THE EFFECT OF TEMPERATURE ON GERM CELLS IN JUVENILE WALLEYE (*Sander vitreus*) WITH IMPLICATIONS FOR THE USE OF HEAT TO INDUCE STERILITY

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AUTHORIZATION TO SUBMIT

THESIS

This thesis of Sean M. Wilson, submitted for the degree of Master of Science with a major in zoology and titled "The effect of temperature on germ cells in juvenile walleye (*Sander vitreus*) with implications for the use of heat to induce sterility" has been reviewed in final form. Permission, as indicated by the signatures and dates given below, is now granted to submit final copies to the College of Graduate Studies for approval.

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Abstract

The walleye (*Sander vitreus*) is a North American freshwater fish that has been introduced into waters outside its native range for sport fishing purposes. To prevent undesirable introductions, fish managers are interested in a reliable method of producing sterile walleye for stocking purposes. The overall goal of this project was to evaluate heat as a potential method for inducing sterility in juvenile walleye. This study consisted of three components, which separately examined the effects of age and salinity on temperature tolerance, the effects of heat on germ cells, and the effects of temperature and diet on juvenile walleye sex ratios.

In the first component of this study, the ultimate upper incipient lethal temperature (UUILT) was determined at four salinities (0.2, 2.5, 5.0, and 10.0 ppt) for two age classes of juvenile walleye (sub-yearling and yearling). Sub-yearling walleye (UUILT of 34.1^{O}C) were found to be more heat tolerant than yearlings (UUILT of 31.6^{O}C). Salinity was not found to have a substantial effect on the UUILT of either age class. Therefore, it should be advantageous to perform high temperature related experiments on sub-yearling walleye, using salt only when necessary to control pathogens.

For the second component of the study, two experiments were conducted to determine the effect of heat on germ cells in sub-yearling walleye. For the first experiment, fish ranging from 75 to 100 mm total length (TL) were exposed to a range of temperatures of 24, 27, 30 or 33^{O}C for a period of 28 days, beginning at 88 days post hatch (dph). For the second experiment, fish were exposed to 33^{O}C for 21 days during one of three overlapping developmental intervals. These intervals began when the fish were 54, 68, or 82 dph. The mean TL of each group was 24, 42, and 57 mm, as measured one day prior to elevating the temperature. A control group was maintained at 20 to 22 ^{O}C for

comparison. The mean gonadosomatic index (GSI) was not different between treatments in either experiment, and germ cells were present in all individuals examined. Therefore, heat is not expected to be useful for developing a practical method of sterilizing walleye.

For the third component, sex ratios were analyzed with respect to thermal history and diet. Sex was determined for the fish sampled from the previous experiments to determine the effects of temperature on sex ratios. Sex was also determined for five groups of walleye reared using different diets. One of these groups was fed a commercial diet, Biokyowa FFK series, during the larval and early juvenile life history periods. Two other groups were fed another commercial diet, Lansy CW series, while the remaining groups were fed cultured zooplankton, all during the same developmental interval. When exposed to water temperatures of 33^{O}C beginning at or before 68 dph and 42 mm TL, all fish were subsequently determined to be male. Conversely, temperature was not found to affect sex ratios in fish treated beginning at 82 dph and 57 mm TL. Groups fed Lansy CW diets during the larval and early juvenile periods were found to contain significantly more males than those fed the Biokyowa FFK diet or zooplankton during the same developmental interval. However, further experiments will be needed to draw definitive conclusions on the effect of temperature and diets on juvenile walleye sex ratios.

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Dr. David Smith not only secured the original funding for this project, but provided technical guidance for wetlab design as well. Mike Peterson and Melanie Drennan each served as wetlab manager for a portion of this project, and both played an important role in the successfulness of walleye husbandry during this project. Nathan Jensen and Jorge Lopez also provided valuable assistance in caring for walleye used in the experiments. Dr. Tim Cavileer, Dr. Jerry Bouma and Paul Wheeler all graciously contributed their time in teaching me laboratory techniques. I am deeply indebted to Jerry Tishmack and the U.S. Fish and Wildlife Service for providing eggs for my research.

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Chapter 3

Introduction

The walleye (*Sander vitreus*) is an important freshwater sport fish in North America (Eschmeyer, 1950; Fenton et al., 1996) with good potential for commercial aquaculture (Summerfelt, 1996). The native range of the walleye (Figure 0.1) lies east of the continental divide and west of the Appalachian Mountains, south to the Gulf Coast in Alabama, north to the St. Lawrence River in eastern Canada, and northwest to the Mackenzie River near the Arctic coast (Colby et al., 1979). However, due to its popularity as a sport fish, the walleye has been introduced into waters in every U.S. state with the exceptions of Alaska, Hawaii, and Florida (Fuller et al., 1999). Due to its status as a top-level predator, fish managers are often concerned about new walleye introductions. Walleye introductions have been found to lead to prey depletions and other significant shifts in the composition of fish assemblages in western reservoirs, often to the detriment of pre-existing fisheries (reviewed by McMahon & Bennett, 1996). A reliable method of producing sterile walleye is desired by fish managers to prevent undesirable introductions.

