and may require additional measurements.

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CHAPTER I – GENERAL INTRODUCTION

The global demand for seafood has been rising steadily over the past few decades (FAO, 2018), reflecting the growth of world population and a continuous increase in fish consumption per capita. Marine fisheries harvests have historically supplied this demand, but harvests reached a maximum of 90 million tons in the 1980's and have remained stable since. Most fisheries stocks are now fully exploited or overfished such that fisheries production cannot provide for the increasing seafood demand. Aquaculture production has been filling the gap between fisheries supply and demand since the 1980's and was reported to have produced 84 million tons in 2019 (OECD/FAO, 2018; FAO, 2020). From the same report (FAO, 2020) aquaculture is predicted to exceed wild fisheries production in 2023 and produce 109 million tons by 2030.

US seafood consumption, much like global consumption, has been increasing continuously, but the domestic supply is insufficient to fill the demand. In 2018, 90% of the seafood consumed by Americans was imported and half of these imports were aquaculture products. The overall seafood trade deficit, which also has been growing, reached $17.1 billion (Liddel and Yencho, 2020) and can be reduced only by developing domestic aquaculture, particularly considering the stagnation of fisheries harvests. However, US aquaculture ranked only 17th in the world in 2014 (Lester et al., 2018) and contributes a negligible fraction of the US consumption. Domestic aquaculture production is largely focused on freshwater species and salmonids. The small contribution of marine species (13.5% of US aquaculture production) consists mostly of shellfish (Montgomery, 2019) and nearly no finfish. However, marine finfish represent

31.4 % of US seafood imports in 2018 (Liddel and Yencho, 2020), which highlights the need to develop an aquaculture industry targeting these species.

A number of factors have slowed the development of marine finfish aquaculture. These factors include but are not limited to a high initial monetary investment needed to establish production units, a complex permitting process, the insufficient technological control of the production for many species of interest, and the extended time delay between investment and financial return. However, some finfish culture industries abroad have successfully overcome these challenges. Aquaculture of the Atlantic Salmon (*Salmo salar*) is an example of a successful marine aquaculture species. Aquaculture production for this species grew from near zero in the mid-eighties to over 84 MT in 2019 and will exceed that of wild fisheries production by 2023 (OECD/FAO, 2018; FAO, 2020). This success was made possible by major technical progress in husbandry that led to the acquisition of the full control of the rearing cycle and to the development of breeding programs (Gjedrem et al., 2012).

New candidate aquaculture species have almost always been selected based on the high market value of their wild counterparts (Quemener, 2002). However, this selection method has its limitations because the technology to mass-produce most marine fishes was not available and proved difficult to develop for many species (Davis et al., 2019). Accordingly, the selection of future candidate finfish species may need to be based partly on biological and technical characteristics to increase the likelihood of success. Characteristics to consider include the feasibility of closing the life cycle in captive conditions, the technological requirements involved in the culture of critical life stages (for example salmonid species can be fed prepared feed as initial food source and require

no live feeds), the growth rate (fast growth rate is expected to lower production costs by shortening the culture cycle), the larval culture survival rates, the suitability for extensive culture practices such as omnivory or planktivory, and the flesh quality and fillet yield, which both influence market potential and outlook. A study by Thouard et al. (1990) evaluated possible candidate aquaculture species in La Martinique by assessing experimentally each species' captive reproduction output, larval qualities, and growth parameters. Alvarez-Lajonchere et al. (2013) used a similar protocol to select future candidate species for intensive RAS (recirculating aquaculture systems) culture in the Caribbean.

Recently, available data on marine finfish species candidate for aquaculture development in the U.S. were reviewed by Davis et al. (2019) and synthesized in a volume of the Journal of the World Aquaculture Society (Rexroad et al., 2021). Several species were identified as species with potential based on the above characteristics, although at varying degrees of technical feasibility or commercial development. Forefront species already produced commercially include the Almaco jack and other *Seriola* species, the Florida pompano (*Trachinotus carolinus*), cobia (*Rachycentron canadum*), and red drum (*Sciaenops ocellatus*) among others. Recently, Tripletail (*Lobotes surinamensis*) was identified as a promising candidate aquaculture fish that requires additional research to reach technical feasibility (VanderKooy, 2016).

Little is known about the biology of Tripletail and its potential for aquaculture. Tripletail is a subtropical and tropical fish that maintains a mostly pelagic lifestyle throughout its lifecycle. The species can be found throughout the western Atlantic Ocean from Massachusetts to Argentina and the eastern Pacific Ocean where it is reported off

Central America. Tripletail is described as a batch spawning finfish species that exhibits an asynchronous oocyte development (Brown-Peterson and Franks, 2001). Like many other marine fishes that broadcast eggs, this species shows a high fecundity with estimates of mean relative batch fecundity of 47.6 ± 18.1 eggs/gram ovary-free body weight^{-1}. Spawning is thought to occur from June through August with highest intensity in July and regression beginning in August (Brown-Peterson and Franks, 2001). Spawning habitats have not been formally described, but Ditty and Shaw (1994) concluded that spawning likely occurred near the outer continental shelf based on the observation of very small larvae (<5.0 mm) in these waters in the Gulf of Mexico. Tripletail larvae were all found in the surface layers of the water and were often associated with *Sargassum* or other flotsam.

Tripletail are highly sought-after by recreational anglers who enjoy their strong fight on the hook and excellent flesh quality (VanderKooy, 2016). In contrast, commercial fishing is undeveloped in the U.S. because of the species' non-gregarious lifestyle which is incompatible with mass harvest (Saillant et al., 2014) and also because the demand was low or inexistent until recently. However, commercial harvests have been increasing recently as consumers became aware of the quality of this fish even though annual landings have averaged only 7,000 lbs. in the US since 2000 (VanderKooy, 2016). Tripletail market value per pound has varied erratically but has been reported as high as $3.65/pound in the southern Atlantic in 2014, although prices in the Gulf of Mexico remained somewhat steady at $1.25/pound (Vanderkooy, 2016). The east coast of Florida accounted for 62% of the total U.S. commercial landings in 2000 (VanderKooy, 2016) suggesting a stronger market or possibly a higher abundance in that

area. The largest landings of Tripletail in the world occur in South American countries (Guyana, Suriname, and Brazil) which have reported up to 3 million tons per year (VanderKooy, 2016). There is now an established demand for Tripletail in the U.S. and, because of the lack of a reliable domestic source, this demand is filled by imports from the eastern Pacific and South America (VanderKooy, 2016). Aquaculture could address this limitation of domestic production and provide a reliable source to retailers and consumers thus creating a hold on domestic markets.

At the beginning of this work, published information on the culture of TripletailI was limited to reports by Franks et al. (2001) and Saillant et al. (2014). Franks et al. (2001) reared wild-caught juvenile Tripletail for 210 days in Recirculating Aquaculture Systems at warm temperatures (25-29ºC) typical of wild habitats. The juveniles reached an average 359 mm and 1,102 g during the period with growth rates in length and weight of 1.4 mm/day and 4.8g/day, respectively. Although this culture trial was performed at very low density, the recorded growth rates are in the range of those reported in some of the fastest marine species candidate for aquaculture such as amberjack or cobia (Davis, 2019). Saillant et al. (2014) conducted preliminary studies of captive reproduction and early larval culture. Captive Tripletail broodstock did not spawn spontaneously, but some females were found at advanced stages of vitellogenesis compatible with hormonal induction of final oocyte maturation and ovulation. These females did release large spawns following hormonal induction. Males, however, did not release sperm following manual stripping and all but one spawn released by females following hormonal induction were unfertilized. The only spawn with fertilized embryos had a low fertility (9.8% fertilized embryos). This work also revealed challenges identifying the sex of

broodfish due to the lack of dimorphic sexual secondary characters and difficulties obtaining gonad biopsies or gamete samples for direct sexing of males and females. Larval culture was attempted by offering rotifers as initial live feed and monitoring the development and growth of the larvae. Unfortunately, only limited feeding and growth was observed, and complete mortality occurred by 10-day post hatch. The cause of mortality was not determined but could be attributed to several extrinsic factors related to husbandry such as sub-optimal environmental conditions, prey density and/or nutritional quality or intrinsic factors related to poor egg quality and lack of success transitioning to exogenous feeding.

The first bottleneck of Tripletail aquaculture is the lack of success producing fertilized spawns from captive brooders. Resolving this issue with captive reproduction is critical to complete the species' life cycle in captivity and secure a source of fry for aquaculture. Disruptions of gametogenesis and spawning are common in aquaculture and have been linked to inhibitions of the production of gonadotropin by the pituitary and/or its release into the bloodstream in farmed conditions (Zohar and Mylonas, 2001). In most cases, disruptions affect the final phases of oocyte maturation in females and/or spermiation in males. These processes can be restored by stimulating the pituitary using Gonadotropin Releasing Hormone (GnRH) administered in a single injection or a slow-release implant (Zohar and Mylonas, 2001). In the case of Tripletail, the observation of oocytes at advanced stages of vitellogenesis compatible with hormonal induction using GnRH by Saillant et al. (2014) suggests that only the final phase of oocytes maturation of females may be inhibited. The failure of males to release sperm following manual stripping indicates that spermiation is inhibited although earlier phases of

spermatogenesis also may be disrupted. The lack of fertility of spawns following GnRH administration indicates that GnRH stimulation is ineffective at inducing spermiation and spawning. Limited effectiveness of GnRH hormonal therapies has been reported in other aquaculture species and was shown to be due to endogenous inhibition of gonadotropin release by dopamine (Chang and Peter, 1983). Production and release of gonadotropin could be restored by administration of dopamine receptor antagonists such as Domperidone or Pimozide (Chang et al., 1984) in conjunction with GnRH. It is, thus, possible that dopamine inhibition is responsible for the lack of success inducing spermiation of Tripletail males and production of fertile spawns. It is also possible that earlier phases of spermatogenesis are inhibited in Tripletail males such that the few days of activity of the GnRH therapy is insufficient to complete the production of sperm in sufficient quantities. In the latter case, repeated stimulations may be necessary to stimulate spermatogenesis and spermiation in large volumes as shown by Passini et al. (2018) who were able to increase spermiation through repeated administration of androgens in *Centropomus undecimalis*.

Another issue related to Tripletail broodstock management is the unavailability of a reliable sexing method. Females at advanced stages of vitellogenesis can be sexed by the collection of an ovarian biopsy using a catheter but immature females cannot be cannulated and therefore cannot be distinguished from males since males typically do not release sperm during manual stripping in captive conditions. Developing a non-lethal sexing method is therefore another important need for Tripletail aquaculture.

This work aims to address these two bottlenecks by testing protocols for hormonal induction of final gamete maturation and spawning as well as a method to identify the phenotypic sex based on measurements of plasma levels of gonadal steroids.

CHAPTER II- INDUCTION OF SPERMATOGENESIS AND FINAL GAMETE MATURATION IN TRIPLETAIL

2.1 Introduction

The first step toward the development of aquaculture for a new species is to achieve a reliable source of seeds for grow out through controlled captive reproduction. Initial attempts to obtain captive spawning usually consist of acclimating broodstock caught in the wild under a natural temperature and photoperiod cycle (Duncan et al., 2013). This protocol is not always successful, and some species show inconsistent or complete lack of spawning. One of the most common causes for the failure of fish to spawn in captivity is a dysfunction of the final phases of oocyte maturation in females or of spermiation in males (Zohar and Mylonas, 2001). This disruption is due to a lack of production and/or release of gonadotropin hormone by the pituitary. Gonadotropin hormones are central components of the brain-pituitary-gonad-axis that controls the reproductive cycle. External cues perceived by the brain trigger the release of gonadotropin releasing hormone (GnRH) by the hypothalamus in the hypophyseal portal system (Yu et al., 1997). GnRH stimulates the pituitary to produce and release gonadotropin hormones (FSH and LH) (Kobayashi et al., 1997) which are transported to the gonad where they “regulate the biosynthesis of gonadal steroid hormones that subsequently control processes of sexual maturation, behavior, and FOMO” (final oocyte maturation and ovulation) (Pham and Le, 2016). FSH regulates vitellogenesis and spermatogenesis while LH controls final maturation (FOMO in females and spermiation in males). The stimulation of gonadotropins by GnRH can be inhibited by dopamine, which is a neurotransmitter that can be released, in particular in situations of stress

(Chabbi and Ganesh, 2015; Weltzien et al., 2009). However, this inhibition has been successfully resolved by co-administration of GnRH with a dopamine-antagonist (Peter et al., 1988; Levavi-Sivan et al., 2010).

There are several hormonal therapies available to induce predictable and sustained spawning from broodstock fish. Heterologous gonadotropins such as HCG (mammalian) gonadotropin or pituitary extracts (usually prepared from carp) administered as intra-muscular injection can be used to stimulate gonads. The main drawbacks to pituitary extracts are their limited availability and the inconsistency of their potency (Yaron et al., 2009). HCG is more readily available but has been reported to induce an immune response in treated fish such that only one induction is possible with this agent (Zohar and Mylonas, 2001). GnRH therapies are not immunogenic in treated fish due to the high degree of conservation of the GnRH peptide, but Zohar et al. (1990) found that native forms of GnRH such as Salmon GnRH (GnRH) were rapidly degraded through cleavage by endogenous peptidases. Cleavage-resistant analogs (GNRHa) bearing amino acid substitutions that decrease the affinity to peptidases were found to remain active for up to twice as long as native GnRHs, thereby significantly increasing the intensity of the gonadotropic stimulation in treated fish. The efficiency of these ‘super-active’ analogs can further be improved through administration in polymer-based delivery systems that can achieve a sustained release in the blood for up to 6 weeks (Mylonas et al., 1995).

Dopamine blockage of GnRH action on the pituitary can occur in species developing stress reactions to captive conditions and/or handling as discussed above. Antidopaminergic compounds that can block dopamine receptors have been used successfully in conjunction with GNRHa to induce maturation and spawning in many

species of fish (e.g., Aizen et al., 2005; Yanong and Martinez, 2009). Domperidone is a dopamine-antagonist that blocks the dopamine D2 receptor and can prevent the inhibitory effect of dopamine on the GnRH release. Domperidone is the preferred dopamine antagonist (DA) for spawn induction in fish because it does not cross the blood-brain barrier and can produce a long-lasting effect (Sloley et al., 1991). Alternative DAs including pimozide and metoclopramide have also been shown to increase LH leading to successful ovulations in finfish species (Peter et al., 1988).

Previous studies of captive maturation and spawning of Tripletail by Saillant et al. (2014) revealed that some females reach advanced gamete maturation stages compatible with hormonal induction of final maturation and spawning. Ovulation was induced through treatments with slow-release implants delivering a Salmon Gonadotropin Releasing Hormone analogue (sGnRHa) or a super active cleavage-resistant GnRHa agonist. Though eggs were released in each of the above experiments, the produced spawns were not fertilized except for one spawn that yielded a 9.8% fertilization rate. The lack of spontaneous spawning (without hormonal stimulation) indicates that FOMO is inhibited in captivity. The failure of males to emit milt during manual stripping suggests that sperm production also is inhibited, although sperm release was not observed after induction either which may indicate that manual stripping for sperm is of limited effectiveness in this species. The lack of fertility of spawns obtained following GnRH stimulation could reflect either that the GnRH treatment is inhibited or that spermatogenesis was not advanced enough to respond with the production of motile sperm in the short time frame of the induction period. According to the latter hypothesis, not only spermiation but also spermatogenesis would be inhibited in captive conditions.

The objective of this chapter was to evaluate the effects of hormonal induction protocols aiming to overcome failure of Tripletail to produce fertilized spawns in tanks spontaneously or in response to GnRH treatments. Spawning agents evaluated included exogenous gonadotropin, GnRH and an antidopaminergic compound. Treatments were characterized by their effects on the maturation of oocytes in females, spermiation in males, spawning activity, and spawn quality.

2.2 Methods

2.2.1 Experimental Design

2.2.1.1 Fish Collection and Husbandry

Broodstock were collected by hook and line sampling during the summer months (July-September) over three years (2017, 2018, and 2020) along the Northern Gulf of Mexico specifically in the Bay St. Louis area of Mississippi and adjacent Louisiana waters and in the Biloxi Bay system north of Horn and Ship Islands, Mississippi. Collected fish were transported to the Thad Cochran Marine Aquaculture Center at the University of Southern Mississippi's Gulf Coast Research Laboratory (USM-GCRL) in Ocean Springs, MS. Upon arrival at the facility, the weight and length of each fish was measured, and a gonad biopsy was taken for sex determination. Biopsies from males were obtained by applying a gentle pressure to the flanks to induce sperm release. Presumptive females (not releasing sperm following manual stripping as above and/or showing a protruding genital papilla) were assessed by taking an ovarian biopsy using a 1.5 mm-diameter Frydman memory catheter inserted into the oviducts. Following this procedure, the fish were subjected to prophylactic treatments to remove external and

internal parasites and a passive integrated transponder (PIT) tag was injected into the dorsal muscular tissue for individual identification.

Broodstock were held in 28 m^3 round fiberglass tanks (diameter of 2.4 m, depth of 1.5 m) provided artificial lighting with LED light fixtures, and temperature control through an Aqualogic HP3 heating and cooling unit. Fish evaluated for hormonal induction treatments were moved to 32 m^3 (diameter of 1.8 m, depth of 3.1 m) cylindrical fiberglass spawning tanks for monitoring during the induction and spawning periods. Spawning tanks were equipped with a side egg collector based on the Cornell dual drain design (Davidson and Summerfelt, 2004) that allows separation of suspended or floating particles (live eggs) present in surface water from sinking particles. Eggs were incubated in seven 120-liter cylindric-conical tanks connected to a recirculating system and maintained at 26ºC with a 300 $mL.min^{-1}$ water flow and very light aeration.

Artificial seawater was prepared by mixing well water and Crystal Sea Marine Mix to maintain a salinity of 30 ± 3 psu which matches conditions in the Tripletail's presumed natural spawning habitat. Temperature and photoperiod followed a cycle that mirrored seasonal variations in Mississippi coastal waters based on National Oceanographic and Atmospheric Association (NOAA) data collected at the buoy station 42067-USM-R1 in 2015 and 2016 except during the coldest months of winter when temperature was maintained at or above a minimum of 20ºC. Temperature below 20ºC was not applied because complete cessation of feeding was observed below this limit during previous Tripletail maturation trials at the TCMAC (Unpublished results). Temperature did not exceed a maximum of 28ºC in summer to extend the period matching early summer conditions (June and July) that correspond to the presumed peak

spawning in the wild (Brown-Peterson and Franks, 2001). Temperature was regulated by flow-through Aqualogic HP3 heater-chiller units monitored remotely. Temperature, dissolved oxygen, salinity, pH, and alkalinity were monitored daily while dissolved levels of ammonia, nitrite, and nitrate were monitored after feeding days. The alkalinity was maintained at 180-240 $mg.L^{-1}$ and the pH at 8.00 ± 0.5 through the additions of sodium bicarbonate (soda ash). Dissolved oxygen was maintained at or above 5.0 ppm. During the maturation period, water was exchanged bi-weekly to maintain nitrate concentration below 200 mg/L. Ammonia and nitrite concentrations were maintained below 0.5 ppm and 1.5 ppm, respectively, through maintenance of a fully operational biofilter. Additional water changes were performed when needed to maintain these parameters in the target range.

Broodfish were fed a mixture of fish, squid, and shrimp (1:1:1) at 3% body weight three times a week during the fall, winter and early spring that correspond to periods of sexual inactivity. During the maturation and spawning season, the feeding rate was increased to 4% body weight three times a week and, once a week, the ration was partially substituted (1% BW) with a supplement prepared as described in Bardon-Albaret and Saillant (2017). The supplement consists of lecithin, vitamin tablets (Sea Tab, Pacific Research Labs, Inc.), fish oil, and ground fish meal mixed in a gelatin-based diet.

The phenotypic sex of all broodfish was determined prior to allocating fish to maturation brood tanks to achieve the desired sex ratio in each group. Sex was determined based on the observation of sperm release for males, observation of eggs in ovarian biopsies for females, confirmed spawning in previous years in a single pair

mating trial where the sex of the second member of the pair was known, or assignment as male or female based on plasma levels of 11 Keto-testosterone and Estradiol as described in chapter III.

2.2.1.2 Broodstock Management

The broodstock used for experiments was held in two to four of the 28 m^3 brood tanks described in the previous section. There were only two spawning tanks available for the project, which prevented simultaneous replication of the tested treatments. Therefore, in each experiment, treatments were compared by conducting sequential one-week long trials where each of the two spawning tanks received one mating group treated with one of the tested hormonal induction treatments. The protocol applied for all weekly trials was identical (except for hormonal induction treatment) leading to the production of multiple 'temporal' replicates of each treatment. Up to four weekly trials were conducted using fish from a given brood tank (Figure 1.1). Brood tanks were stocked with similar biomasses and equal numbers of males and females. Trials were conducted every week by selecting two males and two females at a stage suitable for hormonal induction in one of the brood tanks. Each of the two spawning tanks received one mating pair (one male and one female, Figure 1.1). The following weekly trial employed fish from a second brood tank. Accordingly, each of the two replicate brood tanks used during a given spawning period were handled only every other week, which minimized the stress inflicted on broodstock by reducing handling frequency.

During the 2019 spawning year (experiment 1) there were four brood tanks, all following the same photothermal cycle. Experiments initially employed two of the four

tanks which were examined alternately to select mature males and females for weekly trials as above until no spawning ready females were found in the two tanks. At that point, the other two tanks were used to select fish on a weekly rotation. Because of this management, many females in the second group of tanks remained at spawning temperature for an extended period before being evaluated for spawning trials and were found going through atresia before they could be used for experiments. As a consequence, for the trials conducted in 2020 (experiment 1 below), the photothermal cycle in two of the brood tanks were advanced by one month and it was delayed by one month in the other two tanks. The two-month interval between the two groups of tanks allowed for staggering spawning induction experiments by using fish from the advanced cycle for the initial two months and then use of fish from the delayed cycle once they reached spawning conditions for another two months. This management was designed to allow completion of all spawning trials within two months of fish reaching spawning temperature in their respective groups and avoid premature atresia. For the 2021 trials (experiment 2), there were only two brood tanks due to the smaller number of broodstock available. Both tanks were kept under the same photothermal cycle and were alternated on a weekly basis until no female in pre-spawning condition was found.