Methods that have been identified as having potential to induce sterility in walleye include triploidization, administration of steroid hormones, and exposure to high early rearing temperatures. Of these, triploidization has received the most attention to date. Protocols have been developed to induce triploidy in walleye (Malison et al., 2001), and in saugeye, a hybrid cross between walleye and sauger (*Sander canadense*) (Garcia-Abiado et al., 2001). However, it has been difficult to achieve 100% triploid production consistently. Furthermore, there is evidence that some triploid fish species may have limited reproductive capacity and may not be truly sterile. For example, semen obtained

from triploid male grass carp (*Ctenopharyngodon idella*) was able to produce a small number of viable offspring when used to fertilize eggs from a diploid female (Van Eenennaam et al., 1990). Although triploid walleye are expected to be sterile (Malison et al., 2001), the effects of triploidy on reproductive potential in adult walleye has not been determined.

Administering steroid hormones (i.e. androgens) has been found to be an effective means of inducing sterility in some fish species, including coho salmon (*Oncorhynchus kisutch*) (Piferrer et al., 1994) and common carp (*Cyprinus carpio*) (Basavaraja et al., 1997). Androgens are perhaps more commonly used to masculinize genotypic females (Donaldson & Hunter, 1982). However, when administered at higher dosages than required for masculinization, they can induce sterility in both sexes (Donaldson & Hunter, 1982). Treatment regimes to induce sterility usually begin with immersion of eggs or larvae prior to exogenous feeding. Immersion treatments are commonly followed by oral administration of the androgen in the food after the onset of exogenous feeding. There are several drawbacks to this approach. First, government approval would be required for the technique to be put into commercial production. Second, a reliable and cost effective method to treat steroid contaminated effluents would have to be developed. Finally, public acceptance of food fish treated with a chemosterilant may be problematic.

Temperature induced apoptosis has been well documented in the male germ cells of scrotal mammals (Hikim et al., 2003). However, the disappearance of germ cells due to high temperatures has not been widely studied in fish. Strüssmann et al. (1998) reported losses of germ cells, ranging from partial to complete, in the pejerrey (*Odontesthes bonariensis*), and another atherinid, (*Patagonina hatcheri*) after exposure to heat. In the

pejerrey, gonads that were completely devoid of germ cells had normal appearing somatic cells. Gonads that were partially devoid of germ cells had "cysts" that contained germ cells in association with a normal complement of somatic cells. These "cysts" were surrounded by larger areas that contained only somatic cells. Similar results have also been reported for the tilapia (*Oreochromis niloticus*) (D'Cotta et al., 2001). In both cases, fish were exposed just prior to, or in the early stages of sex differentiation. In walleye, sex differentiation takes place during the early part of the juvenile life history period, typically when they are between 75 and 100 mm in total length (Malison et al., 1990). The loss of germ cells in fishes differs from that in the mammal in that both sexes are affected. The use of heat to induce sterility could provide a technique that circumvents problems associated with other methods. Furthermore, the effects of elevated temperature on developing germ cells have not been studied in the walleye, or any other percid fishes.

Since temperature has been demonstrated to be the primary environmental factor to influence sex determination in fishes (reviews by Strüssmann & Patiño, 1999; Baroiller et al., 1999), its effect on sex ratios in walleye are of additional interest. The use of temperature to redirect sex determination in walleye, such that monosex populations are generated, could have a number of useful applications. For example, reproduction could be controlled by stocking only males. Conversely, since females are known to grow faster and attain larger sizes, stocking all females could be useful for developing trophy fisheries or increasing productivity in commercial aquaculture (Malison et al., 1998).

The overall objective of this project was to study the effects of temperature on developing germ cells in juvenile walleye. The data from this research was used to

determine the potential of developing a practical method for using elevated temperature to induce sterility in this species. In addition, the potential for using temperature to control sex in this species was evaluated.

Figure 0.1. A map of North America, with the shaded area depicting the native distribution of walleye (*Sander vitreus*) based on Colby et al. (1979).

Chapter 1. The effects of age and salinity on the upper lethal temperature limits for juvenile walleye

Introduction

Numerous factors, both biotic and abiotic, have been shown to effect temperature tolerance in fishes. Life history period is one such biotic factor that is known to have a great effect on temperature tolerance in percids (Hokanson, 1977). Balon (1975) defines the juvenile period in fishes as the developmental interval beginning when the fish is fully differentiated and possesses definitive organs, and ends with maturation of the gametes. Since walleye have a protracted juvenile life history period, typically lasting two to three years, it was initially important to determine the effect age (and therefore size) might have on temperature tolerance within this life history period.

Environmental factors, such as photoperiod (Hoar & Robinson, 1959), metabolites (Watenpaugh et al., 1985) and salinity (Arai et al., 1963) have also been demonstrated to influence temperature tolerance in fishes. In noting an interaction between salinity and temperature tolerance in the blackchin tilapia (*Saratherodon melanotheron*), Stauffer et al. (1984) hypothesized that a reduction of osmotic stress would increase the available energy to combat thermal stress, therefore effectively increasing the range of temperatures a species can tolerate. Furthermore, salt has long been used to alleviate the effects of handling stress in fish (Piper et al., 1982), including walleye (Barton & Zitzow, 1995). Salt has also been used to treat certain diseases in fish (Piper et al., 1982; Summerfelt, 1996). Therefore, it was hypothesized that salt (in the form of NaCl) could be used to increase the upper temperature tolerance of juvenile walleye. Prolonging the

exposure to higher temperatures would be more likely to effectively destroy developing germ cells in the gonads during this life history period.