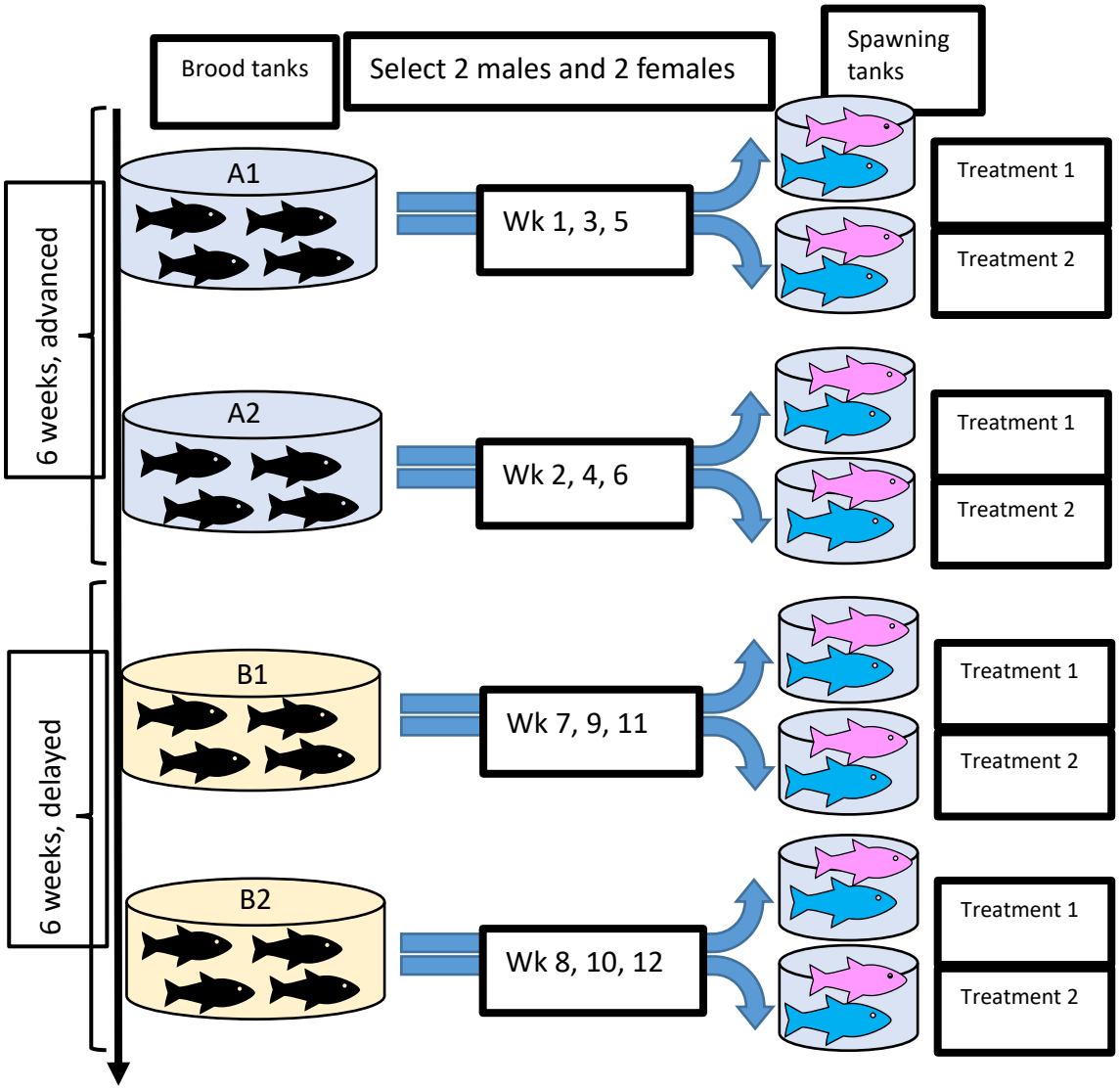


Figure 1.1 *Diagram outlining the management of 2020 broodstock groups and spawning trials for Experiment #1.*

2.2.1.3 Fish Selection, Hormonal Induction and Spawning

Upon reaching spawning temperature (26 ºC), all brood stock fish in each maturation tank were measured (weight and length) and a gonad biopsy was taken from males and females as described in section 2.2.1.1 under anesthesia to determine sexual maturation status. Males were selected for trials if they appeared healthy, and priority

was given to those who released sperm. Tripletail males typically release very small amounts of sperm or no sperm at all during stripping so non-releasing males were used if no male released sperm during the stripping attempt. Ovarian biopsies from females were placed in a petri dish and preserved in a fixative composed of Ethanol Formol Acetic acid (6:3:1) to clear the cytoplasm and determine the oocyte maturation stage. Samples were then observed using a compound microscope (Meiji Techno RZ). Pictures of at least 30 eggs were taken using iSolution Lite x64 digital software for measurements of egg diameter. The camera installed on the compound microscope was a Canon ERC with capturing software EOS Rebel T7i. Females were considered for spawning trials if the biopsy revealed a batch of oocytes ≥400µm in diameter. The oocyte maturation stage was then determined as described by Bardon-Albaret and Saillant (2017) and Żarski et al. (2011). Oocyte development is characterized based on the distribution of oil droplets in the vitellus, their degree of coalescence, and the degree of migration of the germinal vesicle. Stage I: uniform yolk with no visible oil droplets, stage II: several small droplets not well defined and filling the entire cytoplasm, Stage III: oil droplets well defined, peripheral hyalinization, Stage IV: a ring of large droplets centered around the germinal vesicle, often corresponding to migration of the germinal vesicle in some oocytes, and stage V: less than 5 large oil droplets. Females bearing Stage II and III oocytes were selected first for trials as induction of females at those stages leads to the highest rate of ovulation and best egg quality (Żarski et al., 2011). Stage I females were used when no other options were available.

2.2.1.4 Hormonal Induction Treatments

Treatments were applied to small mating pairs, primarily single pairs (one male and one female) selected from one of the brood tanks. The single pair design was chosen to allow for documenting the response of individual males and females to hormonal induction treatments. For each spawning trial, the male and female selected as described in the previous section received treatments while under anesthesia and prior to transfer to the spawning tank.

2.2.1.4.1 Experiment #1

Experiment 1 was conducted during the initial phase of this work (2019 and 2020 spawning seasons) with limited results in 2020 due to campus closure during the Covid-19 pandemic and a disease outbreak (*Amyloodinium ocellatum*) affecting broodstocks. These trials tested the effects of 5 treatments on spawn parameters. These treatments included control (Treatment 0), HCG (Treatment 1), GNRHa (Treatment 2), HCG followed by GNRHa and Domperidone (Treatment 3) and GNRHa and Domperidone (Treatment 4). Control group mating pairs received no hormonal treatment (Treatment 0). Pairs of the Treatment 1 group received a single injection of chorionic gonadotropin hormone (HCG, 1,100 $IU.kg^{-1}$ for females and 550 $IU.kg^{-1}$ males) determined based on successful doses used in other warmwater marine fish species in the laboratory (Bardon-Albaret and Saillant, 2017, unpublished results). Pairs in the Treatment 2 group were administered a GNRHa slow-release implant inserted in the dorsal musculature at a dose of 75 $\mu g.kg^{-1}$ for females and 55 $\mu g.kg^{-1}$ for males. This dose led to the ovulation and release of large batches of eggs in Tripletail females in previous trials (Saillant et al.,

2014). Pairs in the Treatment 3 group received a single injection of HCG according to Treatment 1 protocol, followed, a week later, by a GNRHa implant as in treatment 2, and an injection of 5 $mg.kg^{-1}$ Domperidone. Domperidone was dissolved in a vehicle solution (0.7% NaCl, 0.1M sodium metabisulfite) and administered as a single injection in the dorsal musculature immediately following the GNRHa implants. Treatment 4 received a GNRHa implant as in Treatment 2 administered in conjunction with an injection of Domperidone at a dosage of $5mg.kg^{-1}$.

2.2.1.4.2 Experiment #2

Experiment 2 was completed in 2021 and tested two treatments determined based on the results of experiment 1. These treatments include Treatment 4 from Experiment 1 (GnRHa and 5 $mg.kg^{-1}$ Domperidone) and a second treatment with a higher dose of Domperidone (Treatment 5, GnRHa and 10 $mg.kg^{-1}$ Domperidone).

2.2.1.5 Spawn Quality and Development Monitoring

For each trial, spawns were collected daily and assessed for fecundity, fertilization rate, hatching rate, biometric traits of newly hatched larvae (0 dph), and survival of larvae at 1, 2 and 4 dph.

Egg collectors were monitored for the presence of eggs every 3 hours between 0600 and 2100 beginning 24-hour post injection. Eggs were removed from the egg collector using a 100-µm fine mesh net, transferred into a plastic Cambro in 10-liters of prepared salt water, and provided gentle aeration until fertilization could be assessed. Fecundity and fertilization were assessed by subsampling 3 mL of the spawn three times

with a pipet. Subsamples were visually inspected using a compound microscope, and fertilized eggs and unfertilized eggs were counted. The fertilization rates were determined when the majority of developing eggs had reached at least the 4-cell stage to ensure that all developing embryos were detected.

Egg viability up to 4dph was assessed in static beakers where incubation could be replicated. Embryos were removed upon stocking of incubators through volumetric sampling after mixing and transferred to 1-liter beakers (n ~ 100 embryos per beaker) where they were maintained in static conditions for development as described by Bardon-Albaret and Saillant (2017). In experiment 1, 9 beakers were prepared for each spawn and maintained in identical conditions (30 psu, 26 ºC). The number of surviving larvae and dead eggs or larvae were determined in three replicates at 1-day post hatch (dph), another three replicates at 2 dph, and the remaining 3 replicates at 4 dph. Larvae were not fed. At each counting point, embryos or larvae present in the beaker were sieved through a 200 µm screen and the numbers of unhatched eggs, live, and dead larvae were determined using a dissecting microscope. Groups were discarded after assessment because the stress induced by handling for counting was expected to influence further survival potential of the larvae found alive at a given time point. Monitoring was discontinued at 4 dph when Tripletail yolk reserves are mostly depleted, and surviving larvae are ready to feed thus providing information on the viability of spawns produced from the different treatments to first feeding. In experiment 2, monitoring of survival in static beakers followed the same procedure except that only three replicate beakers were prepared per spawn, and the survival rate was recorded in the same three replicates at 1, 2, and 4 dph by carefully pouring the 1-liter beaker containing larvae into another 1-liter beaker pre-filled with 50-

mL of water over a LED light table, and counting larvae as they exited the origin beaker; Live larvae were detected based on their movement during the transfer. At the end of the monitoring period (4 dph), each replicate beaker was sieved through a 200 µm screen and the numbers of unhatched eggs, live and dead larvae were determined using a dissecting microscope. This protocol change was determined based on observations during experiment 1 that indicated that the viability of embryos and larvae was not affected by the modified counting procedure.

In experiment 2, viable spawns were stocked in 120-liter incubator tanks described in section 2.2.1.1. Embryos or larvae were obtained from the water column after mixing with a beaker to ensure random sampling. Larval measurements were taken at 0-dph and included total length, mouth gape, body depth, and yolk sac diameter. Measurements were obtained through image analysis of pictures taken from a camera connected to a compound microscope as described in section 2.2.1.3. Measurements included mouth gape, body depth measurement (myotome height) taken at the gut origin, and yolk sac diameter. Yolk-sac diameter was defined as the average of two orthogonal diameter measurements.

2.2.1.6 Statistical Analysis

Statistical analyses were performed in the online implementation of SAS® software v. 9.04. The homoscedasticity assumption was examined prior to comparisons of treatment groups using the Brown-Forsythe test (Brown and Forsythe, 1974). The number of spawns post-induction, the total fecundity, and the average fertility were compared among treatments using one-way ANOVAs. Groups were compared *a*

posteriori using the Tukey's Studentized Range test. The larval length, larval depth, yolk-sac diameter of newly hatched larvae, the hatch rate, and larval survival rate at 1, 2, and 4 dph were compared between treatments using three-way ANOVAs accounting for the fixed effects of treatment, the random effect of the mating pair nested within treatment, and the random effect of the spawn nested within mating pair. Percentage data were arcsin (square root) transformed before use in ANOVA when needed to improve homoscedasticity.

The effect of parameters measured on the female including the oocyte diameter and stage at the time of hormonal induction, the number of days at spawning temperature prior to induction, and the number of handling events experienced by the mating pair since the beginning of trials on variation within a treatment was evaluated by computing the Spearman rank correlation coefficient of each spawn parameter with each of these variables. The correlations between all the spawn traits assessed also were assessed using Spearman rank correlations. Correlations were computed across treatment groups on the residuals obtained after ANOVA to correct for treatment effects on the variables.

2.3 Results

2.3.1 Experiment 1

Summary data for the spawning trials implemented during the experiment and results are presented in Table 1.1.

Date	Spawn ID	Treatment	Number of Spawns	Average Fertilization Rate	Total Fecundity
6.18.2019	1	2	2	2.92	1,829,504
6.26.2019	2	1	0	0	0
6.26.2019	3	0	0	0	0
7.2.2019	4	3	3	75.7	817,820
7.19.2019	5	4	1	37.3	276,000
7.26.2019	7	1	0	0	0
7.26.2019	8	1	0	0	0
7.31.2019	9	3	0	0	0
7.31.2019	10	3	0	0	0
8.09.2019	11	4	2	21	1,016,200
08.15.2019	12	4	2	81.3	304,200
08.15.2019	13	2	1	0	207,000
3.15.2020	14	3	2	61.9	454,047
3.15.2020	15	3	1	0.8	276,930
5.10.2020	16	0	0	0	0
5.10.2020	17	2	2	0	887,990
5.16.2020	18	0	0	0	0
5.16.2020	19	1	0	0	0
5.21.2020	20	4	2	34.2	1,575,910
5.21.2020	21	3	0	0	0
6.1.2020	22	2	2	0	466,000
6.1.2020	23	4	3	78.9	1,065,590
6.9.2020	24	0	0	0	0
6.9.2020	25	2	1	0	89,000
6.15.2020	26	0	0	0	0
6.15.2020	27	1	0	0	0
6.20.2020	28	3	1	0	112,054
6.20.2020	29	4	1	0	143,942

Table 1.1 Summary data (number of spawns, fertility, and fecundity) recorded in 29 spawns obtained during experiment 1 trials. Treatment 0 (control), Treatment 1 (hCG), Treatment 2 (GnRHa implant), Treatment 3 (HCG+GnRHa implant+Domperidone), Treatment 4 (GnRHa implant + Domperidone)

2.3.1.1 Treatment effects on spawning activity, fecundity, and fertility

The number of spawns following induction differed significantly between treatments ($F_{4,24} = 8.31$, $P = 0.0002$). Treatments 0 (control) and 1 (hCG) produced no spawns and differed significantly from Treatments 4 (1.00 ± 1.15), 2 (1.60 ± 0.55), and 3 (1.83 ± 0.75).

Total fecundity did not differ significantly between Treatment groups ($F_{4,24} = 1.92$, $P = 0.1398$). The variances in treatment groups were heterogeneous for this trait ($F_{4,24} = 4.76$, $P = 0.0057$) leading to limited power during comparison of groups.

The average fertilization rate was marginally affected by treatments ($F_{2,14} = 3.66$, $P = 0.0573$). A posteriori tests indicated that Treatment 2 pairs (0.9 ± 2.00%) had a lower fertilization rate than those receiving Treatment 3 (41.6 ± 31.7%).

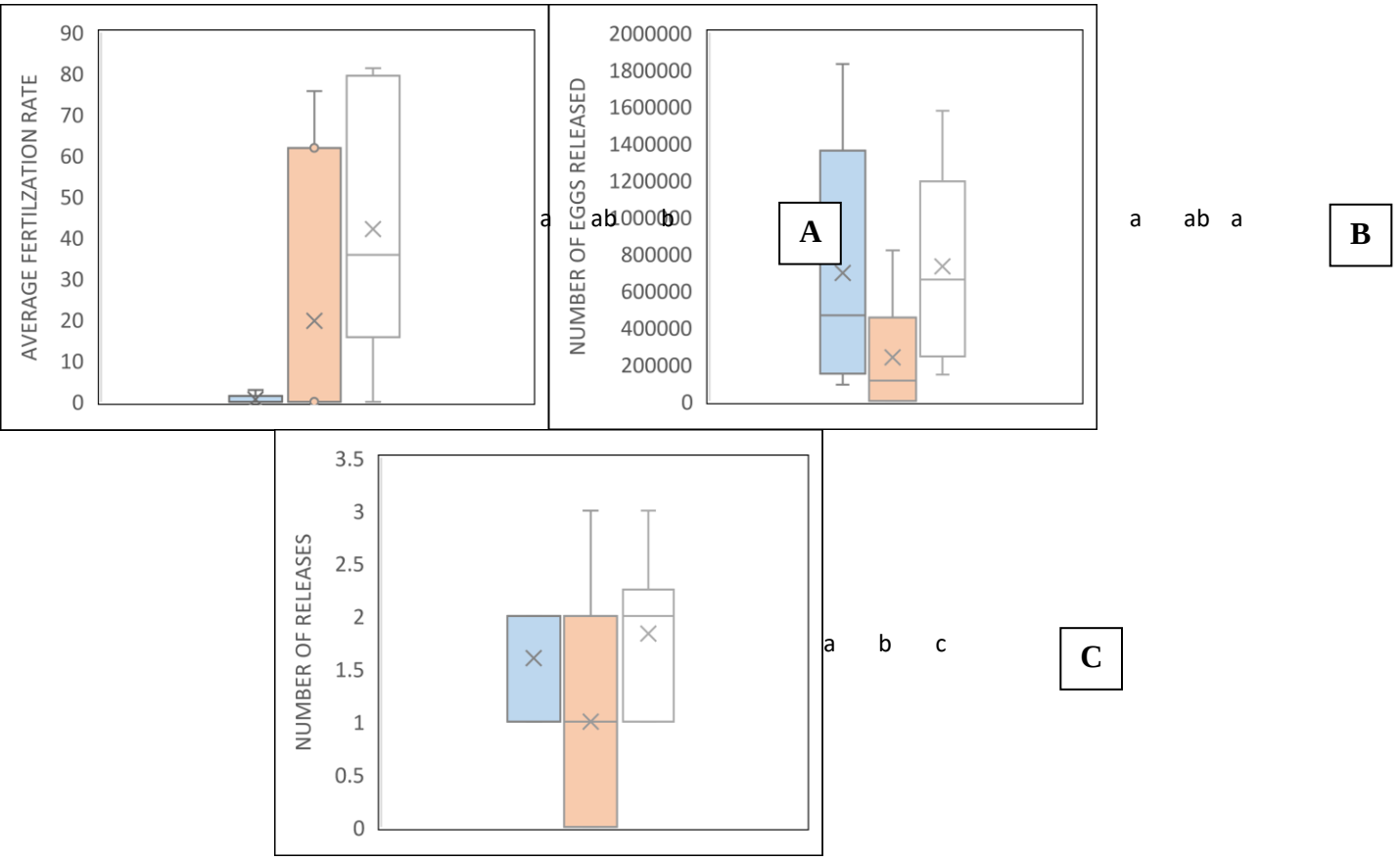


Figure 1.2 *Box and whisker plots (mean, range and quartiles) of A) average fertilization rate, B) total fecundity and C) number of releases in spawns recorded during Experiment 1.* ■ *Treatment 2 (GnRHa),* ■ *Treatment 3 (HCG followed by GnRHa + Domperidone),* □ *Treatment 4 (GnRHa + Domperidone). Treatments 0 (control) and 1 (hCG) produced no spawns and are not shown. See text for details of treatment protocols. Groups labeled with different letters differ significantly from each other.*

2.3.1.2 Treatment effects on hatch rate and survival post hatch

The hatch rate and survival rate at day 1, 2 and 4 post-hatch were measured in replicated static beakers for all spawns that produced fertilized embryos. Treatments 0 (control) and 1 (hCG) did not produce any fertilized spawns and are not included in this analysis. One mating pair produced viable embryos for Treatment 2 and was included in

the analysis. This treatment is, therefore, evaluated without replication in multiple mating pairs.

Results of analysis of survival rates at 1, 2, and 4 dph mirrored those obtained for the hatch rate. Survival differed significantly between treatments for all four time points ($F_{2,36} > 24$, $P = <0.001$), and the three treatments ranked in the same order (Treatment 2 had a lower survival than Treatment 3, which had a lower survival than Treatment 4 (Figure 1.3). The spawns obtained from the Treatment 2 mating pairs had no live larvae at 4 dph (Figure 1.3). The effect of mating pair on survival was also significant for all time points ($F_{6,36} > 27$, $P = <0.001$). The ranking of families was highly conserved between the hatch rate and survival recorded at 4 dph with correlation of mating pair survival scores between time points averaging 0.91 (range 0.86 to 0.98). Finally, survival rates differed significantly among spawns within a mating pair ($F_{6,36} > 41$, $P = <0.001$). For all time points, the survival rate of embryos and larvae was lowest for the first spawn obtained post-induction and increased with the chronological order of the spawn.

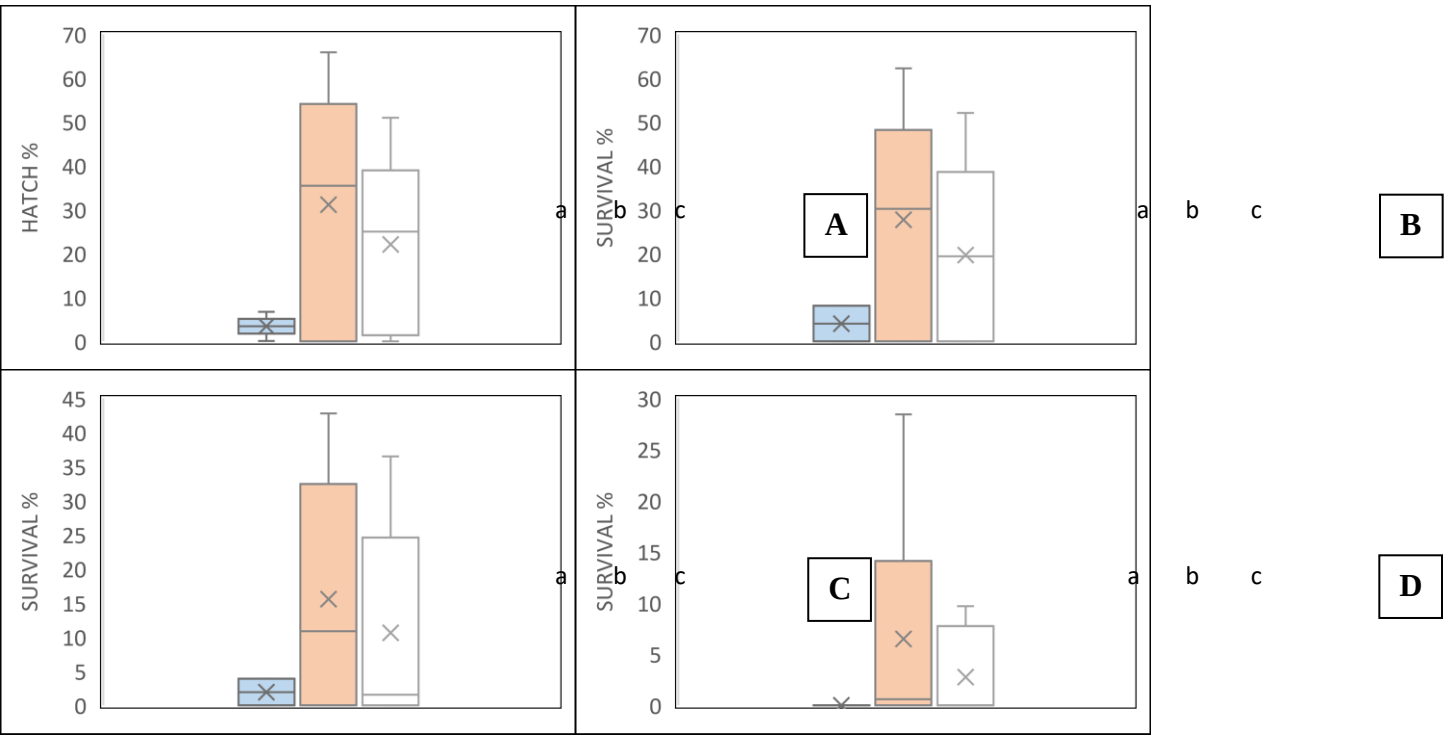


Figure 1.3 *Box and whisker plots (mean, range and quartiles) of A) average hatch rate, survival at B) 1 dph, C) 2 dph and D) 4 dph in spawns recorded during Experiment 1.* ▰ *Treatment 2 (GnRHa),* ▰ *Treatment 3 (hCG GnRHa +Domperidone,* □ *Treatment 4 (GnRHa + Domperidone). See text for details of treatment protocols. Groups labeled with different letters differ significantly from each other.*

2.3.2 Experiment #2

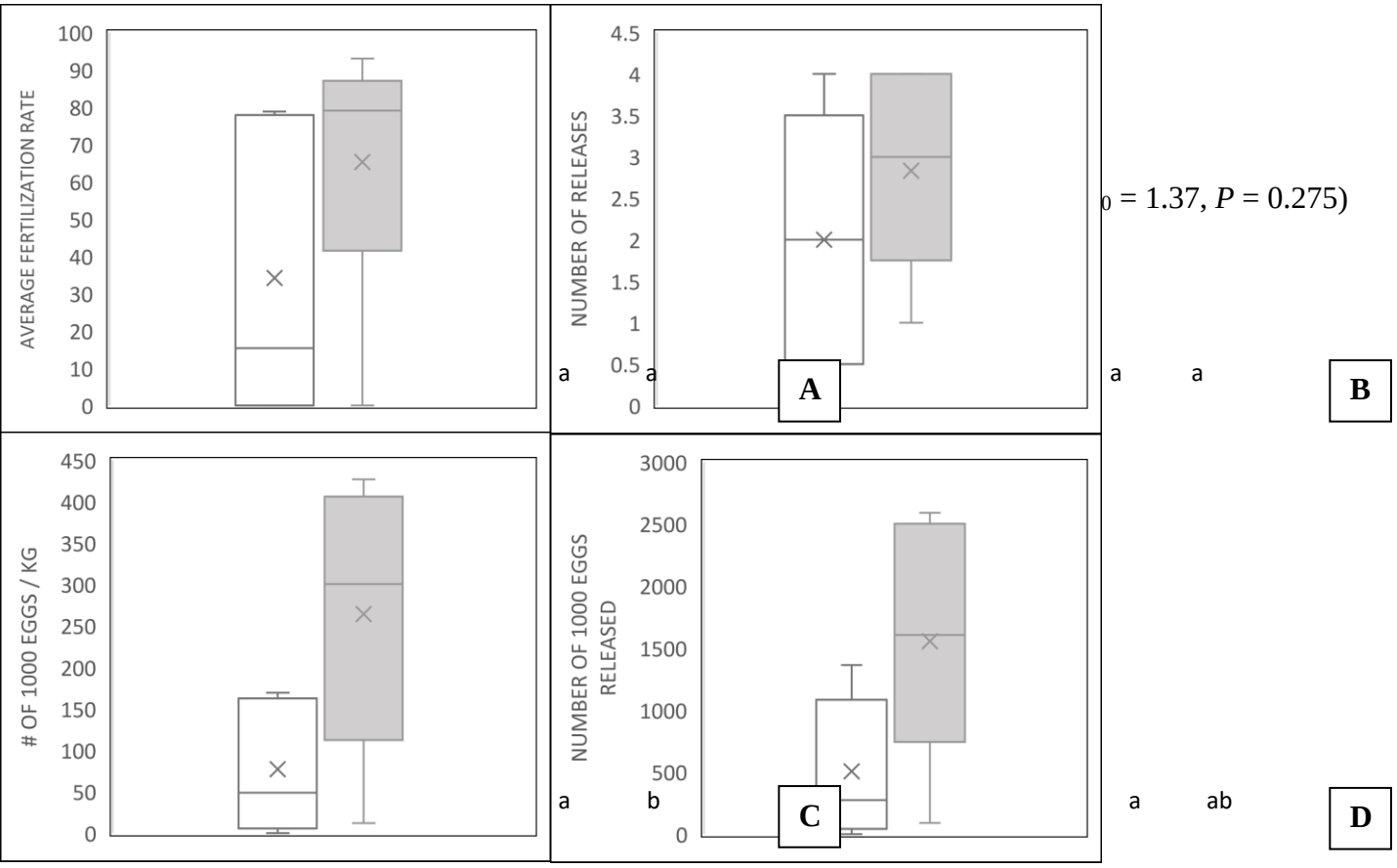
This experiment compared Treatment 4 (GnRHa in combination with 5 mg.kg^{-1} Domperidone) from Experiment 1 with Treatment 5 (GnRHa in combination with 10 mg.kg^{-1} Domperidone). Summary data for the spawning trials implemented during this experiment and results are presented in Table 1.2.

Date	Spawn ID	Treatment	Number of Spawns	Fertilization Rate	Total Fecundity
5.03.2021	30	5	4	74%	2,472,600
5.03.2021	31	4	4	77%	1,364,200
5.10.2021	32	5	2	84%	1,401,800
5.10.2021	33	4	2	15%	276,000
5.17.2021	34	5	4	85%	1,808,840
5.17.2021	35	4	3	79%	806,560
5.24.2021	36	5	1	0%	90,340
5.24.2021	37	4	1	0%	88,760
6.1.2021	38	5	4	55%	2,590,390
6.15.2021	39	5	2	93%	962,500
6.15.2021	40	4	0	0%	0

Table 1.2 Summary data (number of spawns, fertility, and total fecundity) recorded in 11 spawns obtained during experiment 2. Treatment 4 (GnRHa implant+ Domperidone 5 mg.kg^{-1}) and Treatment 5 (GnRHa implant+ Domperidone 10 mg.kg^{-1}).

2.3.2.1 Treatment effects on spawning activity, fecundity, and fertility

Mating groups in Treatments 4 tended to produce fewer spawns than Treatment 5 (2.00 ± 1.58 versus 2.83 ± 1.33 for Treatment 4 and 5 respectively), but the difference between the two groups was not significant ($F_{1,9} = 0.91$, $P = 0.366$). Absolute total fecundity was, on average, almost 3.06 times higher in Treatment 5 than in Treatment 4 ($F_{1,10} = 4.64$, $P = 0.059$). Similarly, relative fecundity for Treatment 5 was 3.4 times higher than Treatment 4 ($F_{1,10} = 5.53$, $P = 0.043$). Fertility was, on average, 1.73 times higher in Treatment 5 than in Treatment 4, but the difference between the two treatments



$_{0}$ = 1.37, *P* = 0.275)

Figure 1.4 *Box and whisker plots (mean, range and quartiles) of A) average fertilization, B) number of releases, C) relative fecundity (number of eggs.kg^{-1} of fish), and D) total fecundity in spawns recorded during Experiment 2. □ Treatment 4 (GnRHa + Domperidone 5 mg.kg^{-1}) and ■ Treatment 5 (GnRHa + Domperidone 10 mg.kg^{-1}). See text for details of treatment protocols. Groups labeled with different letters differ significantly from each other.*

2.3.2.2 Treatment effects on hatch rate and survival post hatch

The hatch rate and survival rate at day 1, 2 and 4 post hatch were measured in replicated static beakers for all spawns that produced fertilized embryos.

The hatch rate differed significantly between treatments ($F_{1,44} = 569$, $P = <0.001$). Treatment 5 hatch rates were, on average, 3.66 times higher than those of spawns from Treatment 4 (Figure 1.5). The hatch rate also varied significantly among mating pairs within treatments ($F_{6,44} = 86$, $P = <0.001$), with hatch rates of mating pairs in Treatment 4 ranging from 0.7 to 42.3% and those of mating pairs in Treatment 5 ranging from 32.5 to 82.0%. Finally, the hatch rate also varied significantly among spawns within pairs ($F_{14,44} = 32$, $P = <0.001$). Across all pairs, the average hatch rate of the first, second, third and fourth spawns were 36.3 ± 10.0%, 50.5 ± 16.8%, 39.2 ± 24.6%, and 74.5 ± 1.8%, respectively.

Results of analysis of survival rates at 1, 2, and 4 dph mirrored those obtained for the hatch rate. Survival differed significantly among treatments for all three time points ($F_{1,44} > 241$, $P = <0.001$), and the two treatments ranked in the same order as hatch rate results (Treatment 4 had a lower survival than Treatment 5, Figure 1.5). The effect of mating pair on survival also was significant for all three time points ($F_{6,44} > 12$, $P = <0.001$). The ranking of families was highly conserved between the hatch rate and survival recorded at 1, 2, and 4 dph, with correlation of mating pair survival scores between time points averaging 0.89 and ranging between 0.82 and 0.95. Finally, survival rates differed significantly among spawns within a mating pair ($F_{14,44} > 8$, $P = <0.001$). The survival rate of embryos and larvae was lowest for the first and third spawns obtained post induction and highest for the fourth spawn when the mating pair spawned four times.

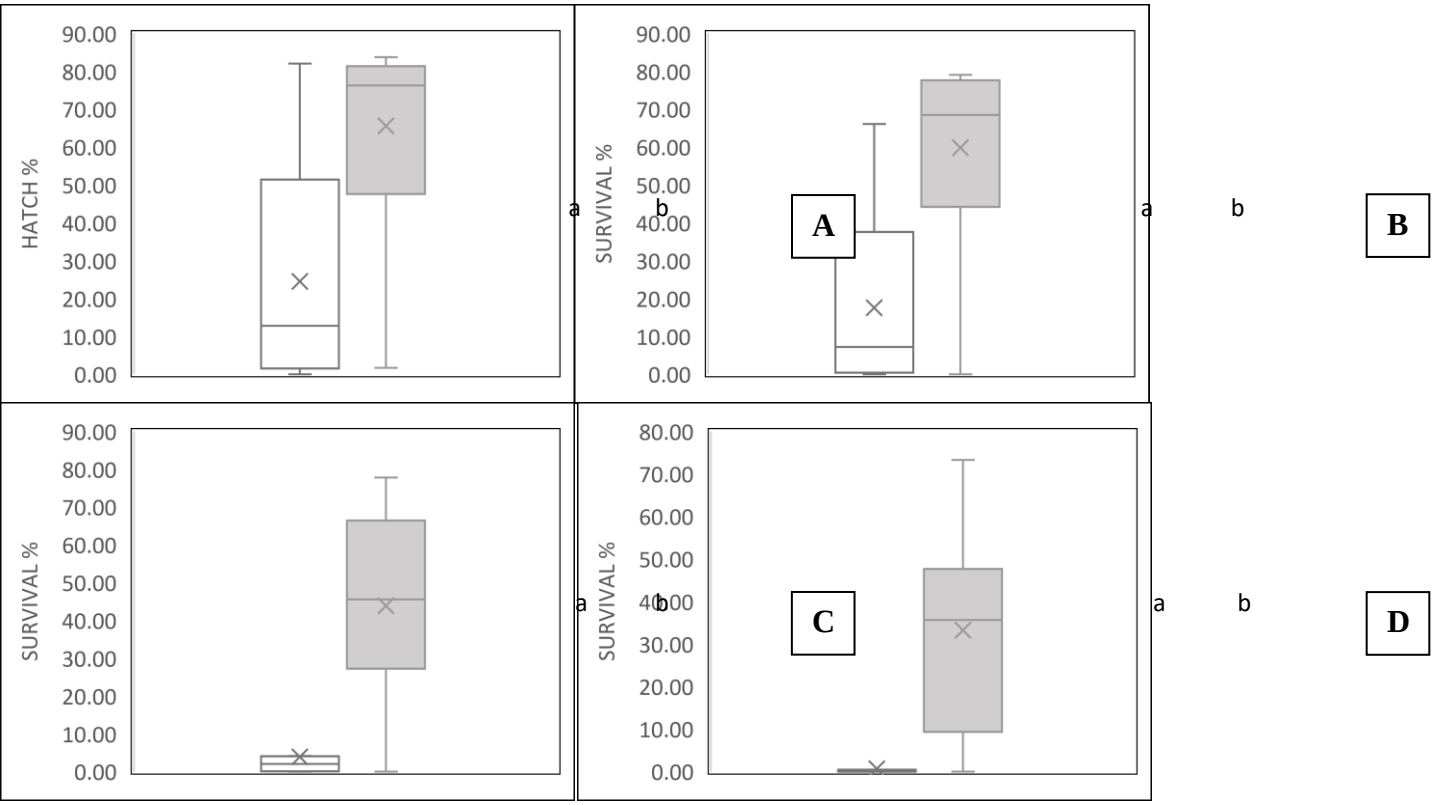
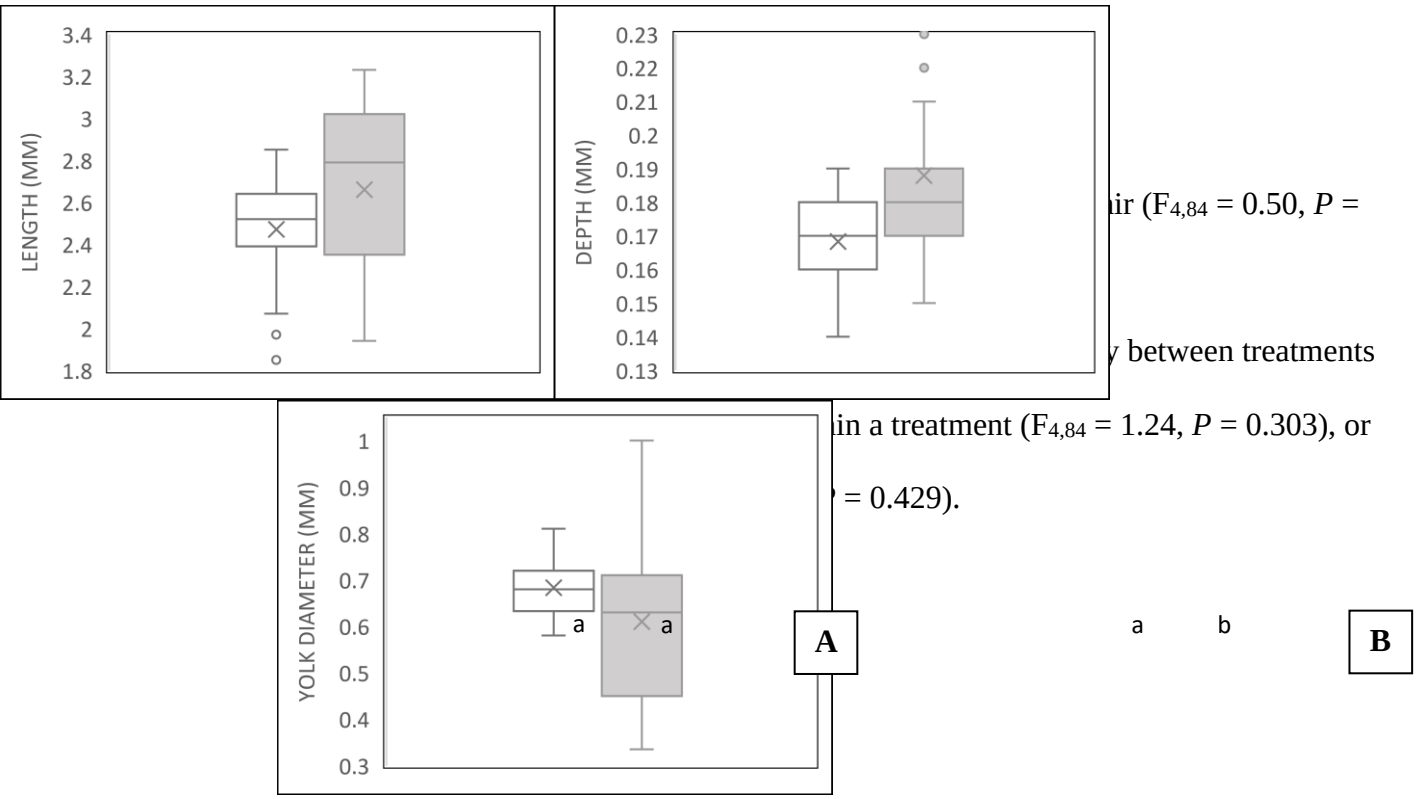


Figure 1.5 Box and whisker plots (mean, range and quartiles) of A) average hatch rate and survival of B) 1-dph, C) 2-dph and D) 4-dph larval fish in spawns recorded during Experiment 2. □ Treatment 4 (GNRHa + Domperidone 5 mg.kg^{-1}) and ■ Treatment 5 (GNRHa implant + Domperidone 10 mg.kg^{-1}). See text for details of treatment protocols. Groups labeled with different letters differ significantly from each other.

2.3.2.3 Treatment effects on newly hatched larvae biometric traits

Larval length did not differ significantly between treatments ($F_{1,84} = 0.49$, $P = 0.487$), mating pairs within a treatment ($F_{4,84} = 1.10$, $P = 0.362$), or spawn within a mating pair ($F_{4,84} = 0.53$, $P = 0.712$).

Larval depth differed significantly between treatments ($F_{1,84} = 9$, $P = 0.004$). Treatment 5 spawns tended to produce larvae with a larger body depth than Treatment 4 (Figure 1.6). Larval depth also varied significantly among mating pairs within a treatment



ir ($F_{4,84}$ = 0.50, *P* =

y between treatments

in a treatment ($F_{4,84}$ = 1.24, *P* = 0.303), or

= 0.429).

Figure 1.5 *Box and whisker plots (mean, range and quartiles) of A) average larval length, B) larval depth and C) yolk diameter of 0-dph larvae in spawns recorded during Experiment 2. □ Treatment 4 (GnRHa + Domperidone 5 mg.kg^{-1}) and ■ Treatment 5 (GnRHa + Domperidone 10 mg.kg^{-1}). See text for details of treatment protocols. Groups labeled with different letters differ significantly from each other.*

2.3.3 Correlations between spawn parameters and female traits

Partial correlations between female parameters and response variables after correcting for effects of treatments are detailed in appendix 2. Because treatments 0, 1 and 2 had few or no data for a large portion of the response variables, correlation analysis was implemented using data from only treatment groups 3, 4, and 5.

2.3.3.1 Correlations of response variables with female parameters

The number of handling events prior to spawning induction and the number of days at spawning temperature prior to induction were positively correlated ($r = 0.59$, $P = 0.003$), and both parameters were negatively correlated with the number of spawns following treatments ($r = -0.42$, $P = 0.0376$), the total fecundity ($r = -0.40$, $P = 0.0498$), and the hatch and survival to 1 dph of the third spawn released by a mating pair ($r = -0.90$, $P = 0.006$).

The oocyte stage at the time of induction was positively correlated with the hatch rate of the first spawn released by a pair and the survival of this spawn to day 4 post hatch ($0.52 < r < 0.59$ $0.32 < P < 0.067$). Oocytes were at the following stages through the research project: 8 no biopsies (17%), 4 immature (8%), 5 stage I (11%), 27 stage II (56%), and 4 stage III (8%).

Female size and condition were significantly correlated with only hatch and survival of the 4th spawn of a pair when a pair produced 4 spawns: Female weight and length were negatively correlated with survival of 4th spawn to 4 dph ($r = -1$, $P < 0.0001$), and female condition was negatively correlated with hatch and survival to 1 and 2 dph of the 4th spawn ($r = -1$, $P < 0.0001$). These estimates were based on only three spawns.

Estimates of correlations between female or parent pair parameters and larval biometric traits were very variable and, in most cases, not significant. Only a small number of spawns were available (n = 6 or less in most cases) to support the estimates.

2.3.3.2 Correlations between response variables

The number of spawns and total fecundity were positively correlated with each other and the fertility of the first spawn ($0.44 < r < 0.79$, $P < 0.049$). The number of spawns produced by a pair also was positively correlated with the hatch rate and survival rate to 2 days post hatch of the first spawn ($0.53 < r < 0.60$, $0.022 < P < 0.050$) while total fecundity was positively correlated with the hatch and survival of the second spawn ($0.60 < r < 0.62$ $0.018 < P < 0.024$).

The latency between induction and the observation of the first spawn was negatively correlated with the number of spawns ($r = -0.75$, $P = 0.0001$), the total fecundity ($r = -0.43$, $P = 0.061$), the fertility of the first three spawns ($-0.81 < r < -0.52$, $0.019 < P < 0.045$), and the hatch and survival rates to 1, 2 and 4 days post hatch of the first spawn ($-0.59 < r < -0.48$, $0.025 < P < 0.084$). Correlations with hatch and survival of subsequent spawns were also in general negative although not significant.

The fertility of the first two spawns and the hatch rates of these spawns were all positively correlated (38 significant correlation estimates out of 44). Correlations with fertility and survival traits of the third and 4th spawn of a pair also tended to be positive, but were lower and more inconsistent, in part due to the lower number of data points available.

2.4 Discussion

In this work, as reported in previous studies of captive maturation of Tripletail (Saillant et al., 2014, 2021), control groups conditioned under a simulated natural photothermal cycle did not spawn spontaneously. Examination of broodfish at different times during the spawning season revealed that, on average, 31% of the males released milt, although, when milt was expressed, it was always in very small quantities (<0.5mL). The proportion of females reaching late stages of vitellogenesis was slightly higher (averaging 44%). The lack of spontaneous spawning, even though some individuals reached advanced stages of maturation in both sexes, is consistent with the hypothesis that the final phases of gamete maturation and spawning are inhibited in captive conditions. Inhibition of this process is common in aquaculture and is due, in most cases, to disruptions of the production and/or release of gonadotropin by the pituitary (Zohar and Mylonas, 2001). This study evaluated the effects of 5 different hormonal therapies aiming to restore gonadotropin stimulation of gamete maturation and spawning.

2.4.1 Effects of GNRH implant

The first hormonal therapy evaluated for Tripletail spawning was a slow release GNRHa implant. These implants deliver a sustained release of GNRHa for two to three weeks (Mylonas et al., 1995), which is expected to restore gonadotropin production and release by the pituitary and induce gamete maturation and spawning in both sexes. In this study, administration of GNRHa slow-release implants led to final maturation, ovulation, and egg release in all 5 females induced with this treatment. However, fertility averaged

0.58% and was 0% for 4 of the 5 spawns even though the average total fecundity was high (~695,899) and 3 out of the 5 females released eggs twice in response to induction. These results are consistent with previous attempts to induce maturation and spawning using GnRH slow-release implants in Tripletail (Saillant et al., 2014, 2021) and indicate that GNRHa implants successfully induce final oocyte maturation and large releases of eggs in females. The lack of fertility can be due to low viability of eggs produced in response to the treatment, lack of sperm production in males, or behavioral issues that would lead to failure of females to release eggs at ovulation or of males to fertilize them. Males did not release milt when they were examined after induction, suggesting that lack of or insufficient spermiation in response to the treatments was an important factor.

Additional treatments were therefore evaluated to overcome the failure of GNRHa implants to induce production of fertilized spawns. The GNRHa stimulation of gonadotropin can be inhibited by dopamine which can be induced by stress (Schang and Peters, 1983; Dufour et al., 2010). Such inhibition was hypothesized to explain the lack of success in inducing sperm production by males and the low fertility of spawns obtained with the GNRHa implants.