Researchers have developed a number of methods to investigate temperature tolerance in fishes. The two most prominent methods are the incipient lethal temperature (ILT) and the critical thermal maximum (CTM) (reviewed by Fry, 1971; Becker & Genoway, 1979; Lutterschmidt & Hutchison, 1997). The ILT method (reviewed by Fry, 1971; Lutterschmidt & Hutchison, 1997) involves a sudden transfer of fish from a pre-selected acclimation temperature to an array of baths, each held at a constant temperature, for a prolonged period of time (2 to 7 days). The ILT is defined as the temperature at which half the test fish survive indefinitely, and is often specified according to the acclimation temperature and the duration of the trial. Typically, a series of acclimation temperatures is used that increases to the point at which they no longer have an effect. The "ultimate" upper incipient temperature (UUILT) is the highest temperature to which a species can acclimate.

The CTM method (reviewed by Becker & Genoway, 1979; Lutterschmidt & Hutchison, 1997) involves a rapid heating of the water, also from a pre-selected acclimation temperature. For this method, a rate of heating is chosen that will allow the body temperature of the test fish to equilibrate with minimal lag time as the temperature is increased, yet not allow the fish to adjust physiologically during the course of the experiment. Typical rates range from 0.5 to 1.5 ^{O}C per minute. Typical endpoints used for this method include a loss of equilibration, the onset of muscular spasms, and sometimes death.

A modification of the CTM method, in which the water is heated at a slow rate (less than or equal to 1^{O}C per day), has been used to estimate the UUILT of fishes (Cocking, 1959; Hokanson & Koenst, 1986). This rate allows fish to adjust physiologically as the temperature is increased, and presumably survive at the highest possible temperature (Cocking, 1959; Fry, 1971). Hokanson and Koenst (1986) used this method to revise previous estimates of the UILT for juvenile walleye, arriving at a value for the UUILT of this species that coincided with previous observations under simulated natural conditions.

In order to design experiments to investigate the effects of elevated temperature on germ cells in juvenile walleye, the UUILT for the life history period and age that will be used needed to be established. Although the UUILT has been previously reported for juvenile walleye (i.e. 34.1 ^{O}C), the precise age and developmental state was not provided (Hokanson & Koenst, 1986). The purpose of this component of the overall study was to first establish the UUILT for two different age classes of juvenile walleye, and second determine if an elevated salt concentration could increase the UUILT.

Methods

Experimental animals

Eyed stage embryos were shipped by overnight air freight from Garrison Dam National Fish Hatchery in North Dakota to the University of Idaho. They were incubated at 15 ^{O}C in a single McDonald jar using domestic water that was dechlorinated by passage through a carbon filter. After hatching, larvae were stocked into 1.5 m circular fiberglass tanks and reared in re-circulated water using methods adapted from Summerfelt (1996). The water temperature was approximately 15 ^{O}C at the beginning and gradually increased to 22 ^{O}C during the larval rearing period. Water temperatures for

juveniles ranged from a high of 23 ^{O}C in the summer, to a low of 14 ^{O}C in the winter. Larvae were fed commercially prepared diets, either FFK B series (Biokyowa, Inc., Chesterfield, MO) or Lansy CW (INVE Aquaculture, Inc., Mountain Green, UT), depending on availability. Juveniles were subsequently transitioned to the WG-9206 grower diet (Barrows & Lellis, 1996) while available. Once the WG-9206 was no longer available, fish were switched to a 5/32 Soft Moist salmon diet (Rangen, Inc., Buhl, ID). Sub-yearling walleye were between two and four months old and averaged 57 mm TL at trial 2 and 95 mm TL at trial 3. Yearling walleye were between twelve and sixteen months old and averaged 235 mm total length (TL) at the time of the experiment. The use of experimental animals for this research was approved by the University of Idaho Animal Care and Use Committee.

Experimental apparatus

Trials were conducted using four independent water re-circulation systems. Each system consisted of three 170-liter tanks and a 75-liter sump. A fluidized bed sand filter was used to control nitrogenous waste, a 40 μm mesh screen was used for water clarification, and a 40 W ultraviolet (UV) sterilizer was used to reduce pathogens. Solid waste was collected in 100 μm filter bags placed over the water return to the sump, and siphoned from the tank bottoms as needed. Temperature was controlled with a 1 kW heater and digital controller placed in the sump. Dissolved oxygen levels were maintained by placing airstones in the tanks and sumps, and monitored using a DO meter (YSI model Y55, Yellow Springs Instrument Co., Inc., Yellow Springs, OH) to ensure that the concentration of dissolved oxygen did not drop below 5 parts per million (ppm) (Piper et al., 1982). Other water quality parameters, specifically ammonia, nitrite, pH

and hardness, were monitored on a weekly basis and always found to be within acceptable limits for walleye culture. Overhead fluorescent lights provided a mean light intensity of 75 lux at the water surface. The photoperiod was maintained at 16 hours light and 8 hours dark using mechanical timers.

Experimental design

Prior to the experiment, fish were moved from the rearing tanks to experimental tanks that were within $1^{O}C$ of the rearing water. Each experimental unit was stocked with ten yearling or twenty sub-yearling fish and the salinity was adjusted to 2.5, 5.0, or 10.0 parts per thousand (ppt). Salinity was modified by adding an appropriate amount of NaCl (99.5% NaCl; Morton International, Inc., Chicago, IL) and checked using a salinity meter (YSI model Y30, Yellow Springs Instrument Co., Inc., Yellow Springs, OH). A control group (0.2 ppt) was maintained with no salt added. The set temperature was then increased by $1^{O}C$ each day until 100% mortality was observed. Temperature was recorded at 30-minute intervals using an Optic StowAway thermal recorder (Onset Computer Corporation, Bourne, MA). Mortality for the previous 24 hr period was recorded at 09:00 hours each morning, just prior to adjusting the temperature. During the experimental treatments, sub-yearling walleye were fed Lansy CW 8/12 to satiation, while yearling walleye were fed the salmon diet, also to satiation. Fish were not fed once the water temperature exceeded $30^{O}C$ (Hokanson & Koenst, 1986) to improve water quality as feeding declined at high temperatures.