2.4.2 Effect of HCG

The first approach to overcome a potential dopaminergic inhibition of GNRHa stimulation was to administer exogenous gonadotropin (mammalian chorionic gonadotropin, hCG). This therapy bypasses the Brain-Pituitary system and directly provides gonadotropin stimulation to the gonads. It has been successfully used to induce final gamete maturation in situations of high stress including in wild-caught red snapper

induced within a few hours of their capture on natural habitats (Minton et al., 1983), but also in captive broodstock of this species (Bardon-Albaret and Saillant, 2017) and others (e.g., *Sander lucioperca*, Křišťan et al., 2013). Induction trials with hCG were, however, largely unsuccessful in this project in which no egg release was observed in any of the mating pairs induced with this treatment (n = 5). No sperm release was observed during examinations of the males after treatment, but two of the females induced with hCG did show an increase in average egg diameter during follow-up ovarian biopsies suggesting a stimulation of gametogenesis had occurred. When hCG was successfully used to stimulate oocyte maturation in other species, the females selected for treatment had gonads in advanced stages of maturation at the time of induction (e.g., oocyte maturation stage 3 or higher in most of the females used by Bardon-Albaret and Saillant, 2017). In this situation, an LH surge lasting for a few hours (e.g., 6 hrs in Iwamatsu, 1978) is sufficient to trigger final maturation and ovulation in females. Similarly, a short surge induced by a single injection of GnRH leads to increased spermiation in males (e.g., Weltzien et al., 2004; Sorbera et al., 1996).

The lack of success of the hCG treatment in Tripletail may be due to the immaturity of gametes at the time of administration of the treatments. Only 8% of ovarian biopsies showed advanced stage oocytes (≥ stage 3). Gonads respond to gonadotropin stimulation via FSH and LH receptors. FSH receptors are located primarily in Sertoli cells in the testis and the granulosa cells in the ovary. They are involved in oocyte growth in females and spermatogenesis in males. LH receptors are located in Leydig cells in testes and theca cells in ovaries where they stimulate the production of the gonadal steroids that control the final maturation events (Levavi-Sivan et al., 2010). FSH

receptors are more abundant during the earlier phases of maturation while LH receptors peak just before oocyte maturation (Kwok et al., 2005). A first hypothesis for the lack of response to hCG is that gonads lacked competent LH receptors to relay the gonadotropin stimulation with the production of maturation inducing steroids and induction of subsequent events involved in final gamete maturation. Eighty six percent of the Tripletail females induced with GNRH implants in this study ovulated within a few days of induction (within 3 days for 80% of the females, within 4 to 5 days for the remaining 20%), indicating that the gonads (at least ovaries) were able to complete final maturation and spawning within a couple of days in response to a surge of gonadotropin. The exogenous gonadotropin used in this study (hCG) has a very long half-life following injection (up to 5 days, Ohta and Tanaka, 1997), thus our treatment should have maintained stimulation for a duration exceeding the gonad response time observed with GNRHa.

Accordingly, the hypothesis of a failure to respond due to lack of competent LH receptors seems unlikely. An alternative explanation is that oocytes may have been over-stimulated by a prolonged exposure to a high dosage of hCG (Zarski et al., 2019). Most oocytes were at stage II (54%) at the time of induction and the latency prior to ovulation in the GnRH implant treatments was long (greater than 50 hours on average) compared to that of red snapper at the same temperature (typically 24 to 32 hours, Bardon-Albaret and Saillant, 2017). Tripletail immature oocytes were therefore exposed to high steroid levels induced by the gonadotropin surge for an extended period as needed to mature to the stage III compatible with rapid ovulation and expulsion from the follicles. Treatment of immature oocytes with hCG may negatively affect egg quality (Zarski et al., 2019). This

may be due in part to the gonadotropin levels used in hCG treatments, which are much higher than the levels of LH induced by GnRH implants and have been shown to induce a stress response in other species of fish treated with hCG (e.g., Falahatkar and Poursaeid, 2014). Such stress may thus have prevented final maturation in our hCG treatment.

2.4.3 Effect of Domperidone administered in conjunction with a GNRH implant

A second approach to overcome dopamine inhibition is to administer dopamine receptors antagonists such as Domperidone or Pimozide (Chang et al., 1984). In this work, a third group of induction protocol tested this strategy by administering GNRHa implants in conjunction with Domperidone. This protocol was first tested using a single injection of 5 $mg.kg^{-1}$ of Domperidone and resulted in a major increase in average fertilization (42.1% fertility in comparison to 0.58% when GNRHa was administered alone), an increase in the average total fecundity (730,307 vs. 695,899), and an increase in the average number of egg releases (1.83 vs. 1.6). The maximum fertilization rate for this treatment reached 81.3% in one spawn. These results support the hypothesis that dopamine inhibition was involved in the lack of fertility of spawns obtained when GNRHa was administered alone. The intensity of dopamine inhibition on maturation and spawning varies widely among species (Dufour et al., 2010) from complete suppression in *Mugil cephalus* (Aizen et al., 2005) to mild reduction in salmonids (Dufour et al., 2010) and no detectable effects in some marine species (e.g., *Micropogonias undulates*, Copeland and Thomas, 1989; *Sparus aurata*, Zohar et al., 1995).

In Tripletail, the inhibition of final maturation appeared to affect primarily males who produced little or no sperm even after administration of a GNRHa implant. In

contrast, females appeared arrested at the end of vitellogenesis but could be induced to ovulate successfully with a simple GNRHa implant. These results suggest that dopamine inhibition is stronger in males. Dopamine inhibition has been related to environmental factors that appear to vary among species (Levavi-Sivan et al., 2010), but are often related to stress in aquaculture conditions (e.g., Chabbi and Ganesh, 2015). The nature of the environmental cue in Tripletail is unclear, but the brain dopaminergic system appears involved in aggressive behaviors (Winberg and Nilsson, 1993). Thus, some form of interactions between fish within brood tanks may be involved. We did not notice aggressive behaviors or injuries of sexually mature fish during this study or in previous studies of Tripletail maturation. Formal studies of the behaviors of brooders of both sexes in tanks will be required to determine if the level of inhibition can be correlated to subtle social interactions.

Fertility tended to increase substantially after the initial egg release. A first hypothesis is that spermatogenesis in males was arrested at an early stage, prior to induction, and the time required to complete a spermatogenesis cycle and produce motile sperm in response to the gonadotropin stimulation was longer than the time to complete final oocyte maturation and egg release such that males could not fertilize the first batch of eggs released by females. The stage of spermatogenesis in non-spermiating males could not be established at the time of induction in this work. All males were examined multiple times within the spawning season and most of them released milt only once, usually in very small quantities. This suggests that spermatogenesis was active, but sperm was produced in too small quantity to allow detection during manual stripping in most cases. The latter scenario is common in other species where gonadotropin release is

inhibited in males (Zohar and Mylonas, 2001). Under this scenario, the low fertility of initial spawns would not have been due to a delayed response of males because males were expected to respond rapidly with sperm production once gonadotropin stimulation was restored. A second hypothesis explaining the lack of fertility of the initial spawns is that the quality of eggs was low which translated in a low fertilization potential.

As discussed above, most of the females selected for treatments were at stage II of oocyte maturation, and induction at those early stages leads to lower success and low egg quality (Zarski et al., 2012, 2019). The role of egg quality in the low fertility of the first spawn post-induction is further supported by the positive correlation between the stage at induction and fertility and viability of the first spawn (i.e., fertility and viability of the first spawn was higher in the few females induced at a more advanced maturation stage) while latency was negatively correlated with these two egg quality parameters. Subsequent egg releases showed much higher fertilization potential (up to 81%). These egg releases involved newly recruited oocytes which experienced a rapid maturation cycle (close to 24 hours) consistent with the natural hormonal stimulation and maturation of successive batches in a batch spawning species and may not have been affected by the over-stimulation of the initial batch. In practice, finer characterization of the pre-maturation stage could help improve the results of the first spawn if females at stage III can be found in captive broodstocks. In this study, however, only 8% of the captive female population was at stage III.

Based on the success obtained with domperidone administration in combination with GnRH implants, two treatments were implemented in 2021 comparing two doses of domperidone (5 and 10 $mg.kg^{-1}$). Because domperidone was administered as a single

injection in this work, a higher dose was expected to maintain stimulation for a longer time and potentially increase the number of releases and egg quality by controlling the dopamine inhibition for a longer period. Treatments with 10 $mg.kg^{-1}$ domperidone (n = 6) led to a significant increase in the number of releases (relative gain +41.5%), which translated in an increase in average total fecundity (+206.6%) and average fertilization rate (+90.6%). This treatment, therefore, appears to increase the spawning output by inducing a longer active spawning phase with, on average, almost one more egg release than females induced with 5 $mg.kg^{-1}$ and larger releases. The positive effect on average fertilization rate reflects the higher fertilization rates of the late releases (2^{nd}, 3^{rd}, and 4^{th}) compared to the first release. The production of an additional egg release with high fertility in the 10 $mg.kg^{-1}$ group effectively reduced the actual contribution of the first egg release to the total output of the females treated with 10 $mg.kg^{-1}$. The 10 $mg.kg^{-1}$ treatment also had a significant positive effect on egg quality as measured by hatch rate and survival post-hatch. This positive effect could be due to a dose effect but also to a longer maintenance of the blockage of dopamine inhibition. The duration of the effect of Domperidone on pituitary dopamine concentration was studied in goldfish treated with 10 $mg.kg^{-1}$ by Sloley et al. (1991) who found that dopamine depletion was maximal at 12 hours post-injection and was maintained for 50 hours or more. While the threshold at which Domperidone effectively inhibits dopamine receptors is not known, the 5 $mg.kg^{-1}$ treatment likely translated into a shorter relief of dopamine inhibition which could explain the additional egg release observed in the 10 $mg.kg^{-1}$ treatment. The positive effect of the 10 $mg.kg^{-1}$ treatment on egg quality also may be due to a stronger blockage of dopamine receptors while dopamine inhibition might have remained partially in effect

in the 5 mg.kg^{-1} treatment leading to disruptions of oocyte maturation. Further study of the effects of Domperidone doses on dopamine receptors over time including a comprehensive characterization of egg quality and viability when comparing treatments would be informative. The present results also suggest that a sustained stimulation with Domperidone would be worth exploring to improve egg quality in the late egg releases expected to display high fertility. The simple induction with Domperidone (without GnRH) was indeed sufficient to restore spawning in goldfish (Omeljaniuk et al., 1987) and gray mullet (Aizen et al., 2005).

In this study, stimulations combining a single injection of Domperidone in combination with a GNRHa implant also were attempted on seven mating pairs a week after treatments with hCG (Treatment 3). The rationale for this attempt was that hCG may have stimulated gametogenesis even if final maturation was not obtained and that the results of the GNRHa and domperidone treatment would be improved in these groups. The results of this combination of treatments (Treatment 3) were variable. Some of the pairs produced multiple spawns with high fertility and viability (2 mating pairs) while others failed to spawn (3 mating sets) or produced one unfertile egg release (2 pairs). The high frequency of negative outcomes is consistent with the hypothesis that the hCG injection incurred a stress and/or an overstimulation which prevented further maturation of immature oocytes following the initial treatment but also after the second induction with Domperidone and GNRHa. This protocol also incurred a second handling of the animal after only a few days to administer the GNRHa implant and the domperidone injection, which likely led to additional stress on the fish and negatively impacted the outcome of the second treatment.

2.4.4 Correlations of spawning success and egg quality with other traits

There was large variation in spawning success and egg quality among mating pairs within each of the treatments. The correlation study showed that, across treatments, the stage at induction, the latency between induction and observation of the first spawn, the number of handling events, and the duration at spawning temperature prior to induction significantly affected the spawning outcomes.

The stage at induction was positively correlated with the hatch and survival of the first spawn of a pair. This correlation likely reflects over-stimulation and/or stress reducing egg quality when immature oocytes were induced as discussed in section 2.4.3. The latency between induction and the observation of the first spawn was negatively correlated with the number of spawns, fecundity, fertility, and egg quality of the first spawn (correlation with the quality of subsequent spawns also was positive although not significant). Latency, therefore, appears to be a good predictor of the success of hormonal induction both in terms of quantity and quality.

The fertility and the hatch rates of the first two spawns were positively correlated with each other and were positively correlated with the number of spawns and/or fecundity, indicating that a positive outcome of the first two egg releases tended to be associated with additional egg releases (3^{rd} and possibly 4^{th}) and overall good performance of the pair. The egg quality parameters of the 3^{rd} and 4^{th} spawns were decoupled from the first two spawns indicating that these later spawns, which were produced after maturation of two initial batches of oocytes, often had good quality even if there were issues with the first two batches due to effects of hormonal stimulation on immature oocytes as discussed earlier.

The number of handling events and the duration at spawning temperature were, logically, correlated with each other since broodfish were examined every other week during the spawning period such that fish induced later in the season had been examined more than those selected for induction at the first scheduled assessment of the stock. These two traits were negatively correlated with fecundity and the viability of the third spawn when one was produced. These results are consistent with the hypothesis that repeated handling stress exhausts the fish leading to a smaller number of egg releases after induction and a lower quality of the final spawn when a third spawn was produced. Accordingly, the approach used in this study where groups of brooders were conditioned under advanced or delayed cycles to minimize the number of handling events and the time spent at spawning temperature prior to induction is recommended for future management of Tripletail broodstock.

2.4.5 Conclusions

In conclusion, this work identified a successful hormonal therapy to restore gonadotropin stimulation in captive Tripletail and obtain large fertile spawns. The most successful protocol used in this work involves inducing males and females with an injection of 10 $mg.kg^{-1}$ domperidone and a GnRH implant. This treatment should be pursued in future work on Tripletail and is expected to lead to the production of multiple large fertile spawns. The third and fourth spawns produced under this treatment appeared to show the highest viability and should be prioritized for larval culture runs.

The hormonal therapy used can be further improved to increase the quality of spawns. Larvae surviving to 4 days post-hatch under their endogenous reserves have a

high likelihood of initiating exogenous feeding. Thus, improving the viability to 4 dph is essential. Even the most successful treatments in this study led to low survival at 4 dph (33.3% average survival at 4 dph of the 10 mg kg^{-1} group); therefore, the induction treatment as well as broodstock management procedures need to be optimized to improve this parameter. Throughout the experiments, the same dosage of GNRHa was used (75 $mg.kg^{-1}$ for females and 55 $mg.kg^{-1}$ for males). Lower doses have been used successfully in marine fish (Zohar and Mylonas, 2001) and may lead to improved egg quality in Tripletail. This work also showed improved results in almost all metrics when the dose of domperidone was increased. Maintaining Domperidone action for a longer period may improve egg quality further and achieve sustained spawning. Evaluation of sustained delivery systems administering Domperidone (e.g., Kumakura et al., 2003) is therefore warranted.

During this work, broodstock were held in a photothermal cycle that simulated the natural conditions in coastal waters of the Northern Gulf of Mexico. No spontaneous spawns were observed over the three-year experimental period or in previous studies of captive maturation, suggesting that maturation and spawning require different environmental conditions. Factors that may act as cues include temperature and photoperiod conditions, but also other less obvious ones such as salinity or sex ratios. Future studies focused on manipulation of such environmental factors may lead to natural spawning without hormonal therapies thereby reducing stress on broodstocks and the overall efforts of the hatchery personnel. This study also showed that handling stress negatively impacted the quality and quantity of spawns. The management implemented during this work where broodstocks are split into multiple tanks that are examined

alternatively to increase time between handling events and limit handling stress is therefore recommended for future studies.

CHAPTER III – DEVELOPMENT OF A SEXING METHOD BASED ON PLASMA LEVELS OF GONADAL STEROIDS

3.1 Introduction

Identifying the phenotypic sex of candidate broodfish is essential to broodstock establishment and management. Knowledge of fish sex allows hatcheries to maintain appropriate sex ratios for maturation and spawning in broodstock tanks. This information also is needed to ensure enough males and females are available in a stock to meet the requirements of breeding designs. Several sexing methods have been used in aquaculture. A common way to determine the sex of fish consists of obtaining a gonadal biopsy and identifying male or female gametes and/or their surrounding tissue through visual observation of the sample. In males, this is achieved through manual stripping and observation of the release of milt, while in females, oocytes can be sampled in an ovarian biopsy using a catheter and identified using a microscope. Ultrasound has been used to visualize gonads and differentiate testes from ovaries in fish with relatively high accuracy (Blythe et al., 1994; Frost et al., 2014; Guitreau et al., 2012). These methods are, however, restricted to the spawning season when gametes are produced, or gonads are large enough to be distinguished via ultrasound. In some species like the Tripletail, milt is rarely obtained by manual stripping and the oviduct is often tightly closed, which prevents effective cannulation of females (Saillant et al., 2014, unpublished results). The aspect of the genital papilla or the shape of the urogenital area (Benz and Jacobs, 1986) also have been used for sexing in some fish species, but these characteristics are difficult to observe outside of the spawning season or in species where the urogenital area is not easily accessible for observation. Some fish species show easily recordable external

secondary sex characters useful for sexing including larger body size in one sex, sex-specific color patterns (Kirpichnikov, 1981), differences in body conformation traits, or differences in behavior between sexes. For example, Echeverria (1986) described four species of rockfish (*Sebastes* sp.) where females were larger than males, presumably favoring higher fecundity in females. The male rockfish exhibited larger pectoral fins and larger standardized eye diameter. These traits were thought to enable the male to compete successfully with females for the capture of prey items. While sexual growth dimorphisms are relatively common in marine fish species, the distribution of sizes or morphometric traits often overlap between sexes, even when average values differ (e.g., Saillant et al., 2001), making sex identification based on size or morphometry alone unreliable. Some marine species like the Tripletail lack any color or clear shape dimorphism between sexes. Alternative measurements that can be used to identify phenotypic sex in these species include markers of a sex genotype or indicators based on the physiology of the gonad such as circulating levels of gonadal sex steroids.

The androgen 11-Ketotestosterone (11-KT) is produced by males during spermatogenesis and is not produced by females in most species (Fostier et al., 1983). In the sea bass *Dicentrarchus labrax*, Prat et al. (1990) recorded 11-KT levels in males reaching up to 4-6 $ng.mL^{-1}$ during the peak spawning season. Baseline (out of spawning season) levels of 11-KT levels were less than 1 $ng.mL^{-1}$ but were still higher in males than in females making this hormone an informative marker of phenotypic sex potentially usable across seasons. Estradiol 17β (E2) is an estrogen steroid hormone produced by the granulosa cells of ovarian follicles (Nelson and Habibi, 2013). Plasma levels of this hormone can reach several $ng.mL^{-1}$ in females during the follicular phase of the

maturation cycle (Prat et al., 1990; Williams and Erickson, 2000). Estradiol is produced by both sexes but is expected to reach higher levels in females during vitellogenesis and, therefore, could be used as a marker to assist with the identification of females during that period. These two hormones and/or their ratios have been used to identify sex in fish species (Chu-Koo et al., 2009; Feist et al., 1990) and, while they may be most effective during the gamete maturation season, they also have been used to sex immature fish (Feist et al., 2004).

Finally, another approach to identify the sex of individuals is to use genetic markers. However, such markers are not available in most species and developing them requires substantial efforts and costs to produce and analyze a genome scan and use it to detect genetic associations with sex. Genetic markers have been used successfully in some fish species (Lin et al., 2017; Liu et al., 2018). However, a useful genetic marker of sex would need to show a complete association between phenotypic sex and sexual genotype, and this may not be the case in species where sex determination is influenced by environmental factors (Baroiller et al., 2009). The role of environmental effects on Tripletail sex determination is not known but, because broodstock management requires identification of functional males and females, this study focused on approaches to determine phenotypic sex.

Considering the lack of sexual dimorphism in Tripletail and the challenges obtaining gonad biopsies from males and females even in the peak of spawning season, circulating levels of gonadal sex steroids are a potential valuable tool to identify the sex of candidate broodstock. Preliminary data obtained by Saillant et al. (2014) suggested higher levels of 11-KT in males, but the data were insufficient to assess the value of this

hormone for sexing due to the small sample size. The objective of this chapter is to formally evaluate assays of plasma levels of E2 and 11-KT for the identification of the sex of candidate broodstock males and females.

3.2 Methods and Materials

3.2.1 Experimental Design

Plasma levels of 11-KT and E2 were measured in 149 adult Tripletail of reproductive size (457 mm and above) maintained at the TCMAC facilities for use as broodstock. The phenotypic sex of 103 of the individuals assayed (44 males and 59 females) was validated through observation of gametes in a gonad biopsy (observation of sperm release of males during manual stripping or of oocytes in an ovarian biopsy obtained by cannulation in females), confirmation of spawning of candidate brooder in a past single pair mating set where the sex of the second member of the pair was known, or direct observation of gonads after dissection. Some of the males and females were kept during multiple reproductive seasons and could be sampled a second time. Because re-sampling occurred in a different reproductive season when individuals were potentially at a different maturation stage, these samples were considered independent samples for the characterization of their sex group in the experiment bringing the number of confirmed male and female samples to 59 and 87, respectively. Blood samples were drawn when temperatures were 25ºC or higher corresponding to conditions when Tripletail mature and spawn in the wild (Brown-Peterson and Franks, 2001). The results obtained on confirmed male and female samples established a baseline distribution of the two hormone levels in

males and females at maturation and spawning temperature. This baseline was used as a training dataset to develop the discriminant sexing function. The sex of the remaining 46 Tripletail of reproductive size also sampled in the same temperature conditions could not be identified by observation of gametes or confirmed spawning as above. These individuals were sexed using the sexing function developed using the training set.

At least 1 mL of blood was drawn from veins located in the posterior part of the kidney under anesthesia. Syringes were rinsed with a 10 $mg.ml^{-1}$ heparin solution prior to the blood draw to prevent coagulation. The samples were mixed thoroughly in the syringe and placed on ice until lab processing. Within two hours of collection, the blood samples were centrifuged at 1,200 g for 15 minutes at 4 ºC, plasma was removed from the precipitate, and the supernatant frozen at -20 ºC until hormone assays.

Plasma levels of 11-KT and E2 were measured using a competitive ELISA assay (Cayman Chemicals ELISA kit) following instructions provided by the manufacturer. Steroids were extracted prior to assay. Estradiol was extracted using a methanol precipitation protocol (Cayman Chemicals, 2019) and 11-KT was extracted 4 times in ethyl acetate/hexane solvent (Metcalfe et al., 2018; Cayman Chemicals, 2017). The ELISA assays are based on the competition of evaluated steroids (11-KT/E2) present in samples and a tracer (acetylcholinesterase/AChE-11-KT or AChE-E2 conjugate). Samples and a known quantity of tracer were incubated in plates coated with a limited amount of anti-11-KT or anti-E2 antiserum. Plates were washed to remove unbound steroids and tracer, then Ellman's reagent was added to wells. Ellman's reagent contains the substrate of the AChE enzyme and its reaction with the tracer produces a colored compound that can be quantified by absorbance using a spectrophotometer. The amount

of tracer detected is inversely related to the amount of hormone present in the sample allowing determination of the steroid content of the sample. Absorbance was read at 412 and 414 nm for the 11-KT and E2 assay, respectively, using a Biotek Synergy 2 spectrophotometer (Biotek) and concentrations were determined using a standard curve based on known quantities of 11-KT/E2 as reference. The 11-KT ELISA (Cayman Chemicals Inc, 2017) kit has a range of 0.78-100 $pg.ml^{-1}$ and a sensitivity of ~1.3 $pg.mL^{-1}$. The assay for Estradiol (Cayman Chemicals Inc, 2019) has a range of 0.61-10,000 $pg.mL^{-1}$ and an expected sensitivity of 20 $pg.mL^{-1}$. The Estradiol assay-kit was re-designed by the provider during this project due to a critical shortage of the antibody used in the initial version (Cayman Chemicals Inc., 2019). Samples assayed in 2018 and spring 2019 were assayed with the initial version of the kit while those obtained in summer 2019 and 2020 were assayed using the new assay. Because the redesign of the assay kit was not anticipated, there was no opportunity to run multiple samples using the two assays to assess the correlation of results from the two assays and develop a conversion factor. The two assays were therefore evaluated separately below and referred to as E2A and E2B assays.

The 11-KT and E2 assays were validated for use in Tripletail by measuring levels of hormones in serial dilutions of multiple Tripletail plasma samples and dilutions of the hormone standard provided with the kit and testing the parallelism of the linearized absorbance curves. Inter-assay reproducibility was evaluated by repeating assays for a subset of the samples on different assay kit plates. The number of samples with repeated measurements was 39 for the E2A assay, 14 for the E2B assay and 47 for the 11-KT assay, respectively.

3.2.2 Statistical Analysis

E2 and 11-KT amounts were determined using their respective assay standard curve after linearization of the data by log-logit transformation.

Assays were validated for use in Tripletail by estimating the repeatability of measurements of the same sample over multiple assay kit plates and by testing the parallelism of logit-transformed absorbance reading curves obtained for serial dilutions of samples and for the hormone standard. The significance of departure from parallelism was tested as the interaction between the covariate (dilution factor) and the main factor (individual sample or hormone standard) in an analysis of covariance in SAS PROC GLM. The repeatability of measurements was estimated as the intraclass correlation obtained from a decomposition of variance accounting for individual samples and repeated measurements within a sample. Variance components were estimated in PROC MIXED and the between individuals (σ_b) and within individuals (σ_w) components were used to estimate repeatability as:

$$R = \frac{\sigma_b}{\sigma_b + \sigma_w}$$

The hormone levels were compared between males and females using a Student t-test. Discriminant analysis was used to optimize a linear combination of hormone levels and other variables for sex identification using a stepwise discriminant analysis in PROC STEPDISC of SAS. Variables considered as entry variables were ln(weight), ln (Length), condition coefficient k, estimated as the residual of the regression of ln (Weight) on ln

(Length), the Estradiol dosage, and the 11-KT dosage. Variables were entered and retained from the model at a significance level of 0.15.

The set of variables identified in stepdisc was used in PROC DISCRIM of SAS to optimize the functions and assess their performance. A quadratic discriminant function was used to account for unequal variances between groups. Functions were optimized separately for the sub-datasets assayed using the E2A and E2B tests. For each sub-dataset, the performance of the obtained functions was evaluated by cross-validation where each sample was assigned a sex based on a discriminant function optimized using all the other samples. The percent correct and incorrect classification was determined for each sex for each of the sub-dataset considered. The values of the functions built with the E2A and E2B sub-datasets as training sets to assign sex in samples assayed with the other kit also was evaluated during cross-validation. The optimized function was used to assign sex to the samples of unknown or unconfirmed sex.

Finally, correlation between variables measured at the time of sample collection and measured levels of E2 or 11-KT were tested using Spearman rank correlation in PROC CORR of SAS. Variables considered included the temperature at the time the blood sample was taken, the number of days the individual had spent at spawning temperature during the on-going reproductive season prior to dosage, and the number of times the sampled individual had spawned in the facilities prior to sampling.

3.3 Results

3.3.1 Assay Validation

The repeatability estimates for the 11-KT, E2A and E2B assays were 0.70 (0.67-0.72), 0.18 (0.07-0.97), and 1, respectively, indicating high reproducibility of the new E2 assay (E2B), moderate to good reproducibility of the 11-KT assay and poor reproducibility of the old Estradiol assay (E2A).

Analysis of the absorbance curves obtained from serial dilutions of Tripletail samples indicated that in both assays the slope associated with the dilution factor was highly significant ($F_{1,12}$ = 756 for 11-KT, $F_{1,18}$ = 1,734 for E2b, $P < 0.001$), and the linear response to the dilution factor explained a high percentage of the variance ($R^2 > 0.986$ for the E2B assay, $R^2 > 0.969$ for the 11-KT assay). The test of heterogeneity of slopes was significant for the 11-KT assay ($F_{2,12}$ = 8.9, $P = 0.004$) and the E2B assay ($F_{4,18}$ = 44.2, $P < 0.0001$) indicating slopes were heterogeneous in both assays (Figures 1.7, 1.8). Serial dilutions could not be tested to evaluate parallelism in the E2A assay.

The heterogeneity of slopes in the 11-KT assay was due primarily to a difference between the slope of the kit standard and the slopes of the Tripletail serially diluted samples. Serial dilutions of Tripletail samples showed a slower decrease in absorbance with increasing (log) dilution factor (Figure 1.7). However, the heterogeneity of slopes was not significant when the kit standard was not included in the comparison, indicating that responses to dilution were parallel for the two tested Tripletail samples ($F_{1,6}$ = 0.01, $P = 0.924$). The parallelism of slopes among Tripletail samples indicates that 11-KT levels measured in samples can be compared to rank samples, but the lack of parallelism with the kit standard indicates that absolute concentrations are incorrect.

Lack of parallelism in the E2B assay also was due in large part to different slopes between serially diluted samples and hormone kit standard. Serially diluted samples tended to show a steeper decrease of absorbance than the kit standard indicating possible under-estimation of hormone levels. Slope heterogeneity was still significant when the hormone kit standard data were omitted, indicating that slopes varied among the 4 serially diluted samples ($F_{3,12}$ = 8.88, P = 0.002), although when the absorbance range was restricted to values between 20% and 80% of the binding capacity of the antibody, the heterogeneity of slopes of the 4 samples was only marginally significant ($F_{3,8}$ = 4.33, P = 0.043).

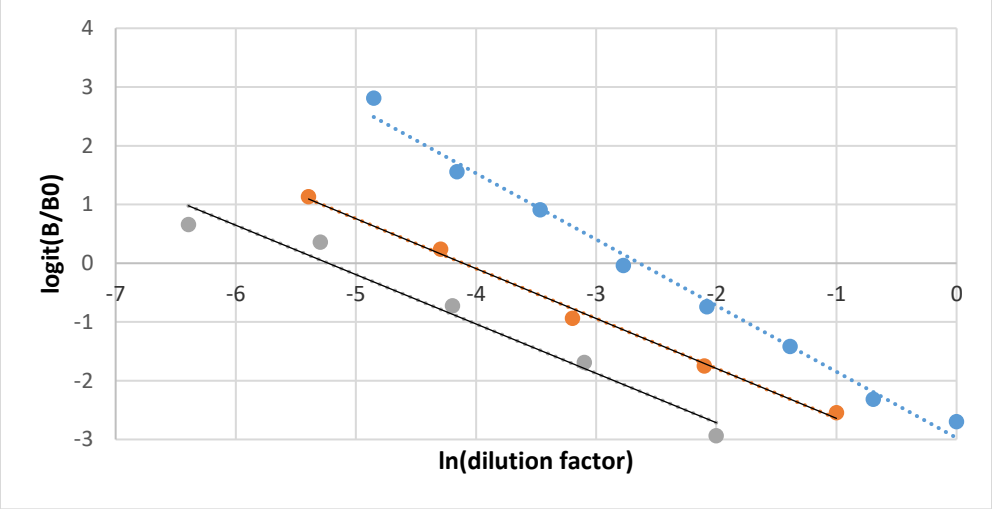


Figure 1.6 *Slopes of logit transformed absorbance measurements as a function of the dilution factor obtained during 11-KT ELISA assays of serial dilutions of two test Tripletail plasma samples (●, ●) and of the assay kit standard (●) for validation of the 11-KT assay.*

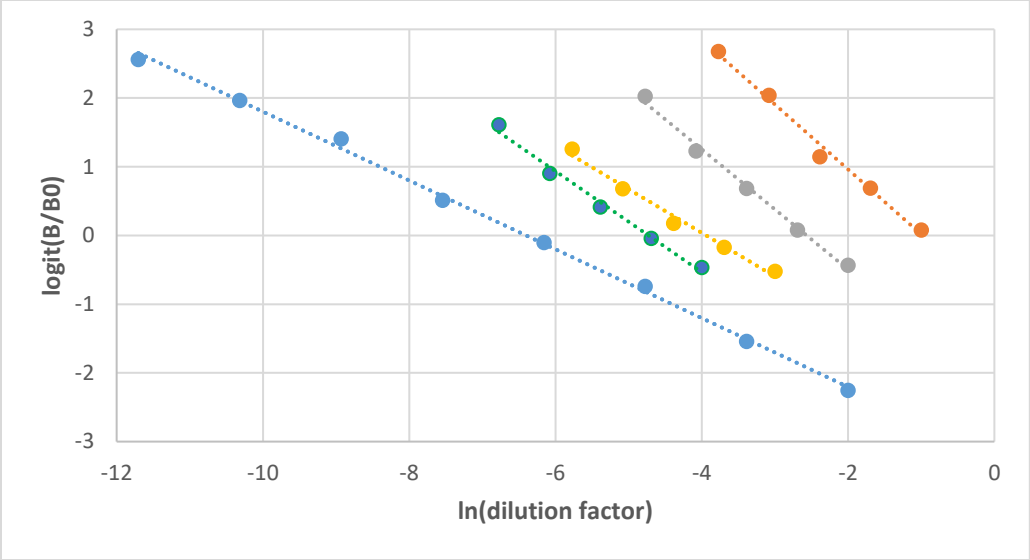


Figure 1.7 *Slopes of logit transformed absorbance measurements as a function of the dilution factor obtained during Estradiol 17β ELISA assays of serial dilutions of four test Tripletail plasma samples (●,●,●,●) and of the assay kit standard (●) for validation of the Estradiol 17β assay.*

3.3.2 Discriminant Analysis

The frequency distribution of 11-KT and Estradiol values in samples of confirmed males, confirmed females, and fish with unconfirmed sex are presented on Figures 1.8a, b, and c. Diagram c distinguished the E2 assay results obtained using the E2A and E2B kits for females and shows concentrations tended to be higher with the B kit. The 11-KT and E2 values differed significantly between sexes ($F_{1,146}$ = 14.81 P = 0.0002 for 11-KT, $F_{1,161}$ = 7.32 P = 0.0076 for E2).

Discriminant analyses were conducted using two training sets. First, all the data available from individuals of confirmed sex were used. Because of the low repeatability estimated for the E2A assay, a second analysis was conducted using the sub-dataset consisting of samples assayed with the E2B assay only as training set. Cross validation

results were better when using only the E2B sub-dataset as training set and results below are detailed only for the functions obtained with this training set.

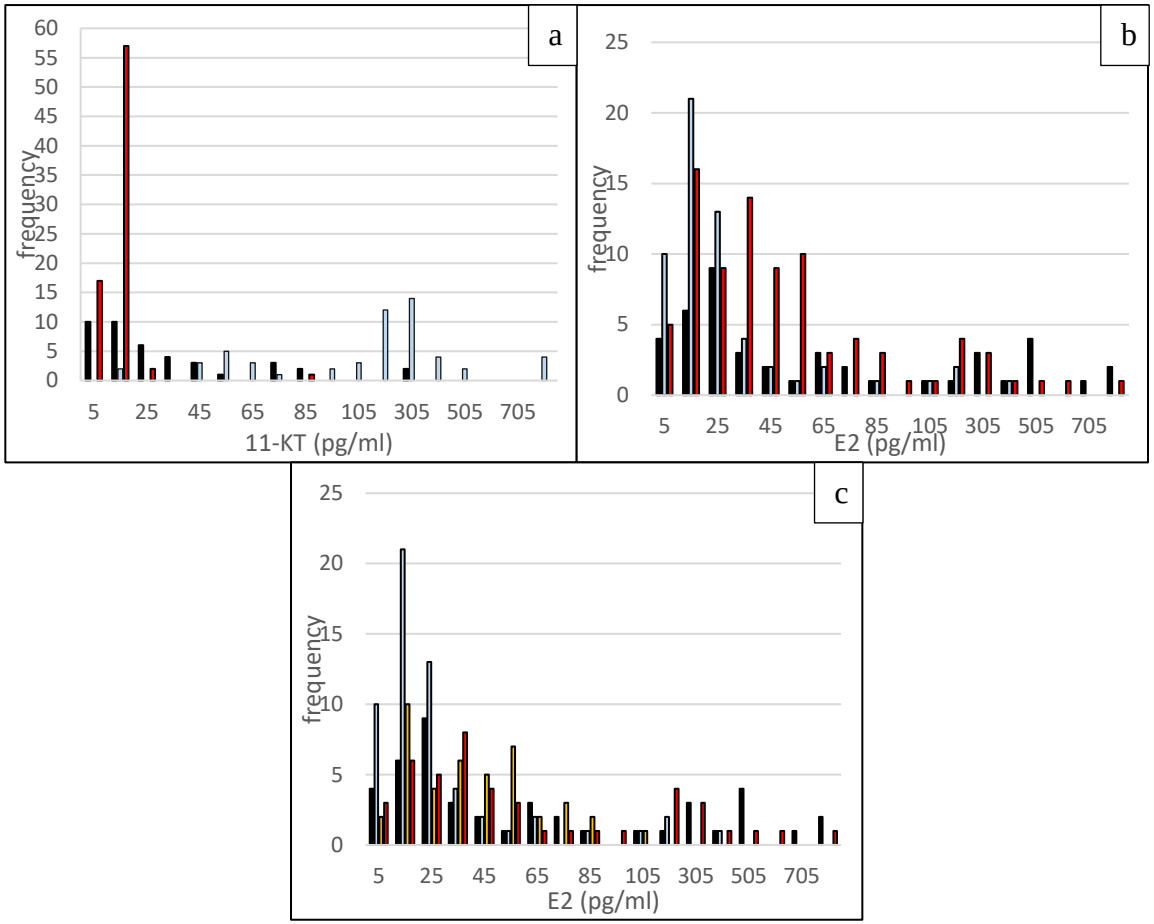


Figure 1.8 *Frequency distributions of (a) 11-KT levels, (b) E2 levels in confirmed females (■), males (■), and individuals with unconfirmed sex (■). In (c) E2 levels in females assayed using the E2A (■) and E2B (■) kits are shown separately.*

The variables retained by stepwise discriminant analysis included the two hormone measurements (11-KT and E2) and body weight. The percentage of assignment errors for training set samples during cross validation of the function obtained using 11-KT levels only was only 1.19% and did not improve when E2 levels or fish weight was included in the function. The assignment error during cross validation corresponded to one individual sexed as female in the field but assigned as a male based on hormone level measurements (11-KT measurement was 79.81 $pg.mL^{-1}$). This assignment conflict likely corresponds to a sexing error in the field, a hypothesis supported by the very low measurement of Estradiol recorded on this individual (2.38 $pg.mL^{-1}$, well below the sensitivity of the assay).

Individuals with sex confirmed but assayed using the E2A Estradiol kit were used as a test dataset. When 11-KT only was included in the sexing function, the error rate during cross-validation was higher (7.83% error overall). Errors corresponded to a high proportion of males incorrectly classified as females by the function. When E2 and Weight were included in the discriminant function, the rate of incorrect assignments for the test dataset decreased to 5.10% but at the expense of the total number of sexed individuals (*i.e.*, 7 individuals were not assigned a sex).

The function obtained was used to assign sex to all undetermined samples with steroid measurements available (14 males and 11 females).

Table 1.3 Summary of assignments results obtained during cross validation of the training dataset and classification of the remaining dataset (individuals with sex confirmed) using the optimized quadratic discriminant function. The prior probability of assignment to both sexes was 0.5. Cross validation results were identical for functions optimized using 11-KT, 11-KT and E2B, or 11-KT, E2B and Weight as input variables.

Number of observations and percent classified into sex			
Cross validation results of training dataset			
From sex	1	2	Error rate
1=male	33 (100%)	0 (0%)	0.00%
2=female	1 (2.38%)	41 (97.62%)	2.38%
Total	34 (45.33%)	41 (54.67%)	1.19%
Assignment of test dataset using 11-KT			
From sex	1	2	Error rate
1=male	26 (86.67%)	4 (13.33%)	13.33%
2=female	1 (2.33%)	42 (97.67%)	2.33%
Total	27 (36.99%)	46 (63.01%)	7.83%
Assignment of test dataset using 11-KT + E2			
From sex	1	2	Error rate
1=male	26 (89.66%)	3 (10.34%)	10.34%
2=female	1 (2.44%)	40 (97.56%)	2.44%
Total	27 (38.57%)	43 (61.43%)	6.39%
Assignment of test dataset using 11-KT + E2+ weight			
From sex	1	2	Error rate
1=male	24 (92.31%)	2 (7.69%)	7.69%
2=female	1 (2.50%)	39 (97.50%)	2.50%
Total	25 (37.88%)	41 (62.12%)	5.10%

3.3.3 Correlations

Correlation coefficients between levels of E2 and 11-KT and parameters recorded at the time of sample collection (body weight, length, condition coefficient, sex, temperature at sampling, number of days at spawning temperature prior to sample collection, and number of spawns in captivity prior to sampling) are reported in Appendix E.

11-KT levels were slightly positively correlated with condition coefficient ($r = 0.169$, $P = 0.028$), negatively with E2 levels ($r = -0.227$, $P = 0.003$), and positively correlated with temperature at sampling ($r = 0.162$, $P = 0.054$) and the number of days spent at or above 25ºC before sampling during the active spawning season ($r = 0.298$, $P = 0.0003$). E2 levels were negatively correlated with the temperature at sampling ($r = -0.439$, $P < 0.0001$).

3.4 Discussion

3.4.1 ELISA Assay Validation

The slopes of the absorbance curves obtained during analysis of serially diluted Tripletail samples with the 11-KT assay differed from those obtained with the kit hormone standard; the increase of absorbance with increasing dilution was slower for the Tripletail samples suggesting that hormone levels were over-estimated by applying the kit standard curve. Measurements recorded for serially diluted samples were all within the range of the kit standard, but most values were not within 20% and 80% of the maximum binding capacity of the antibody, which is the most reliable range for measurements (Cayman chemicals, 2019). The potential improvement of parallelism by restricting the analysis to this interval could not be evaluated in this work because removing values outside the 20%-80% maximum binding capacity of the antibody interval would have left only one or two dilution points per sample, which was insufficient to estimate the slope of the dilution curve. I also note that the overall range of 11-KT concentrations that can be measured with the assay would be very limited if

absorbance values only in this interval were retained (3.13-25 ng.mL^{-1} instead of 0.78-100 ng.mL^{-1}). The dilution curves of the two samples were, however, parallel, suggesting that values can be ranked and used as an index to compare individuals, which was the main objective of this study. The estimate of repeatability for the 11KT assay was 70%, and while this value is commonly considered 'moderate to good' (Koo and Li, 2016), it indicates some variation among assay plates is occurring. Strategies to mitigate repeatability issues include repeating readings on multiple plates or loading calibration samples on each plate and applying a correction to measurements recorded on individual plates based on values obtained for the calibration samples. These strategies may be evaluated in future studies, but would both incur additional costs, which would need to be compared to the gain in successful assignments during sex identification. Levels of 11-KT measured were almost all below 1 ng.mL^{-1}. These levels are low compared to values reported in reproductive adult males in other species where levels close to 1 ng.mL^{-1} or above are common during the maturation and spawning seasons (e.g., Prat et al., 1990, Metcalfe et al., 2018, Chu-Koo et al., 2009; Feist et al., 1990). Levels of 11-KT are usually elevated during spermatogenesis as this hormone plays a major role in the control of this phase in fish (Fostier et al., 1983). Male Tripletail produce little or no sperm in captivity (Saillant et al., 2014, 2021), and the low 11-KT levels recorded in this study are, thus, consistent with limited stimulation of spermatogenesis in captive conditions.

The repeatability of the E2A assay could not be estimated accurately, but the point estimate was very low suggesting the assay was unreliable. The estimate of repeatability for the E2B assay was 1 based on available data indicating that Estradiol levels measurements were highly reproducible. However, the analysis of parallelism

showed that absorbance values in serial dilutions of Tripletail samples increased faster than those in the ELISA kit hormone standard suggesting levels in Tripletail samples may be underestimated by the assay. Serial dilution curves of Tripletail samples were not parallel to each other either, indicating that measurements in different samples could not be compared reliably. However, parallelism was much improved when compared values were within 20% and 80% of the maximum binding capacity of the antibody with only a marginal departure from slope homogeneity. Restriction to 20%-80% of the maximum binding capacity of the antibody would limit the range of Estradiol levels that can be measured with confidence (9.77-625 $pg.ml^{-1}$ in the conditions of this study) but would allow for a more reliable comparison of samples, including for the purpose of sex identification. The lack of parallelism in ELISA assays can be due to (i) different immuno-affinities of the antibody to Estradiol contained in Tripletail samples versus to the hormone molecule used in assay development and calibration, or (ii) effects of the sample matrix on the immuno-affinity during assays (Tu and Bennett, 2017). The hypothesis that the matrix containing the samples interacted with the assay (e.g., due to contaminants) seems more plausible considering that Estradiol is highly conserved across vertebrates (Baker et al., 2009). Estradiol was extracted from plasma samples prior to dosage, which, in principle, should have removed any contaminant. The protocol used for extraction followed the directions of Cayman (2019) and is a single methanol extraction. Alternative extraction protocols for Estradiol use methylene chloride or diethyl ether. These solvents were found to recover a lower fraction of this hormone in human samples (Carter and Sluss, 2013), but might be more effective at removing contaminants in

extracts. This hypothesis could be evaluated by assessing the parallelism of serial dilution curves for Tripletail samples using alternative extraction methods.

3.4.2 Sexing Outcomes

The stepwise discriminant analysis retained 11-KT, E2, and body weight as the most effective variables contributing to a discriminant function for sex identification. The overall error rate obtained during cross validation of the optimized function was 1.19% and was due to the mis-assignment of one individual, likely corresponding to a sexing error in the field considering the high level of 11-KT recorded for that individual. Cross-validation of the training dataset suggested that 11-KT could be used alone because the sexing error rate obtained when using only this hormone was the same as the error rate of the composite discriminant function that incorporated E2 and/or weight. However, application of the sexing function to the test dataset led to a higher error rate due, in large part, to the incorrect classification of some of the males as females because their levels of 11-KT were low. Therefore, addition of other criteria to the sexing function would be helpful to allow identification of males with low 11-KT values because low levels of this hormone are likely to occur in captive conditions where spermatogenesis and spermiation are inhibited (Saillant et al., 2021, this thesis).