Two trials were conducted for each age class. Each trial, consisting of one true replicate of each treatment, was considered a temporal replicate for the analysis. Only one trial was conducted using sub-yearling and yearling fish simultaneously. An

additional trial was conducted separately for each of the sub-yearling and yearling age classes.

Analysis

Fry (1971) defines the UUILT as "the highest incipient lethal temperature an organism can attain." An incipient lethal level refers to the threshold between a range that will not affect the life span of an organism and a range that will result in death after a period of time. This is usually reported as a median lethal dose (LD_{50}), which refers to the value that will result in the death of half the population (Fry, 1971). For this analysis, dosage, in terms of temperature, was determined by calculating the mean temperature for each 24-h period with data from the thermal recorder in each tank. This temperature was then matched to the cumulative mortality in that tank for the same period. The untransformed data were fit to the standard four-parameter Hill equation (Motulsky and Christopoulos, 2003) using Prism 4.0 software (GraphPad Software, Inc., San Diego, CA). The equation used follows:

$$C_M = \frac{1}{1 + \left(\frac{TL_{50}^{Hillslope}}{T^{Hilslope}} \right)}$$

Where:

C_M = Cumulative Mortality

T = Mean temperature for the corresponding period

TL_{50} = Temperature at which 50% cumulative mortality occurs

To compare differences in the UUILT at each level of salinity, the data from both trials of a given age class were treated as replicates and fit to the model. Prism 4.0 software was used to perform an *F*-test to determine whether the TL_{50} was significantly

different between treatments. To compare differences in the UUILT between age classes, levels of salinity that were not significantly different were used as replicates and fit to the model for each age class. Differences with $p \leq 0.05$ were considered statistically significant.

Results

Effect of age

The UUILT for sub-yearling walleye (34.1^{O}C) was significantly ($p \leq 0.05$) higher than the UUILT for yearling walleye (31.4^{O}C) (Table 1.1). Leaving out the data from the 10 ppt treatments, in which a disease was prevalent among fish of both age classes in trials 2 and 3, produced a better fit to the model (Fig. 1.1) but did not change the estimated UUILT significantly for either age class. The disease observed at 10 ppt in trials 2 and 3 was not diagnosed, but the affected fish exhibited a dermal necrosis and experienced earlier mortality compared to groups in which the disease was not observed. For the trial in which sub-yearling and yearling fish were compared simultaneously, the UUILT was 3.2^{O}C higher for sub-yearling walleye as opposed to yearling walleye (Table 1.1). Furthermore, mortality of the sub-yearling fish was never more than 10% by 32^{O}C, a temperature above which 100% mortality always occurred in the yearling fish (Fig. 1.1).

Effect of salinity

Estimates of the UUILT among salinity groups for yearling walleye ranged from a low of 32.7^{O}C to a high of 35.1 ^{O}C (Table 1.1). The differences between the UUILT at different levels of salinity in the sub-yearling fish were not significant ($p \leq 0.05$) (Fig.1.2). However, when using the data from trials 2 and 3 as replicates, the data for 10 ppt could not be fit to the model. This was due to the disease that was encountered in

these groups, the effects of which were confounded with salinity at this level. In contrast, estimates of the UUILT among salinity groups for yearling walleye ranged from a low of 31.0 ^{O}C to a high of 31.7^{O}C (Table 1.1). When analyzing the data using trials as replicates, the UUILT for yearling fish at 2.5 ppt was significantly ($p \leq 0.05$) lower than that at the other salinities (Table 1.1). This can be seen when comparing the curves obtained by fitting the data to the Hill equation (Fig. 1.3).

Discussion

Effect of age

Age was found to have a profound effect on temperature tolerance in juvenile walleye during this study. Our data clearly show that sub-yearling fish were more heat tolerant than yearling fish. Hokanson and Koenst (1986) previously reported a UUILT of 34.1^{O}C for juvenile Minnesota walleyes. This value does not differ significantly from our data for sub-yearling walleye derived from North Dakota. The precise age of the fish used by Hokanson and Koenst (1986) was not stated, but they were likely obtained as sub-yearlings for research that began the following January, therefore it is probable that they were in between the ages of the fish used in this study.

This shift in the UUILT between age classes of juvenile walleye may relate to Coutant's (1987) idea of habitat partitioning, based on preference to different water temperatures. In one study, young-of-the-year walleye were found to inhabit shallow near shore waters in typical Minnesota lakes in association with young-of-the-year yellow perch (*Perca flavescens*) (Maloney & Johnson, 1955). Another study found that walleye fingerlings stocked in a centrarchid dominated impoundment in Illinois were not a significant portion of the diet of larger walleye (Santucci & Wahl, 1993), suggesting

that age classes of juvenile walleye were segregated in the study area. A similar segregation of year classes has also been reported for a related species, the Eurasian perch (*Perca fluviatilis*) (Wang & Eckmann, 1994). As Coutant (1987) points out, this spatial segregation would be beneficial for the offspring of a voracious predator, such as the walleye.