Sexing errors on the test dataset were partially reduced by including E2 and weight in the discriminant function, but the gain was small and may not justify running a second ELISA assay to measure E2. The limited improvement of sex identification when E2 was included in the test dataset may have been due to the low reliability of the old E2 assay (E2A) which was used in these samples and had a low repeatability. In addition, the

overall low rate of sexing errors on the training dataset when 11KT was used alone in itself limited the range of improvement that E2 could bring to the sexing function (only a few individuals were left mis-classified after assignment with 11-KT). However, when E2 and weight were used without 11-KT, assignment success was poor (32% error rate in the training dataset and 48% in the test dataset, respectively), highlighting the limited power of E2 and weight for sex assignment as compared to 11-KT. The new E2 assay (E2B) is more repeatable and may bring more improvement in sexing outcomes if the parallelism can be improved and more variance in E2 levels can be revealed, which would allow differentiating females showing high levels.

The discrimination power of both hormones could in theory be improved by targeting the appropriate period of the reproductive cycle. While Estradiol and 11-KT tend to be dimorphic between sexes all year round in other species, the differences are more pronounced during vitellogenesis and spermatogenesis for E2 and 11-KT, respectively (Kucherka et al., 2006). However, the levels of these two hormones can peak at different times in some species (e.g., Kohn et al., 2013), and plasma levels also may vary within the day due to the pulsatility of gonadotropin stimulation (e.g., Metcalfe et al., 2018; Zohar and Billard, 1984), which complicates the design of an optimal sampling strategy. The disruptions of the hormonal cycle in captive Tripletail also may render hormone levels unpredictable, further impacting the sexing outcomes at a specific time. Another approach to improve sex assignment accuracy would be to improve the precision of the 11-KT measurement by using a calibration sample run on all the assay plates or by replicating assays on multiple plates, although these options would increase the costs and efforts involved in sexing samples as discussed in section 3.4.1. Sexing accuracy also

could be improved by taking a second measurement on samples showing values close to the threshold for differentiating males and females or taking a second blood sample on these individuals.

Some studies used direct dosage of sex steroids without extraction to simplify assays and reduce costs (e.g., Mills et al., 2010). While this approach would reduce lab work and assay turn-around time, and minimize equipment and consumables needed, it also would likely affect the repeatability, sensitivity, and accuracy of assays as discussed by Metcalfe et al. (2018) and lead to increased error rates during sexing.

The females were on average larger, which led to the selection of body weight as a sexing criterion during stepwise discriminant analysis, but the size differences were moderate, and the usefulness of this trait may be questionable in this study because broodstock assessed for sexing were all wild-caught and of unknown age. Accordingly, differences in sizes between sexes may simply be due to an artifact during broodstock collections and in any case cannot be related to a faster growth rate of females. Fish were selected at a relatively small size for use as broodstock because females tolerate better handling involved in assessment and spawning (Saillant et al., 2021). However, future domestication efforts for this species will involve monitoring groups of the same age in standard conditions and, if differences in growth rates between sexes do occur in captive condition, the size of fish and possibly other morphometric measurements, may be informative and contribute to a phenotypic sexing function.

Sex identification could also include genetic markers of sex in the future if some are discovered in Tripletail. Identification of sex markers will require understanding the mechanism of sex determination in this species. Sex determination in fish is often

influenced by environmental factors (Baroiller et al., 2009) and, while some sex markers have been identified in some species, comprehensives studies conducted on some of the major aquaculture species have revealed complex multifactorial mechanisms (Martinez et al., 2014) that could be incompatible with genetic sexing in a simple assay. Approaches that specifically target the phenotypic sex such as levels of gonadal steroids were therefore preferred in this study, but genetic markers should be investigated as genomic tools currently in development for Tripletail become available.

3.4.3 Correlations

E2 tended to decrease with temperature at sampling. E2 is high during vitellogenesis and could be low if females are sampled during the final stages of maturation (which was not the case in this study as no female reached final oocyte maturation prior to induction during the project). While most females assayed could not be staged in this work, females with low E2 likely were not engaged in vitellogenesis. Females sampled at higher temperature included those sampled late in the season who may have discontinued vitellogenesis because of the stress incurred by repeated handling, which would explain lower levels of E2.

11-KT was positively correlated to temperature at sampling and the number of days at spawning temperature. The positive relationship with temperature and duration of exposure to spawning conditions may indicate that males slowly started maturing when exposed to spawning conditions for prolonged periods. Because the percentage of females found in vitellogenesis decreases with duration of exposure to spawning temperature as discussed above and in chapter I, one strategy could be to separate males

from females and bring them to warm temperature earlier than females to promote initiation of spermatogenesis prior to spawning trials.

CHAPTER IV – GENERAL CONCLUSION

This objective of this work was to contribute to the development of captive spawning in Tripletail by addressing two key bottlenecks. Initial attempts to acclimate Tripletail to captivity and condition them for maturation and spawning were unsuccessful. Hormonal therapies attempted prior to this project involved GnRH slow-release implants that restored final maturation and ovulation in females but produced unfertile spawns due, in part, to the lack of spermiation in males. Thus, the first bottleneck to hatchery production of Tripletail was that there was no effective protocol to produce fertile spawns from captive held brooders. The first objective of this work was therefore to test alternative hormonal therapies to restore spermiation in males and obtain fertile spawns.

Experiments conducted as part of chapter II of this thesis evaluated different hormonal therapies aiming to overcome Dopamine inhibition of GnRH stimulation as the dopaminergic pathway was hypothesized to be responsible for failure of males to respond to GnRH induction and ultimately for the low or lack of fertility of spawns. The first therapy evaluated was an administration of chorionic gonadotropin (hCG) as a single injection at a dose used successfully in other marine species (e.g., Bardon-Albaret and Saillant, 2017). No spawn was obtained using this approach, possibly due to over-stimulation of the gonads by the exogenous gonadotropin. However, induction with a GnRH analogue implant combined with a dopamine antagonist (Domperidone) led to fertilized spawns, showing that dopaminergic inhibition played a major role in the lack of

success inducing spawning with GNRHa implants alone. The protocol drastically improved the fertility, with some spawns displaying over 80% fertilization. Hatch rate and survival post-hatch were also higher, suggesting that egg quality was improved when Domperidone was used. The second experiment in Chapter II tested a higher dose of Domperidone in combination with the GNRHa implant. This treatment was highly successful and led to further increase of the number of egg releases, fecundity, fertility, hatch rate, and survival post hatch. The best treatment among the 5 therapies tested was thus the combination of the GNRHa implant and a 10 $mg.kg^{-1}$ injection of Domperidone.

Some aspects of the current protocol for maturation and spawning of captive Tripletail may be further improved. All spawning induction trials implemented in this study employed the same dosage of GNRHa, a dosage that is in the middle of the range of doses used in fish aquaculture (Mylonas and Zohar, 2000). Increasing the dosage could increase fecundity and the number of spawns, but also may lead to over-stimulation, a potential concern considering the lack of success of inductions with hCG. Decreasing the dose of GNRHa could lead to improved egg quality and larval survival to first feeding as reported in other species (e.g., Wright-Moore et al., 2019) and may be worth exploring. Hatch rates and survival post-hatch were indeed still low, even with the best treatment tested in this study. There was a positive effect of Domperidone dosage on all metrics (spawning output, fertility, and viability). Further improvement could be achieved by increasing the dose. However, the positive effect of the higher dose of Domperidone in this work seemed to be related to the longer duration of the blockage of Dopamine receptors achieved. A higher dosage administered as a single injection as in this study may only remove dopamine inhibition for a few additional hours unless extremely high

doses are used. An alternative approach would be to administer Domperidone or another dopamine antagonist in a sustained release implant (Kumakura et al., 2003) which would block dopamine receptors for a substantially longer time and possibly lead to maturation and spawning without even the need for a GnRH implant (Aizen et al., 2005). Efforts also could be directed toward removing the cause of the Dopamine inhibition in culture conditions. Dopamine inhibition has been related to stress conditions (Bhat and Ganesh, 2020). Accordingly, removing sources of stress may lead to maturation, possibly without the need for induction. As discussed in chapter II, some subtle behavioral interactions or other source of stress not detected in this study may lead to the inhibition of maturation and spawning. A formal behavioral study may help understand interaction dynamics between and within sexes in a spawning tank. Handling events negatively affected spawns in this study as illustrated by negative correlations between the number of handling events and the fecundity and viability of the late spawns produced by females. Decreasing the number of handling events could therefore have a positive impact on spawning output of this species. Stress also could be reduced by minimizing vibrations and noise in facilities during the broodstock holding phases.

Finally, environmental conditions during maturation may deserve additional investigations. Tripletail broodstock were conditioned under a natural cycle of temperature and photoperiod for the Mississippi Gulf coast. The presumed peak of the spawning period in the wild is in early summer (July according to Brown-Peterson and Franks, 2001) in the northern Gulf of Mexico. Accordingly, 27ºC was used for spawning temperature during this study. However, Ditty and Shaw (1994) found many larval Tripletail at the surface off the continental shelf in warmer temperatures (> 29ºC) in the

Gulf of Mexico, suggesting that the optimal temperature for maturation and spawning may be warmer than 27ºC. This hypothesis could be examined by monitoring maturation and spawning of mating sets conditioned at temperatures above 27ºC.

While achieving maturation and spontaneous spawning without the need for handling and hormonal induction is an important objective for enabling large-scale production in hatcheries, the methods implemented in this study offer some advantages for investigators aiming to domesticate Tripletail and develop a breeding program. In this work, single pairs isolated in a spawning tank could be induced to spawn and produce fertile and viable embryos reliably using the hormonal therapies discussed above. This approach could be extended to enable spawning of several pairs at the same time, which would allow comparison of aquaculture traits in a common garden design (e.g., Saillant et al., 2007). From a commercial perspective, using multiple males and females during a single spawning event would allow for higher throughput and efficiency.

Finally, during most of the spawning trials conducted during this work, the first spawn produced by females was either unfertilized or of poor quality resulting in poor hatch and survival of larvae. This result may be due, in part, to the fact that the oocyte stage at the time of induction was only 1 or 2, which led to a long induction period (Zarski et al., 2019). Based on these observations, the second or third spawns should be given priority for stocking into larval production to increase the odds of obtaining a high hatch rate and viability to first feeding.

The second bottleneck addressed by this study was the challenge identifying the sex of candidate broodstocks in the absence of clear secondary sexual characters. Results obtained in chapter III showed that the levels of 11-KT, an androgen specific to the male

(Fostier et al., 1983), effectively discriminated between sexes, and cross validation suggested this hormone could be used alone to assign sexes in this species with high success rates. The method could be improved to reduce further the rate of sexing errors which affect primarily males with low 11-KT values. This could be achieved by improving the accuracy of the 11-KT measurements through repeated assays on the same sample or by resampling individuals for which measured levels are close to the threshold for sexing. The currently available commercial kit for Estradiol 17β had excellent repeatability, but hormone levels may be under-estimated due to contaminants, and the assay contributed little to sex identification. While the assay could be optimized to better recover the hormone present in samples and distinguish females more accurately, these improvements may have little effect on sexing error rates considering the high rate of assignment with 11-KT alone. 11-KT values were positively correlated with temperature at sampling and number of days at spawning temperature, which may indicate that males slowly mature with increased exposure to higher temperatures and are better sexed with high levels of 11-KT late in the season. Inversely, the level of E2 was negatively correlated with duration of spawning temperature. If groups of candidate brooders held in facilities are sampled at the same time for sexing, sampling in the late phases of the spawning season would more effectively recover high levels of 11-KT in males and, in these conditions, the Estradiol assay would provide less information.

While females in the broodstocks examined for hormonal sexing were larger, and body weight contributed to the sexing functions, fish size likely will be of limited use in practical sexing of wild caught broodstock because they are of potentially different ages and are purposely selected within a size range. However, a more in-depth examination of

morphological features may be warranted to identify subtle sexual dimorphisms that may have been left undiscovered (*e.g.*, Im et al., 2016). Genetic markers of sex also should be pursued because they could provide a cost-effective option for sexing if the sex determination system in Tripletail is genetic and involves one or a few loci compatible with a rapid assay.

Appendix A– Quarantine Protocol

The protocol to introduce new fish to the broodstock included immersion for 5 minutes in freshwater followed by 24-hour immersion in a Praziquantel solution (5 mg praziquantel per liter saltwater following the instructions of the Aquascience product instructions) to remove external parasites. Upon removal of each fish from the Prazi bath, a passive integrated transponder (PIT) tag for individual identification was implanted. Fish were then transferred to quarantine facilities where copper sulphate was maintained at 0.15-0.20 mg/L for no less than 28 days to ensure that all stages of *Amyloodinium ocellatum* were eradicated before transferring fish to bio-secure facilities.

During this period, fish were held in either 7 m^3 or 45 m^3 round fiberglass tanks connected to a recirculating life support system featuring a biofilter, protein skimmer, and temperature control. Environmental parameters (temperature and photoperiod) mimicked the natural cycle in Mississippi coastal waters. Salinity was maintained at 30 ± 3 psu throughout this period. Quarantine Tripletail were fed frozen food (cut Squid, Mackerel, Shrimp) at a rate of 3% of body weight 3 times per week. Water quality (temperature, salinity, dissolved oxygen concentration, pH, and alkalinity) was monitored daily. Dissolved ammonia, nitrite, and nitrate were measured on feeding days. Procedures for monitoring culture parameters and maintenance of recirculating systems are detailed in the Standard Operating Procedures attached as Appendix 1. Upon completion of quarantine, animals were sedated with Methane Tricaine Sulfonate (100mg/L) and moved to brood stock holding tanks.

Appendix B – Spawning Correlation Table

Spearman Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Samples

	AvgFem Wt2	AvgFem LTH2	FemCond2	Odiam2	Stage2	Hand event2	Dayat Spawn2	NR2	TotalFec2	Lat12	Fert12	Fert22	Fert32	Fert42
AvgFemLTH2 AvgFemLTH	0.89604 <.0001 24	1.00000 24	-0.05196 0.8230 21	-0.32731 0.1849 18	-0.33814 0.1699 18	0.49238 0.0145 24	0.36513 0.0794 24	-0.00669 0.9752 24	0.02463 0.9090 24	-0.09309 0.6963 20	-0.07089 0.7665 20	-0.13201 0.6528 14	-0.45455 0.3055 7	0.00000 1.0000 4
FemCond2 FemCond	0.24156 0.2915 21	-0.05196 0.8230 21	1.00000 21	-0.02941 0.9139 16	-0.05618 0.8363 16	-0.05047 0.8280 21	-0.23779 0.2993 21	-0.02276 0.9220 21	0.06112 0.7924 21	0.26690 0.2843 18	-0.55606 0.0166 18	-0.30769 0.3064 13	0.11595 0.8268 6	0.40000 0.6000 4
Odiam2 Odiam	-0.33953 0.1681 18	-0.32731 0.1849 18	-0.02941 0.9139 16	1.00000 18	0.35307 0.1507 18	-0.15144 0.5486 18	0.08174 0.7471 18	0.42833 0.0762 18	0.25194 0.3132 18	-0.41098 0.0902 18	0.20752 0.4086 18	0.47253 0.1030 13	0.27028 0.5577 7	-0.80000 0.2000 4
Stage2 Stage	-0.29900 0.2281 18	-0.33814 0.1699 18	-0.05618 0.8363 16	0.35307 0.1507 18	1.00000 18	-0.24172 0.3339 18	0.13075 0.6051 18	0.09654 0.7032 18	0.04296 0.8656 18	-0.12064 0.6335 18	0.31338 0.2054 18	0.38367 0.1956 13	0.41194 0.3585 7	. . 4
Handevent2 Handevent	0.45657 0.0249 24	0.49238 0.0145 24	-0.05047 0.8280 21	-0.15144 0.5486 18	-0.24172 0.3339 18	1.00000 24	0.58784 0.0025 24	-0.42657 0.0376 24	-0.40472 0.0498 24	0.21649 0.3593 20	-0.19967 0.3987 20	-0.50456 0.0658 14	-0.54272 0.2081 7	-0.31623 0.6838 4
DayatSpawn2 DayatSpawn	0.30410 0.1485 24	0.36513 0.0794 24	-0.23779 0.2993 21	0.08174 0.7471 18	0.13075 0.6051 18	0.58784 0.0025 24	1.00000 24	-0.38340 0.0644 24	-0.42379 0.0390 24	-0.00632 0.9789 20	0.10157 0.6700 20	-0.03528 0.9047 14	-0.27524 0.5502 7	-0.31623 0.6838 4
NR2 NR	-0.06334 0.7687 24	-0.00669 0.9752 24	-0.02276 0.9220 21	0.42833 0.0762 18	0.09654 0.7032 18	-0.42657 0.0376 24	-0.38340 0.0644 24	1.00000 24	0.78899 <.0001 24	-0.75351 0.0001 20	0.47598 0.0339 20	0.28910 0.3161 14	-0.07282 0.8767 7	. . 4

Table 1.4 Continued

Spearman Correlation Coefficients Prob > |r| under H0: Rho=0 Number of Samples														
	AvgFem Wt2	AvgFem LTH2	FemCond2	Odiam2	Stage2	Hand event2	Dayat Spawn2	NR2	TotalFec2	Lat12	Fert12	Fert22	Fert32	Fert42
TotalFec2 TotalFec	-0.01133 0.9581 24	0.02463 0.9090 24	0.06112 0.7924 21	0.25194 0.3132 18	0.04296 0.8656 18	-0.40472 0.0498 24	-0.42379 0.0390 24	0.78899 <.0001 24	1.00000 24	-0.42601 0.0611 20	0.44478 0.0494 20	0.27033 0.3499 14	-0.07207 0.8780 7	-0.80000 0.2000 4
Lat12 Lat1	-0.06309 0.7916 20	-0.09309 0.6963 20	0.26690 0.2843 18	-0.41098 0.0902 18	-0.12064 0.6335 18	0.21649 0.3593 20	-0.00632 0.9789 20	-0.75351 0.0001 20	-0.42601 0.0611 20	1.00000 20	-0.52067 0.0186 20	-0.54179 0.0454 14	-0.80904 0.0275 7	-0.31623 0.6838 4
Fert12 Fert1	-0.24002 0.3081 20	-0.07089 0.7665 20	-0.55606 0.0166 18	0.20752 0.4086 18	0.31338 0.2054 18	-0.19967 0.3987 20	0.10157 0.6700 20	0.47598 0.0339 20	0.44478 0.0494 20	-0.52067 0.0186 20	1.00000 20	0.40924 0.1462 14	-0.50452 0.2482 7	0.40000 0.6000 4
Fert22 Fert2	-0.09011 0.7593 14	-0.13201 0.6528 14	-0.30769 0.3064 13	0.47253 0.1030 13	0.38367 0.1956 13	-0.50456 0.0658 14	-0.03528 0.9047 14	0.28910 0.3161 14	0.27033 0.3499 14	-0.54179 0.0454 14	0.40924 0.1462 14	1.00000 14	0.01802 0.9694 7	-0.40000 0.6000 4
Fert32 Fert3	0.01802 0.9694 7	-0.45455 0.3055 7	0.11595 0.8268 6	0.27028 0.5577 7	0.41194 0.3585 7	-0.54272 0.2081 7	-0.27524 0.5502 7	-0.07282 0.8767 7	-0.07207 0.8780 7	-0.80904 0.0275 7	-0.50452 0.2482 7	0.01802 0.9694 7	1.00000 7	0.40000 0.6000 4
Fert42 Fert4	0.80000 0.2000 4	0.00000 1.0000 4	0.40000 0.6000 4	-0.80000 0.2000 4	. . 4	-0.31623 0.6838 4	-0.31623 0.6838 4	. . 4	-0.80000 0.2000 4	-0.31623 0.6838 4	0.40000 0.6000 4	-0.40000 0.6000 4	0.40000 0.6000 4	1.00000 4
Hatcha2 Hatcha	-0.20530 0.4814 14	-0.00663 0.9821 14	-0.39438 0.2046 12	0.23757 0.4345 13	0.56558 0.0440 13	0.05842 0.8427 14	0.14602 0.6184 14	0.53190 0.0503 14	0.19205 0.5107 14	-0.56870 0.0338 14	0.92495 <.0001 14	0.66550 0.0182 12	-0.05798 0.9131 6	0.50000 0.6667 3
Surva12 Surva1	-0.20455 0.4830 14	0.01125 0.9695 14	-0.41288 0.1822 12	0.15043 0.6237 13	0.54958 0.0517 13	-0.02142 0.9421 14	0.07659 0.7947 14	0.56051 0.0371 14	0.24501 0.3985 14	-0.59404 0.0251 14	0.90135 <.0001 14	0.66903 0.0174 12	-0.05798 0.9131 6	0.50000 0.6667 3

Table 1.4 Continued

Spearman Correlation Coefficients Prob > |r| under H0: Rho=0 Number of Samples														
	AvgFem Wt2	AvgFem LTH2	FemCond2	Odiam2	Stage2	Hand event2	Dayat Spawn2	NR2	TotalFec2	Lat12	Fert12	Fert22	Fert32	Fert42
Surva22 Surva2	-0.25400 0.3809 14	-0.00675 0.9817 14	-0.43423 0.1584 12	0.23958 0.4305 13	0.59490 0.0320 13	0.00000 1.0000 14	0.14192 0.6284 14	0.60308 0.0224 14	0.21803 0.4540 14	-0.56908 0.0337 14	0.86089 <.0001 14	0.64086 0.0247 12	-0.26090 0.6175 6	-0.50000 0.6667 3
Surva42 Surva4	-0.14360 0.6243 14	0.05361 0.8556 14	-0.46788 0.1251 12	0.04181 0.8921 13	0.52237 0.0670 13	0.17392 0.5521 14	0.03049 0.9176 14	0.41487 0.1402 14	0.35535 0.2125 14	-0.47838 0.0836 14	0.84457 0.0001 14	0.45534 0.1369 12	-0.01471 0.9779 6	0.50000 0.6667 3
Hatchb2 Hatchb	-0.09461 0.7477 14	-0.09471 0.7474 14	-0.44017 0.1323 13	0.20633 0.4988 13	0.40471 0.1701 13	-0.08577 0.7706 14	-0.15784 0.5899 14	0.23919 0.4102 14	0.61606 0.0190 14	-0.26631 0.3574 14	0.37225 0.1900 14	0.58526 0.0279 14	0.19821 0.6701 7	0.00000 1.0000 4
Survb12 Survb1	-0.08581 0.7705 14	-0.07709 0.7934 14	-0.43466 0.1377 13	0.16781 0.5837 13	0.40471 0.1701 13	-0.11198 0.7031 14	-0.19757 0.4984 14	0.17461 0.5505 14	0.59846 0.0238 14	-0.22477 0.4398 14	0.34141 0.2322 14	0.58526 0.0279 14	0.19821 0.6701 7	0.00000 1.0000 4
Survb22 Survb2	-0.09492 0.7469 14	-0.01547 0.9581 14	-0.38122 0.1987 13	0.29835 0.3221 13	0.38579 0.1929 13	0.00239 0.9935 14	-0.25692 0.3752 14	0.29998 0.2974 14	0.62032 0.0179 14	-0.29171 0.3116 14	0.28508 0.3232 14	0.54967 0.0417 14	-0.01802 0.9694 7	-0.60000 0.4000 4
Survb42 Survb4	-0.05111 0.8622 14	0.01112 0.9699 14	-0.42623 0.1464 13	0.27579 0.3617 13	0.45893 0.1147 13	-0.03490 0.9057 14	-0.19176 0.5114 14	0.29716 0.3022 14	0.60226 0.0227 14	-0.40472 0.1512 14	0.44162 0.1139 14	0.62004 0.0180 14	0.07207 0.8780 7	-0.60000 0.4000 4
Hatchc2 Hatchc	0.60714 0.1482 7	0.14415 0.7578 7	0.48571 0.3287 6	-0.10714 0.8192 7	-0.20412 0.6606 7	-0.89642 0.0063 7	-0.76376 0.0457 7	0.43301 0.3318 7	0.10714 0.8192 7	-0.66815 0.1009 7	-0.46429 0.2939 7	0.60714 0.1482 7	0.34236 0.4523 7	0.40000 0.6000 4
Survc12 Survc1	0.50000 0.2532 7	0.00000 1.0000 7	0.31429 0.5441 6	0.03571 0.9394 7	-0.20412 0.6606 7	-0.89642 0.0063 7	-0.70921 0.0743 7	0.28868 0.5301 7	0.07143 0.8790 7	-0.80178 0.0301 7	-0.57143 0.1802 7	0.75000 0.0522 7	0.39641 0.3786 7	0.20000 0.8000 4

Table 1.4 Continued

Spearman Correlation Coefficients Prob > |r| under H0: Rho=0 Number of Samples														
	AvgFem Wt2	AvgFem LTH2	FemCond2	Odiam2	Stage2	Hand event2	Dayat Spawn2	NR2	TotalFec2	Lat12	Fert12	Fert22	Fert32	Fert42
Survc22 Survc2	0.52254 0.2289 7	0.02727 0.9537 7	-0.14494 0.7841 6	- 0.73877 0.0579 7	0.41194 0.3585 7	- 0.13065 0.7801 7	0.15597 0.7384 7	-0.07282 0.8767 7	-0.03604 0.9389 7	- 0.33710 0.4597 7	0.34236 0.4523 7	- 0.05406 0.9084 7	0.39091 0.3859 7	0.80000 0.2000 4
Survc42 Survc4	0.40769 0.3639 7	0.03740 0.9366 7	-0.14494 0.7841 6	- 0.37062 0.4131 7	0.42366 0.3435 7	- 0.33076 0.4687 7	- 0.11323 0.8090 7	0.29957 0.5139 7	0.25944 0.5742 7	- 0.41603 0.3532 7	0.07412 0.8745 7	- 0.03706 0.9371 7	0.65446 0.1107 7	0.80000 0.2000 4
Hatchd2 Hatchd	- 0.50000 0.6667 3	-0.50000 0.6667 3	-1.00000 <.0001 3	- 0.50000 0.6667 3	. . 3	0.86603 0.3333 3	0.86603 0.3333 3	. . 3	0.50000 0.6667 3	0.00000 1.0000 3	0.50000 0.6667 3	- 0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3
Survd12 Survd1	- 0.50000 0.6667 3	-0.50000 0.6667 3	-1.00000 <.0001 3	- 0.50000 0.6667 3	. . 3	0.86603 0.3333 3	0.86603 0.3333 3	. . 3	0.50000 0.6667 3	0.00000 1.0000 3	0.50000 0.6667 3	- 0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3
Survd22 Survd2	- 0.50000 0.6667 3	-0.50000 0.6667 3	-1.00000 <.0001 3	- 0.50000 0.6667 3	. . 3	0.86603 0.3333 3	0.86603 0.3333 3	. . 3	0.50000 0.6667 3	0.00000 1.0000 3	0.50000 0.6667 3	- 0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3
Survd42 Survd4	- 1.00000 <.0001 3	-1.00000 <.0001 3	-0.50000 0.6667 3	0.50000 0.6667 3	. . 3	0.00000 1.0000 3	0.00000 1.0000 3	. . 3	1.00000 <.0001 3	- 0.86603 0.3333 3	- 0.50000 0.6667 3	0.50000 0.6667 3	1.00000 <.0001 3	-0.50000 0.6667 3
AvgLtha2 AvgLtha	- 0.08571 0.8717 6	-0.23191 0.6584 6	0.20000 0.7471 5	0.25714 0.6228 6	0.82808 0.0418 6	0.16903 0.7489 6	- 0.08571 0.8717 6	-0.06172 0.9075 6	0.02857 0.9572 6	- 0.82808 0.0418 6	0.08571 0.8717 6	0.02857 0.9572 6	1.00000 <.0001 3	. . 1
AvgDepa2 AvgDepa	- 0.46382 0.3542 6	-0.41176 0.4173 6	-0.30779 0.6144 5	0.46382 0.3542 6	0.52511 0.2847 6	0.42875 0.3963 6	- 0.20292 0.6998 6	0.23483 0.6542 6	0.02899 0.9565 6	- 0.52511 0.2847 6	0.17393 0.7417 6	0.05798 0.9131 6	0.00000 1.0000 3	. . 1

Table 1.4 Continued

Spearman Correlation Coefficients Prob > |r| under H0: Rho=0 Number of Samples														
	AvgFem Wt2	AvgFem LTH2	FemCond2	Odiam2	Stage2	Hand event2	Dayat Spawn2	NR2	TotalFec2	Lat12	Fert12	Fert22	Fert32	Fert42
AvgYolka2 AvgYolka	-0.14286 0.7872 6	0.11595 0.8268 6	-0.30000 0.6238 5	0.31429 0.5441 6	-0.62106 0.1882 6	0.16903 0.7489 6	0.20000 0.7040 6	0.61721 0.1917 6	0.02857 0.9572 6	0.82808 0.0418 6	-0.42857 0.3965 6	-0.42857 0.3965 6	-1.00000 <.0001 3	. . 1
AvgLthb2 AvgLthb	-0.20000 0.7040 6	-0.65714 0.1562 6	0.10000 0.8729 5	-0.25714 0.6228 6	0.39279 0.4411 6	-0.30861 0.5518 6	0.25714 0.6228 6	-0.33947 0.5104 6	-0.82857 0.0416 6	-0.41404 0.4144 6	-0.14286 0.7872 6	0.37143 0.4685 6	0.50000 0.6667 3	1.00000 . 2
AvgDepb2 AvgDepb	0.54286 0.2657 6	0.25714 0.6228 6	0.50000 0.3910 5	-0.65714 0.1562 6	0.13093 0.8047 6	0.00000 1.0000 6	-0.42857 0.3965 6	-0.46291 0.3552 6	-0.08571 0.8717 6	-0.82808 0.0418 6	0.20000 0.7040 6	0.25714 0.6228 6	0.50000 0.6667 3	1.00000 . 2
AvgYolkb2 AvgYolkb	-0.31429 0.5441 6	0.08571 0.8717 6	-0.70000 0.1881 5	0.02857 0.9572 6	0.13093 0.8047 6	0.77152 0.0723 6	0.31429 0.5441 6	-0.18516 0.7254 6	0.60000 0.2080 6	0.41404 0.4144 6	0.77143 0.0724 6	-0.08571 0.8717 6	-1.00000 <.0001 3	-1.00000 . 2
AvgLthc2 AvgLthc	-0.50000 0.6667 3	-0.50000 0.6667 3	-1.00000 . 2	-0.50000 0.6667 3	. . 3	0.00000 1.0000 3	-0.50000 0.6667 3	0.86603 0.3333 3	0.50000 0.6667 3	0.00000 1.0000 3	0.50000 0.6667 3	-0.50000 0.6667 3	0.50000 0.6667 3	1.00000 . 2
AvgDepc2 AvgDepc	-0.50000 0.6667 3	-0.50000 0.6667 3	-1.00000 . 2	-0.50000 0.6667 3	. . 3	0.00000 1.0000 3	-0.50000 0.6667 3	0.86603 0.3333 3	0.50000 0.6667 3	0.00000 1.0000 3	0.50000 0.6667 3	-0.50000 0.6667 3	0.50000 0.6667 3	1.00000 . 2
AvgYolkc2 AvgYolkc	-1.00000 <.0001 3	-1.00000 <.0001 3	1.00000 . 2	0.50000 0.6667 3	. . 3	-0.86603 0.3333 3	-1.00000 <.0001 3	0.86603 0.3333 3	1.00000 <.0001 3	-0.86603 0.3333 3	-0.50000 0.6667 3	0.50000 0.6667 3	1.00000 <.0001 3	-1.00000 . 2

Table 1.4 Continued

Spearman Correlation Coefficients Prob > |r| under H0: Rho=0 Number of Samples															
	Hatcha2	Surva12	Surva22	Surva42	Hatchb2	Survb12	Survb22	Survb42	Hatchc2	Survc12	Survc22	Survc42	Hatchd2	Survd12	Survd22
AvgFemLTH2 AvgFemLTH	-0.0066 0.9821 14	0.01125 0.9695 14	-0.0067 0.9817 14	0.05361 0.8556 14	-0.0947 0.7474 14	-0.0770 0.7934 14	-0.0154 0.9581 14	0.01112 0.9699 14	0.14415 0.7578 7	0.00000 1.0000 7	0.02727 0.9537 7	0.03740 0.9366 7	- 0.50000 0.6667 3	- 0.50000 0.6667 3	- 0.50000 0.6667 3
FemCond2 FemCond	-0.3943 0.2046 12	-0.4128 0.1822 12	-0.4342 0.1584 12	-0.4678 0.1251 12	-0.4401 0.1323 13	-0.4346 0.1377 13	-0.3812 0.1987 13	-0.4262 0.1464 13	0.48571 0.3287 6	0.31429 0.5441 6	-0.1449 0.7841 6	-0.1449 0.7841 6	- 1.00000 <.0001 3	- 1.00000 <.0001 3	- 1.00000 <.0001 3
Odiam2 Odiam	0.23757 0.4345 13	0.15043 0.6237 13	0.23958 0.4305 13	0.04181 0.8921 13	0.20633 0.4988 13	0.16781 0.5837 13	0.29835 0.3221 13	0.27579 0.3617 13	- 0.10714 0.8192 7	0.03571 0.9394 7	- 0.73877 0.0579 7	- 0.37062 0.4131 7	- 0.50000 0.6667 3	- 0.50000 0.6667 3	- 0.50000 0.6667 3
Stage2 Stage	0.56558 0.0440 13	0.54958 0.0517 13	0.59490 0.0320 13	0.52237 0.0670 13	0.40471 0.1701 13	0.40471 0.1701 13	0.38579 0.1929 13	0.45893 0.1147 13	-0.2041 0.6606 7	-0.2041 0.6606 7	0.41194 0.3585 7	0.42366 0.3435 7	. . 3	. . 3	. . 3
Handevent2 Handevent	0.05842 0.8427 14	-0.0214 0.9421 14	0.00000 1.0000 14	0.17392 0.5521 14	-0.0857 0.7706 14	-0.1119 0.7031 14	0.00239 0.9935 14	-0.0349 0.9057 14	-0.8964 0.0063 7	-0.8964 0.0063 7	-0.1306 0.7801 7	-0.3307 0.4687 7	0.86603 0.3333 3	0.86603 0.3333 3	0.86603 0.3333 3
DayatSpawn2 DayatSpawn	0.14602 0.6184 14	0.07659 0.7947 14	0.14192 0.6284 14	0.03049 0.9176 14	-0.1578 0.5899 14	-0.1975 0.4984 14	-0.2569 0.3752 14	-0.1917 0.5114 14	-0.7637 0.0457 7	-0.7092 0.0743 7	0.15597 0.7384 7	-0.1132 0.8090 7	0.86603 0.3333 3	0.86603 0.3333 3	0.86603 0.3333 3
NR2 NR	0.53190 0.0503 14	0.56051 0.0371 14	0.60308 0.0224 14	0.41487 0.1402 14	0.23919 0.4102 14	0.17461 0.5505 14	0.29998 0.2974 14	0.29716 0.3022 14	0.43301 0.3318 7	0.28868 0.5301 7	-0.0728 0.8767 7	0.29957 0.5139 7	. . 3	. . 3	. . 3
TotalFec2 TotalFec	0.19205 0.5107 14	0.24501 0.3985 14	0.21803 0.4540 14	0.35535 0.2125 14	0.61606 0.0190 14	0.59846 0.0238 14	0.62032 0.0179 14	0.60226 0.0227 14	0.10714 0.8192 7	0.07143 0.8790 7	-0.0360 0.9389 7	0.25944 0.5742 7	0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3

Table 1.4 Continued

Spearman Correlation Coefficients Prob > |r| under H0: Rho=0 Number of Samples															
	Hatcha2	Surva12	Surva22	Surva42	Hatchb2	Survb12	Survb22	Survb42	Hatchc2	Survc12	Survc22	Survc42	Hatchd2	Survd12	Survd22
Lat12 Lat1	-0.5687 0.0338 14	-0.5940 0.0251 14	-0.5690 0.0337 14	-0.4783 0.0836 14	-0.2663 0.3574 14	-0.2247 0.4398 14	-0.2917 0.3116 14	-0.4047 0.1512 14	-0.6681 0.1009 7	-0.8017 0.0301 7	-0.3371 0.4597 7	-0.4160 0.3532 7	0.00000 1.0000 3	0.00000 1.0000 3	0.00000 1.0000 3
Fert12 Fert1	0.92495 <.0001 14	0.90135 <.0001 14	0.86089 <.0001 14	0.84457 0.0001 14	0.37225 0.1900 14	0.34141 0.2322 14	0.28508 0.3232 14	0.44162 0.1139 14	-0.4642 0.2939 7	-0.5714 0.1802 7	0.34236 0.4523 7	0.07412 0.8745 7	0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3
Fert22 Fert2	0.66550 0.0182 12	0.66903 0.0174 12	0.64086 0.0247 12	0.45534 0.1369 12	0.58526 0.0279 14	0.58526 0.0279 14	0.54967 0.0417 14	0.62004 0.0180 14	0.60714 0.1482 7	0.75000 0.0522 7	-0.0540 0.9084 7	-0.0370 0.9371 7	- 0.50000 0.6667 3	- 0.50000 0.6667 3	- 0.50000 0.6667 3
Fert32 Fert3	-0.0579 0.9131 6	-0.0579 0.9131 6	-0.2609 0.6175 6	-0.0147 0.9779 6	0.19821 0.6701 7	0.19821 0.6701 7	-0.0180 0.9694 7	0.07207 0.8780 7	0.34236 0.4523 7	0.39641 0.3786 7	0.39091 0.3859 7	0.65446 0.1107 7	0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3
Fert42 Fert4	0.50000 0.6667 3	0.50000 0.6667 3	-0.5000 0.6667 3	0.50000 0.6667 3	0.00000 1.0000 4	0.00000 1.0000 4	-0.6000 0.4000 4	-0.6000 0.4000 4	0.40000 0.6000 4	0.20000 0.8000 4	0.80000 0.2000 4	0.80000 0.2000 4	0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3
Hatcha2 Hatcha	1.00000 14	0.98210 <.0001 14	0.96404 <.0001 14	0.90698 <.0001 14	0.75263 0.0047 12	0.70000 0.0113 12	0.71253 0.0093 12	0.80424 0.0016 12	-0.0285 0.9572 6	-0.2000 0.7040 6	0.55078 0.2574 6	0.51610 0.2946 6	1.00000 . 2	1.00000 . 2	1.00000 . 2
Surva12 Surva1	0.98210 <.0001 14	1.00000 14	0.98161 <.0001 14	0.92351 <.0001 14	0.72664 0.0074 12	0.67726 0.0155 12	0.69504 0.0121 12	0.80142 0.0017 12	-0.0285 0.9572 6	-0.2000 0.7040 6	0.55078 0.2574 6	0.51610 0.2946 6	1.00000 . 2	1.00000 . 2	1.00000 . 2
Surva22 Surva2	0.96404 <.0001 14	0.98161 <.0001 14	1.00000 14	0.90360 <.0001 14	0.65609 0.0205 12	0.59260 0.0423 12	0.67376 0.0163 12	0.75887 0.0042 12	-0.2571 0.6228 6	-0.3714 0.4685 6	0.28989 0.5774 6	0.27323 0.6004 6	1.00000 . 2	1.00000 . 2	1.00000 . 2

Table 1.4 Continued

Spearman Correlation Coefficients Prob > |r| under H0: Rho=0 Number of Samples															
	Hatcha2	Surva12	Surva22	Surva42	Hatchb2	Survb12	Survb22	Survb42	Hatchc2	Survc12	Survc22	Survc42	Hatchd2	Survd12	Survd22
Surva42 Surva4	0.90698 <.0001 14	0.92351 <.0001 14	0.90360 <.0001 14	1.00000 14	0.83003 0.0008 12	0.78890 0.0023 12	0.81564 0.0012 12	0.90585 <.0001 12	-0.1449 0.7841 6	-0.3188 0.5379 6	0.51471 0.2961 6	0.46203 0.3563 6	1.00000 . 2	1.00000 . 2	1.00000 . 2
Hatchb2 Hatchb	0.75263 0.0047 12	0.72664 0.0074 12	0.65609 0.0205 12	0.83003 0.0008 12	1.00000 14	0.99119 <.0001 14	0.93481 <.0001 14	0.92997 <.0001 14	0.21429 0.6445 7	0.32143 0.4821 7	0.39641 0.3786 7	0.40769 0.3639 7	0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3
Survb12 Survb1	0.70000 0.0113 12	0.67726 0.0155 12	0.59260 0.0423 12	0.78890 0.0023 12	0.99119 <.0001 14	1.00000 14	0.93039 <.0001 14	0.92997 <.0001 14	0.21429 0.6445 7	0.32143 0.4821 7	0.39641 0.3786 7	0.40769 0.3639 7	0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3
Survb22 Survb2	0.71253 0.0093 12	0.69504 0.0121 12	0.67376 0.0163 12	0.81564 0.0012 12	0.93481 <.0001 14	0.93039 <.0001 14	1.00000 14	0.96208 <.0001 14	0.03571 0.9394 7	0.25000 0.5887 7	0.12613 0.7876 7	0.03706 0.9371 7	0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3
Survb42 Survb4	0.80424 0.0016 12	0.80142 0.0017 12	0.75887 0.0042 12	0.90585 <.0001 12	0.92997 <.0001 14	0.92997 <.0001 14	0.96208 <.0001 14	1.00000 14	0.07143 0.8790 7	0.21429 0.6445 7	0.19821 0.6701 7	0.22237 0.6317 7	0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3
Hatchc2 Hatchc	-0.0285 0.9572 6	-0.0285 0.9572 6	-0.2571 0.6228 6	-0.1449 0.7841 6	0.21429 0.6445 7	0.21429 0.6445 7	0.03571 0.9394 7	0.07143 0.8790 7	1.00000 7	0.92857 0.0025 7	0.37839 0.4026 7	0.48181 0.2736 7	- 1.00000 <.0001 3	- 1.00000 <.0001 3	- 1.00000 <.0001 3
Survc12 Survc1	-0.2000 0.7040 6	-0.2000 0.7040 6	-0.3714 0.4685 6	-0.3188 0.5379 6	0.32143 0.4821 7	0.32143 0.4821 7	0.25000 0.5887 7	0.21429 0.6445 7	0.92857 0.0025 7	1.00000 7	0.34236 0.4523 7	0.33356 0.4647 7	- 0.50000 0.6667 3	- 0.50000 0.6667 3	- 0.50000 0.6667 3
Survc22 Survc2	0.55078 0.2574 6	0.55078 0.2574 6	0.28989 0.5774 6	0.51471 0.2961 6	0.39641 0.3786 7	0.39641 0.3786 7	0.12613 0.7876 7	0.19821 0.6701 7	0.37839 0.4026 7	0.34236 0.4523 7	1.00000 7	0.78536 0.0364 7	1.00000 <.0001 3	1.00000 <.0001 3	1.00000 <.0001 3

Table 1.4 Continued

Spearman Correlation Coefficients Prob > |r| under H0: Rho=0 Number of Samples															
	Hatcha2	Surva12	Surva22	Surva42	Hatchb2	Survb12	Survb22	Survb42	Hatchc2	Survc12	Survc22	Survc42	Hatchd2	Survd12	Survd22
Survc42 Survc4	0.51610 0.2946 6	0.51610 0.2946 6	0.27323 0.6004 6	0.46203 0.3563 6	0.40769 0.3639 7	0.40769 0.3639 7	0.03706 0.9371 7	0.22237 0.6317 7	0.48181 0.2736 7	0.33356 0.4647 7	0.78536 0.0364 7	1.00000 7	1.00000 <.0001 3	1.00000 <.0001 3	1.00000 <.0001 3
Hatchd2 Hatchd	1.00000 . 2	1.00000 . 2	1.00000 . 2	1.00000 . 2	0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3	-1.0000 <.0001 3	-0.5000 0.6667 3	1.00000 <.0001 3	1.00000 <.0001 3	1.00000 3	1.00000 <.0001 3	1.00000 <.0001 3
Survd12 Survd1	1.00000 . 2	1.00000 . 2	1.00000 . 2	1.00000 . 2	0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3	-1.0000 <.0001 3	-0.5000 0.6667 3	1.00000 <.0001 3	1.00000 <.0001 3	1.00000 <.0001 3	1.00000 3	1.00000 <.0001 3
Survd22 Survd2	1.00000 . 2	1.00000 . 2	1.00000 . 2	1.00000 . 2	0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3	-1.0000 <.0001 3	- 0.50000 0.6667 3	1.00000 <.0001 3	1.00000 <.0001 3	1.00000 <.0001 3	1.00000 <.0001 3	1.00000 3
Survd42 Survd4	1.00000 . 2	1.00000 . 2	1.00000 . 2	1.00000 . 2	1.00000 <.0001 3	1.00000 <.0001 3	1.00000 <.0001 3	1.00000 <.0001 3	-0.5000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3
AvgLtha2 AvgLtha	0.48571 0.3287 6	0.48571 0.3287 6	0.65714 0.1562 6	0.48571 0.3287 6	0.48571 0.3287 6	0.48571 0.3287 6	0.42857 0.3965 6	0.48571 0.3287 6	0.50000 0.6667 3	0.50000 0.6667 3	1.00000 <.0001 3	0.86603 0.3333 3	. . 0	. . 0	. . 0
AvgDepa2 AvgDepa	0.55078 0.2574 6	0.55078 0.2574 6	0.81168 0.0499 6	0.55078 0.2574 6	0.55078 0.2574 6	0.55078 0.2574 6	0.63775 0.1731 6	0.55078 0.2574 6	0.86603 0.3333 3	0.86603 0.3333 3	0.00000 1.0000 3	-0.5000 0.6667 3	. . 0	. . 0	. . 0
AvgYolka2 AvgYolka	-0.4285 0.3965 6	-0.4285 0.3965 6	-0.2571 0.6228 6	-0.4285 0.3965 6	-0.6000 0.2080 6	-0.6000 0.2080 6	-0.3714 0.4685 6	-0.6000 0.2080 6	-0.5000 0.6667 3	-0.5000 0.6667 3	-1.0000 <.0001 3	-0.8660 0.3333 3	. . 0	. . 0	. . 0