While differences in temperature tolerance between life history periods are routinely considered, the effect of age on temperature tolerance within a life history period is seldom evaluated. Our data demonstrates that in species such as the walleye, temperature tolerance can change over the course of a single life history period. The superior heat tolerance of sub-yearling walleye may be a benefit when conducting experiments to determine the effects of temperature on gonadal development in this species.

Effect of salinity

Initially, it was hypothesized that temperature tolerance in the walleye would increase as salinity increased to the point that the fish became isosmotic with their environment, and then decrease thereafter. However, the direct effects of salinity on the UUILT in this study were negligible. The difference in the estimated UUILT was typically just a few tenths of a degree Celsius, and was only significantly different at 2.5 ppt in the yearling fish. Furthermore, a decrease in the UUILT for water that is slightly more saline than the controls is not consistent with our hypothesis. No explanation can currently be offered as to why the UUILT would be lower at 2.5 ppt compared to both higher and lower salinities.

Although salinity may not have had a large direct effect on temperature tolerance in this study, salinity does have the potential for a number of indirect effects. For example,

salt is commonly used to alleviate the effects of handling stress on walleye and other freshwater teleosts (Barton & Zitzow, 1995). Salt may also be used to combat certain diseases, such as columnaris, which is common in cultured walleye. The reduction of stress and disease could indirectly increase the survival of these fish at extreme temperatures. Furthermore, our data suggests that salinities up to 5 ppt are unlikely to have a direct negative effect on temperature tolerance in sub-yearling walleye, the age class that is most commonly cultured at present (Fenton et al., 1996; Summerfelt, 1996). Therefore, while the addition of salt will not increase the temperature that walleye can be exposed to in subsequent studies, its use in treating pathogens may be valuable.

Table 1.1. Ultimate Upper Incipient Lethal Temperatures (UUILT) in degrees Celsius calculated for sub-yearling and yearling walleye exposed to different salinities. The UUILT, along with the corresponding standard error (S.E.), is indicated for each level of salinity within each age class. An overall UUILT, calculated using data from all salinities that were not significantly different, is also provided for each age class.

Salinity	Sub-yearling		Yearling	
	UUILT	S.E.	UUILT	S.E.
Control	34.2	0.151	31.6	0.056
2.5 ppt	34.3	0.106	31.0*	0.010
5.0 ppt	34.6	0.203	31.5	0.023
10.0 ppt	NV	NV	31.7	0.288
Overall	34.1**	0.155	31.6**	0.084

*Indicates a value that is significantly different from the other levels of salinity at $p < 0.05$.

**Indicates values that are significantly different between age classes at $p < 0.05$.

NV indicates that no value is available because the data could not be fit to the model for this level of salinity.