Table 1.4 Continued

Spearman Correlation Coefficients Prob > |r| under H0: Rho=0 Number of Samples															
	Hatcha2	Surva12	Surva22	Surva42	Hatchb2	Survb12	Survb22	Survb42	Hatchc2	Survc12	Survc22	Survc42	Hatchd2	Survd12	Survd22
AvgLthb2 AvgLthb	-0.0857 0.8717 6	-0.0857 0.8717 6	0.14286 0.7872 6	-0.1159 0.8268 6	-0.0285 0.9572 6	-0.0285 0.9572 6	-0.1428 0.7872 6	-0.0857 0.8717 6	0.50000 0.6667 3	0.50000 0.6667 3	1.00000 <.0001 3	0.00000 1.0000 3	. . 1	. . 1	. . 1
AvgDepb2 AvgDepb	0.08571 0.8717 6	0.08571 0.8717 6	0.02857 0.9572 6	0.11595 0.8268 6	0.37143 0.4685 6	0.37143 0.4685 6	0.14286 0.7872 6	0.42857 0.3965 6	0.50000 0.6667 3	0.50000 0.6667 3	1.00000 <.0001 3	0.00000 1.0000 3	. . 1	. . 1	. . 1
AvgYolkb2 AvgYolkb	0.77143 0.0724 6	0.77143 0.0724 6	0.60000 0.2080 6	0.72471 0.1032 6	0.71429 0.1108 6	0.71429 0.1108 6	0.65714 0.1562 6	0.60000 0.2080 6	-1.0000 <.0001 3	-1.0000 <.0001 3	-0.5000 0.6667 3	-0.8660 0.3333 3	. . 1	. . 1	. . 1
AvgLthc2 AvgLthc	1.00000 . 2	1.00000 . 2	1.00000 . 2	1.00000 . 2	0.50000 0.6667 3	0.50000 0.6667 3	-0.5000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3	-0.5000 0.6667 3	0.50000 0.6667 3	1.00000 <.0001 3	1.00000 . 2	1.00000 . 2	1.00000 . 2
AvgDepc2 AvgDepc	1.00000 . 2	1.00000 . 2	1.00000 . 2	1.00000 . 2	0.50000 0.6667 3	0.50000 0.6667 3	-0.5000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3	-0.5000 0.6667 3	0.50000 0.6667 3	1.00000 <.0001 3	1.00000 . 2	1.00000 . 2	1.00000 . 2
AvgYolkc2 AvgYolkc	1.00000 . 2	1.00000 . 2	1.00000 . 2	1.00000 . 2	1.00000 <.0001 3	1.00000 <.0001 3	0.50000 0.6667 3	1.00000 <.0001 3	1.00000 <.0001 3	0.50000 0.6667 3	-0.5000 0.6667 3	0.50000 0.6667 3	- 1.00000 . 2	- 1.00000 . 2	- 1.00000 . 2

Table 1.4 Continued

Spearman Correlation Coefficients Prob > |r| under H0: Rho=0 Number of Samples										
	Survd42	AvgLtha2	AvgDepa2	AvgYolka2	AvgLthb2	AvgDepb2	AvgYolkb2	AvgLthc2	AvgDepc2	AvgYolkc2
AvgFemLTH2 AvgFemLTH	-1.00000 <.0001 3	-0.23191 0.6584 6	-0.41176 0.4173 6	0.11595 0.8268 6	-0.65714 0.1562 6	0.25714 0.6228 6	0.08571 0.8717 6	-0.50000 0.6667 3	-0.50000 0.6667 3	-1.00000 <.0001 3
FemCond2 FemCond	-0.50000 0.6667 3	0.20000 0.7471 5	-0.30779 0.6144 5	-0.30000 0.6238 5	0.10000 0.8729 5	0.50000 0.3910 5	-0.70000 0.1881 5	-1.00000 . 2	-1.00000 . 2	1.00000 . 2
Odiam2 Odiam	0.50000 0.6667 3	0.25714 0.6228 6	0.46382 0.3542 6	0.31429 0.5441 6	-0.25714 0.6228 6	-0.65714 0.1562 6	0.02857 0.9572 6	-0.50000 0.6667 3	-0.50000 0.6667 3	0.50000 0.6667 3
Stage2 Stage	. . 3	0.82808 0.0418 6	0.52511 0.2847 6	-0.62106 0.1882 6	0.39279 0.4411 6	0.13093 0.8047 6	0.13093 0.8047 6	. . 3	. . 3	. . 3
Handevent2 Handevent	0.00000 1.0000 3	0.16903 0.7489 6	0.42875 0.3963 6	0.16903 0.7489 6	-0.30861 0.5518 6	0.00000 1.0000 6	0.77152 0.0723 6	0.00000 1.0000 3	0.00000 1.0000 3	-0.86603 0.3333 3
DayatSpawn2 DayatSpawn	0.00000 1.0000 3	-0.08571 0.8717 6	-0.20292 0.6998 6	0.20000 0.7040 6	0.25714 0.6228 6	-0.42857 0.3965 6	0.31429 0.5441 6	-0.50000 0.6667 3	-0.50000 0.6667 3	-1.00000 <.0001 3
NR2 NR	. . 3	-0.06172 0.9075 6	0.23483 0.6542 6	0.61721 0.1917 6	-0.33947 0.5104 6	-0.46291 0.3552 6	-0.18516 0.7254 6	0.86603 0.3333 3	0.86603 0.3333 3	0.86603 0.3333 3
TotalFec2 TotalFec	1.00000 <.0001 3	0.02857 0.9572 6	0.02899 0.9565 6	0.02857 0.9572 6	-0.82857 0.0416 6	-0.08571 0.8717 6	0.60000 0.2080 6	0.50000 0.6667 3	0.50000 0.6667 3	1.00000 <.0001 3

Table 1.4 Continued

Spearman Correlation Coefficients Prob > |r| under H0: Rho=0 Number of Samples										
	Survd42	AvgLtha2	AvgDepa2	AvgYolka2	AvgLthb2	AvgDepb2	AvgYolkb2	AvgLthc2	AvgDepc2	AvgYolkc2
Lat12 Lat1	-0.86603 0.3333 3	-0.82808 0.0418 6	-0.52511 0.2847 6	0.82808 0.0418 6	-0.41404 0.4144 6	-0.82808 0.0418 6	0.41404 0.4144 6	0.00000 1.0000 3	0.00000 1.0000 3	-0.86603 0.3333 3
Fert12 Fert1	-0.50000 0.6667 3	0.08571 0.8717 6	0.17393 0.7417 6	-0.42857 0.3965 6	-0.14286 0.7872 6	0.20000 0.7040 6	0.77143 0.0724 6	0.50000 0.6667 3	0.50000 0.6667 3	-0.50000 0.6667 3
Fert22 Fert2	0.50000 0.6667 3	0.02857 0.9572 6	0.05798 0.9131 6	-0.42857 0.3965 6	0.37143 0.4685 6	0.25714 0.6228 6	-0.08571 0.8717 6	-0.50000 0.6667 3	-0.50000 0.6667 3	0.50000 0.6667 3
Fert32 Fert3	1.00000 <.0001 3	1.00000 <.0001 3	0.00000 1.0000 3	-1.00000 <.0001 3	0.50000 0.6667 3	0.50000 0.6667 3	-1.00000 <.0001 3	0.50000 0.6667 3	0.50000 0.6667 3	1.00000 <.0001 3
Fert42 Fert4	-0.50000 0.6667 3	. . 1	. . 1	. . 1	1.00000 . 2	1.00000 . 2	-1.00000 . 2	1.00000 . 2	1.00000 . 2	-1.00000 . 2
Hatcha2 Hatcha	1.00000 . 2	0.48571 0.3287 6	0.55078 0.2574 6	-0.42857 0.3965 6	-0.08571 0.8717 6	0.08571 0.8717 6	0.77143 0.0724 6	1.00000 . 2	1.00000 . 2	1.00000 . 2
Surva12 Surva1	1.00000 . 2	0.48571 0.3287 6	0.55078 0.2574 6	-0.42857 0.3965 6	-0.08571 0.8717 6	0.08571 0.8717 6	0.77143 0.0724 6	1.00000 . 2	1.00000 . 2	1.00000 . 2
Surva22 Surva2	1.00000 . 2	0.65714 0.1562 6	0.81168 0.0499 6	-0.25714 0.6228 6	0.14286 0.7872 6	0.02857 0.9572 6	0.60000 0.2080 6	1.00000 . 2	1.00000 . 2	1.00000 . 2

Table 1.4 Continued

Spearman Correlation Coefficients Prob > |r| under H0: Rho=0 Number of Samples										
	Survd42	AvgLtha2	AvgDepa2	AvgYolka2	AvgLthb2	AvgDepb2	AvgYolkb2	AvgLthc2	AvgDepc2	AvgYolkc2
Surva42 Surva4	1.00000 . 2	0.48571 0.3287 6	0.55078 0.2574 6	-0.42857 0.3965 6	-0.11595 0.8268 6	0.11595 0.8268 6	0.72471 0.1032 6	1.00000 . 2	1.00000 . 2	1.00000 . 2
Hatchb2 Hatchb	1.00000 <.0001 3	0.48571 0.3287 6	0.55078 0.2574 6	-0.60000 0.2080 6	-0.02857 0.9572 6	0.37143 0.4685 6	0.71429 0.1108 6	0.50000 0.6667 3	0.50000 0.6667 3	1.00000 <.0001 3
Survb12 Survb1	1.00000 <.0001 3	0.48571 0.3287 6	0.55078 0.2574 6	-0.60000 0.2080 6	-0.02857 0.9572 6	0.37143 0.4685 6	0.71429 0.1108 6	0.50000 0.6667 3	0.50000 0.6667 3	1.00000 <.0001 3
Survb22 Survb2	1.00000 <.0001 3	0.42857 0.3965 6	0.63775 0.1731 6	-0.37143 0.4685 6	-0.14286 0.7872 6	0.14286 0.7872 6	0.65714 0.1562 6	-0.50000 0.6667 3	-0.50000 0.6667 3	0.50000 0.6667 3
Survb42 Survb4	1.00000 <.0001 3	0.48571 0.3287 6	0.55078 0.2574 6	-0.60000 0.2080 6	-0.08571 0.8717 6	0.42857 0.3965 6	0.60000 0.2080 6	0.50000 0.6667 3	0.50000 0.6667 3	1.00000 <.0001 3
Hatchc2 Hatchc	-0.50000 0.6667 3	0.50000 0.6667 3	0.86603 0.3333 3	-0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3	-1.00000 <.0001 3	0.50000 0.6667 3	0.50000 0.6667 3	1.00000 <.0001 3
Survc12 Survc1	0.50000 0.6667 3	0.50000 0.6667 3	0.86603 0.3333 3	-0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3	-1.00000 <.0001 3	-0.50000 0.6667 3	-0.50000 0.6667 3	0.50000 0.6667 3
Survc22 Survc2	0.50000 0.6667 3	1.00000 <.0001 3	0.00000 1.0000 3	-1.00000 <.0001 3	1.00000 <.0001 3	1.00000 <.0001 3	-0.50000 0.6667 3	0.50000 0.6667 3	0.50000 0.6667 3	-0.50000 0.6667 3

Table 1.4 Continued

Spearman Correlation Coefficients Prob > |r| under H0: Rho=0 Number of Samples										
	Survd42	AvgLtha2	AvgDepa2	AvgYolka2	AvgLthb2	AvgDepb2	AvgYolkb2	AvgLthc2	AvgDepc2	AvgYolkc2
Survc42 Survc4	0.50000 0.6667 3	0.86603 0.3333 3	-0.50000 0.6667 3	-0.86603 0.3333 3	0.00000 1.0000 3	0.00000 1.0000 3	-0.86603 0.3333 3	1.00000 <.0001 3	1.00000 <.0001 3	0.50000 0.6667 3
Hatchd2 Hatchd	0.50000 0.6667 3	. . 0	. . 0	. . 0	. . 1	. . 1	. . 1	1.00000 . 2	1.00000 . 2	-1.00000 . 2
Survd12 Survd1	0.50000 0.6667 3	. . 0	. . 0	. . 0	. . 1	. . 1	. . 1	1.00000 . 2	1.00000 . 2	-1.00000 . 2
Survd22 Survd2	0.50000 0.6667 3	. . 0	. . 0	. . 0	. . 1	. . 1	. . 1	1.00000 . 2	1.00000 . 2	-1.00000 . 2
Survd42 Survd4	1.00000 3	. . 0	. . 0	. . 0	. . 1	. . 1	. . 1	-1.00000 . 2	-1.00000 . 2	1.00000 . 2
AvgLtha2 AvgLtha	. . 0	1.00000 6	0.81168 0.0499 6	-0.77143 0.0724 6	0.60000 0.2848 5	0.50000 0.3910 5	-0.20000 0.7471 5	. . 1	. . 1	. . 1
AvgDepa2 AvgDepa	. . 0	0.81168 0.0499 6	1.00000 6	-0.40584 0.4247 6	0.50000 0.3910 5	0.10000 0.8729 5	-0.10000 0.8729 5	. . 1	. . 1	. . 1
AvgYolka2 AvgYolka	. . 0	-0.77143 0.0724 6	-0.40584 0.4247 6	1.00000 6	-0.50000 0.3910 5	-0.70000 0.1881 5	0.20000 0.7471 5	. . 1	. . 1	. . 1

Table 1.4 Continued

Spearman Correlation Coefficients Prob > |r| under H0: Rho=0 Number of Samples										
	Survd42	AvgLtha2	AvgDepa2	AvgYolka2	AvgLthb2	AvgDepb2	AvgYolkb2	AvgLthc2	AvgDepc2	AvgYolkc2
AvgLthb2 AvgLthb	. . 1	0.60000 0.2848 5	0.50000 0.3910 5	-0.50000 0.3910 5	1.00000 6	0.37143 0.4685 6	-0.60000 0.2080 6	. . 1	. . 1	. . 1
AvgDepb2 AvgDepb	. . 1	0.50000 0.3910 5	0.10000 0.8729 5	-0.70000 0.1881 5	0.37143 0.4685 6	1.00000 6	-0.14286 0.7872 6	. . 1	. . 1	. . 1
AvgYolkb2 AvgYolkb	. . 1	-0.20000 0.7471 5	-0.10000 0.8729 5	0.20000 0.7471 5	-0.60000 0.2080 6	-0.14286 0.7872 6	1.00000 6	. . 1	. . 1	. . 1
AvgLthc2 AvgLthc	-1.00000 . 2	. . 1	. . 1	. . 1	. . 1	. . 1	. . 1	1.00000 3	1.00000 <.0001 3	0.50000 0.6667 3
AvgDepc2 AvgDepc	-1.00000 . 2	. . 1	. . 1	. . 1	. . 1	. . 1	. . 1	1.00000 <.0001 3	1.00000 3	0.50000 0.6667 3
AvgYolkc2 AvgYolkc	1.00000 . 2	. . 1	. . 1	. . 1	. . 1	. . 1	. . 1	0.50000 0.6667 3	0.50000 0.6667 3	1.00000 3

Table 1.4 Correlation table of all treatments with multiple variables. Correlation number values include Spearman's Rank Correlation Coefficient (-1.0-1.0), Significance values (Prob > |r| under H0: Rho=0) and Number of Observations.

Appendix C – Spawning Results Experiment #1

Date	Treat-ment	Spawn #	Spawn ID	Spawn Date	Spawn Fert %	Spawn Fecund	Total Fert %	Total Fecund
6.18.19	2	1	A	6.20.19	8.85%	595254	2.92%	1829504
6.18.19	2	1	B	6.21.19	0.06%	1234250		
6.26.19	1	2	A	N/A	0.00%	0	0.00%	0
6.26.19	0	3	A	N/A	0.00%	0	0.00%	0
7.2.19	3	4	A	7.4.19	63.06%	293250	75.70%	817820
7.2.19	3	4	B	7.5.19	83.49%	324570		
7.2.19	3	4	C	7.6.19	81.60%	200000		
7.19.19	4	5	A	7.22.19	37.32%	276000	37.32%	276000
7.26.19	1	7	A	N/A	0.00%	0	0.00%	0
7.26.19	1	8	A	N/A	0.00%	0	0.00%	0
7.31.19	3	9	A	N/A	0.00%	0	0.00%	0
7.31.19	3	10	A	N/A	0.00%	0	0.00%	0
8.09.19	4	11	A	8.11.19	17.55%	393675	21.01%	1016200
8.09.19	4	11	B	8.12.19	23.20%	622525		
8.15.19	4	12	A	8.17.19	72.65%	93600	81.33%	304200
8.15.19	4	12	B	8.18.19	85.19%	210600		
8.15.19	2	13	A	8.18.19	0.00%	207000	0.00%	0
3.15.20	3	14	A	3.19.20	3.89%	50427	61.93%	454047
3.15.20	3	14	B	3.20.20	69.18%	403620		
3.15.20	3	15	A	3.20.20	0.83%	276930	0.83%	276930
5.10.20	0	16	A	N/A	0.00%	0	0.00%	0
5.10.20	2	17	A	5.14.20	0.00%	450340	0.00%	887990
5.10.20	2	17	B	5.15.20	0.00%	437650		
5.16.20	0	18	A	N/A	0.00%	0	0.00%	0
5.16.20	1	19	A	N/A	0.00%	0	0.00%	0
5.21.20	4	20	A	5.24.20	10.41%	431400	34.25%	1575910

Table 1.5 Continued

Date	Treat-ment	Spawn #	Spawn ID	Spawn Date	Spawn Fert %	Spawn Fecund	Total Fert %	Total Fecund
5.21.20	4	20	B	5.24.20	58.09%	1144510		
5.21.20	3	21	A	N/A	0.00%	0	0.00%	0
6.1.20	2	22	A	6.04.20	0.00%	192000	0.00%	466000
6.1.20	2	22	B	6.05.20	0.00%	274000		
6.1.20	4	23	A	6.03.20	59.62%	451830	78.90%	1065590
6.1.20	4	23	B	6.04.20	82.97%	206110		
6.1.20	4	23	C	6.05.20	94.11%	407650		
6.9.20	0	24	A	N/A	0.00%	0	0.00%	0
6.9.20	2	25	A	6.12.20	0.00%	89000	0.00%	89000
6.15.20	0	26	A	N/A	0.00%	0	0.00%	0
6.15.20	1	27	A	N/A	0.00%	0	0.00%	0
6.20.20	3	28	A	6.23.20	0.00%	112054	0.00%	112054
6.20.20	4	29	A	6.23.20	0.00%	143942	0.00%	143942

Table 1.5 Experiment 1 spawning results in chronological order. Treatment 0 (control), Treatment 1 (HCG-only), Treatment 2 (GnRHa-only), Treatment 3 (HCG primed followed by GnRHa/Domperidone), Treatment 4 (GnRHa/Domperidone)

Appendix D – Spawning Results Experiment #2

Date	Treatment	Spawn #	Spawn ID	Spawn Date	Spawn Fert %	Spawn Fecundity	Total Fert %	Total Fecundity
5.03.2021	5	30	A	5.05.2021	0.00%	530000	73.95%	2472600
5.03.2021	5	30	B	5.06.2021	95.04%	1348200		
5.03.2021	5	30	C	5.07.2021	94.37%	413000		
5.03.2021	5	30	D	5.08.2021	86.77%	181400		
5.03.2021	4	31	A	5.05.2021	15.34%	173400	76.96%	1364200
5.03.2021	4	31	B	5.06.2021	83.95%	633440		
5.03.2021	4	31	C	5.07.2021	89.57%	263160		
5.03.2021	4	31	D	5.08.2021	86.95%	294200		
5.10.2021	5	32	A	5.12.2021	83.75%	941400	84.18%	1401800
5.10.2021	5	32	B	5.13.2021	85.06%	460400		
5.10.2021	4	33	A	5.13.2021	0.00%	88800	15.36%	276000
5.10.2021	4	33	B	5.16.2021	22.65%	187200		
5.17.2021	5	34	A	5.19.2021	85.42%	305140	85.11%	1808840
5.17.2021	5	34	B	5.20.2021	81.12%	905000		
5.17.2021	5	34	C	5.21.2021	89.74%	423200		
5.17.2021	5	34	D	5.22.2021	94.02%	175500		
5.17.2021	4	35	A	5.19.2021	31.77%	103860	78.76%	806560
5.17.2021	4	35	B	5.20.2021	79.61%	279500		
5.17.2021	4	35	C	5.21.2021	89.74%	423200		
5.24.2021	5	36	A	5.27.2021	0.00%	90340	0.00%	90340
5.24.2021	4	37	A	5.28.2021	0.00%	88760	0.00%	88760
6.1.2021	5	38	A	6.3.2021	81.85%	373100	55.32%	2590390
6.1.2021	5	38	B	6.4.2021	83.42%	1148300		
6.1.2021	5	38	C	6.5.2021	28.54%	594840		
6.1.2021	5	38	D	6.6.2021	0.00%	474150		
6.15.2021	5	39	A	6.17.2021	93.45%	397000	93.03%	962500
6.15.2021	5	39	B	6.18.2021	92.74%	565500		
6.15.2021	4	40	A	N/A	0.00%	0	0.00%	0

Table 1.6 Experiment 2 spawning results in chronological order. Treatment 4 (GnRHa/Domperidone 5mg/kg) and Treatment 5 (GnRHa/Domperidone 10mg/kg)

Appendix E – Discriminant Function Optimized using the E2B sub-dataset

The discriminant function optimized is defined by the posterior probability of membership in each sex Pr (j|x)

$$\Pr(j|x) = \frac{\exp\left(-\frac{1}{2}D_j^2(x)\right)}{\sum_k \exp\left(-\frac{1}{2}D_j^2(x)\right)}$$

where the generalized squared distance function $D_j^2(x)$ is defined as

$$D_j^2(x) = \left(x - \bar{x_j}\right)' cov_j^{-1}\left(x - \bar{x_j}\right) + \ln(cov_j)$$

x represents the vector of measurements in an individual and cov_j is the covariance matrix within groups (males and females).

Generalized squared distance to sex ($D_j^2(x)$)

From Sex	1	2
11-KT		
1=Male	13.96737	34251
2=Female	14.23091	2.19242
E2B, 11-KT		
	22.203379	39697
	23.64975	12.33212
Weight, E2B, 11-KT		
	26.01242	40572
	28.54711	16.10059

– IRB Approval Letter (Remove if not required)

THE UNIVERSITY OF
SOUTHERN MISSISSIPPI.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

118 College Drive #5116 | Hattiesburg, MS 39406-0001
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NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER:	1710301
PROJECT TITLE:	Development of aquaculture and stock enhancement for marine finfish at the Thad Cochran Marine Aquaculture Center
PROPOSED PROJECT DATES:	11/2017 – 09/2020
PROJECT TYPE:	Modification
PRINCIPAL INVESTIGATOR(S):	Eric Saillant
DEPARTMENT:	Division of Coastal Sciences
FUNDING AGENCY/SPONSOR:	N/A
IACUC COMMITTEE ACTION:	Designated Review Approval
PROTOCOL EXPIRATON DATE:	September 30, 2020

November 3, 2017

Jake Schaefer, PhD
IACUC Chair

Date

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