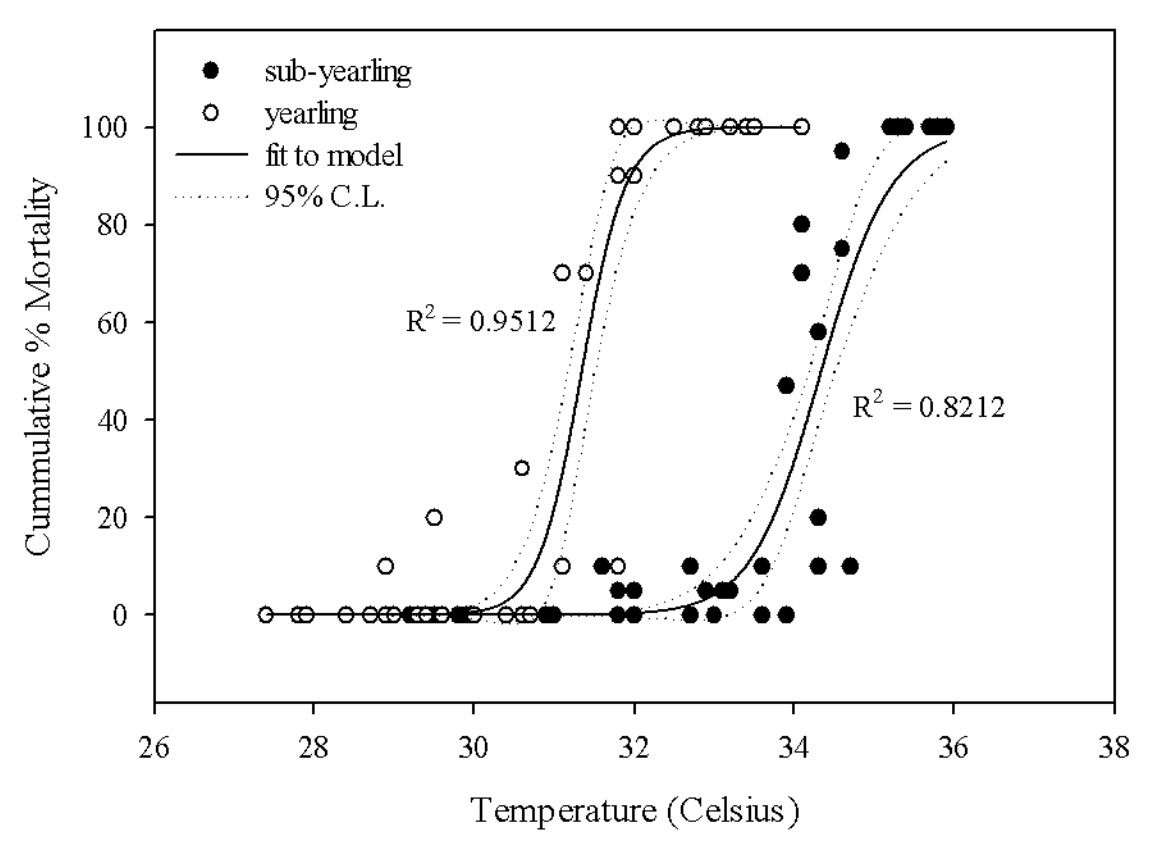

Figure 1.1. The comparative relationship between water temperature and cumulative % mortality in yearling and sub-yearling walleye. The curves shown were produced using a four parameter Hill equation that was fit to the data for each age class as indicated. Data for 10 ppt was excluded due to a disease outbreak at this salinity, however, the inclusion of this data did not change the estimates of the UUILT significantly.

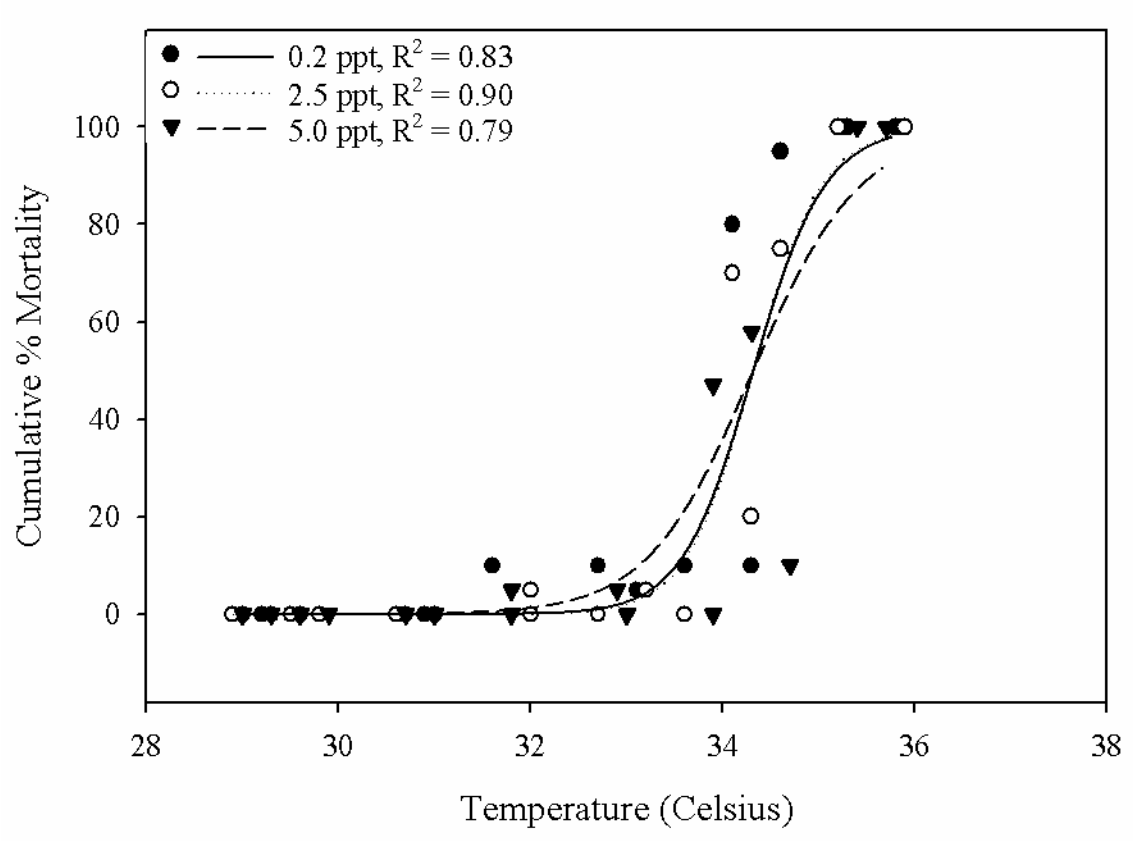

Figure 1.2. The relationship between water temperature and cumulative % mortality in sub-yearling walleye exposed to different salinities. The curves shown were produced using a four parameter Hill equation that was fit to the data from each of the three treatment groups as indicated.

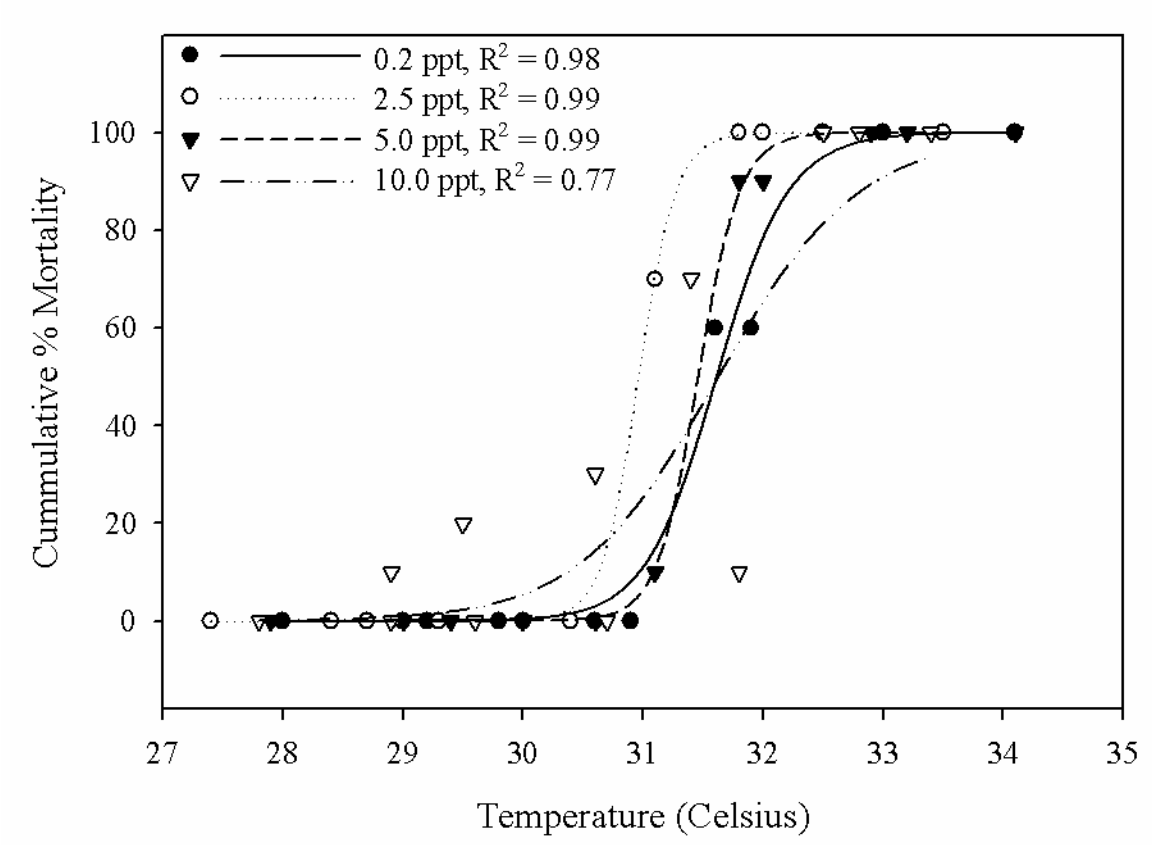

Figure 1.3. The relationship between water temperature and cumulative % mortality in yearling walleye exposed to different salinities. The curves shown were produced using a four parameter Hill equation that was fit to the data from each of the four treatment groups as indicated.

Chapter 2. The effects of temperature on germ cells in juvenile walleye

Introduction

Temperature is the predominant environmental factor known to affect sex differentiation in fishes (reviews by Strüssmann & Patiño, 1999; Baroiller et al., 1999). A number of fish species are now known to exhibit temperature dependent sex determination (TSD). For example, Strüssmann et al. (1997) found that temperature influences the outcome of sex differentiation in the pejerrey (*Odontesthes bonariensis*), in that sex ratios become predominantly male at higher temperatures. However, these authors further noted that extreme high temperatures led to the complete disappearance of the germ cells (i.e. sterility).

Unlike heat induced germ cell loss in scrotal mammals, female and male germ cells appear to be equally sensitive to elevated temperature in fishes (Strüssmann & Patiño, 1999). The temperatures required to cause degeneration of the germ cells are quite high, often close to the upper lethal temperature (Strüssmann et al, 1998; Strüssmann & Patiño, 1999), and degeneration is initiated more rapidly as the temperature increases (Ito et al., 2003). Although germ cell degeneration can be initiated over a range of developmental periods, the sensitivity of germ cells appears to change with developmental stage of the germ cells (Strüssmann et al, 1998; Strüssmann & Patiño, 1999). Strüssmann and Patiño (1999) reported that the ovaries of largemouth bass (*Micropterus salmoides*) contained perinucleolar stage oocytes, but lacked oogonia and oocytes up to the pachytene stage when exposed to elevated temperatures after a period of gonadal development at normal temperatures. Similar results have been reported for the pejerrey (Strüssmann et al,

1998). These authors speculated that the more advanced oocytes had passed an unknown threshold in development before the heat exposures, and had thus become resistant to subsequent elevations in temperature. In the few species that have been examined, temperatures required to induce germ cell loss do not appear to affect the somatic cells in the gonad (Strüssmann et al., 1998; Ito et al., 2003). A practical method of using elevated temperature to induce sterility in juvenile walleye would be useful to fish managers who wish to control reproduction in this species.

In order to test the effects of elevated temperature on developing germ cells in this species, two variables need to be addressed. These variables are the temperature and the timing, in relation to the development of the germ cells, that the temperature is applied. In regards to temperature, it is likely that there is a threshold temperature above which germ cells will be destroyed. Furthermore, it is likely that the temperature sensitivity of germ cells changes with development (Strüssmann et al., 1998). Therefore, any experiments designed to gain an understanding of temperature effects on germ cells must consider these variables. The purpose of this component of the overall study was to determine the effect of temperature on developing germ cells in juvenile walleye during their first year of life. This was accomplished by designing separate experiments to first examine the effect of a range of temperatures over a single time interval, and then to examine the effect of a single temperature over several overlapping time intervals. The data from these experiments were then used to evaluate temperature as a means of inducing sterility during this life history period.

Methods

Experimental animals

Embryos were obtained and reared as described previously (see Chapter 1). For the first experiment, larvae were fed the FFK B series diet (Biokyowa, Inc., Chesterfield, MO) and subsequently transitioned to WG-9206 grower diet (Nelson and Sons, Inc., Murray, UT). For the second experiment, larvae were fed Lansy CW diet (INVE Aquaculture, Inc., Mountain Green, UT), and subsequently transitioned to an extruded salmon diet that is very similar to the WG-9206 diet (Nelson and Sons, Inc., Murray, UT). Different diets were used for the second experiment because the diets used for the first experiment were no longer commercially available. The use of experimental animals for this research was approved by the University of Idaho Animal Care and Use Committee.

Experimental apparatus

The experimental apparatus used was the same as that described in Chapter 1. However, for the critical developmental interval experiment, the number of independent systems was increased from four to eight. This was accomplished by isolating the third tank from each of the original systems and plumbing it as an individual system. For this, identical sumps, sand filters, and mechanical filters were used, along with a similar pump and 25 W UV sterilizer. Only the first tank was used on the original systems, but all systems were otherwise operated in the same manner as before. In addition to the 1 kW heaters used to elevate temperature, a central chiller system was installed to maintain control temperatures during the summer when ambient air temperatures increased. This consisted of using a drop in chiller (Frigid Units, Inc., Toledo, OH) to chill water in a 200

liter barrel. A centrifugal pump was used to circulate the chilled water through individual coils constructed from 6 m of 9.5 mm inside diameter (ID) stainless steel tubing. Flow through each of the coils was regulated with a 12.7 mm ID needle valve.

Critical temperature experiment

The first experiment evaluated the effect of temperature over a single developmental interval. For this experiment, three different treatment groups were exposed to temperatures of 27, 30, or 33 ^{O}C. A control group was held at ambient temperature, which averaged 24 ^{O}C. Each tank was stocked with 30 sub-yearling fish ranging from 75 to 100 mm total length (TL). The temperature was increased in the heat treatments by 1^{O}C each day until the target temperature was reached. The beginning of the warm up period for each group was started so that each treatment reached its target temperature on the same day, when the fish were 88 days post hatch (dph). The target temperatures were held for 28 days, and then decreased by 1^{O}C each day until they had been returned to ambient temperature. Figure 2.1 shows the temperature profiles for this experiment. Fish were subsequently reared for 20 weeks until they reached a TL $\geq$ 150 mm at which point they were euthanized and the gonads sampled.

Critical developmental interval experiment

The second experiment evaluated the effect of a single temperature over three overlapping developmental intervals (Figure 2.2). For this experiment, three treatment groups were exposed to 33 ^{O}C for one of three consecutive 21-day intervals. A control group was not exposed to an elevated temperature and maintained at an ambient temperature ranging from 20 to 22 ^{O}C. Each tank was stocked with an equal mass of sub-yearling fish with a mean TL of 24 mm. The temperature was increased from the control

temperature to 33 ^{O}C over an interval of 7 days. After the treatment interval, temperatures were returned to the control over a period of five days. Temperature profiles for this experiment are shown in Figure 2.3. The mean TL of fish used in each treatment was 24, 42, and 57 mm, as measured one day prior to the warm-up period. The treatment temperature (i.e., 33 ^{O}C) was reached when the fish were 54, 68, or 82 dph. After the experiment, all fish were subsequently reared for 10 weeks until they reached a TL ≥ 100 mm at which point they were euthanized and the gonads sampled.

Analysis

For sampling, fish were euthanized with a lethal dose of MS-222, the TL measured to the nearest mm , and whole body weight (BW) determined to the nearest hundredth of a gram using an electronic balance (model XL-3KD, Denver Instrument Company, Denver, CO.). The gonads were then excised, weighted to the nearest ten thousandth of a gram (GW) using an electronic balance (model A-200DS, Denver Instrument Company, Denver, CO.), and immediately fixed in 10% buffered formalin. After 24 hours, the formalin was drawn off and replaced with 70% ethanol. The gonadosomatic index (GSI) was calculated for each individual fish by the following formula:

$$GSI = 100\frac{GW}{(BW - GW)}$$

Where:

GW = gonad weight

BW = body weight

For the critical temperature experiment, a transformation of the mean GSI of each treatment group was compared using an analysis of variance (ANOVA), with a

significance level of $p \leq 0.05$. For this analysis a new GSI was computed using log(GW), in place of GW as the numerator in the previous equation, to obtain an index with a normal distribution and a homogenous variance. A split plot ANOVA model was then created, using treatments as a whole plot, and sex within the treatments as a split plot (Oehlert, 2000). The same index was compared to analyze the data from the critical developmental interval experiment; however, a complete randomized block design was employed. For this model, the original experimental systems were treated as one block, while the new systems split off each of the old ones was treated as the second block. All statistical analyses were performed using SAS 8.02 software (SAS Institute, Inc., Cray, NC).

For the histological analysis, gonad samples were cleared in xylene, dehydrated through a graded series of ethanol, embedded in paraffin and sectioned at 3-5 μm. Three sagital sections from each fish, separated by 10 μm, were mounted on a glass slide and stained with hematoxylin and eosin for examination via light microscopy. Each fish was then scored for the presence or absence of germ cells observable on the sections.

Results

Determination of the critical temperature

Eight mortalities were encountered at the highest treatment temperature ($33^{O}C$), six of which occurred during the last six days of the exposure. No mortality occurred in any of the other temperature treatment groups during the experiment, however, significant mortalities occurred during the grow-out period in several groups, leading to an imbalance in final sample sizes. When the gonads were examined the mean GSI for male walleye ranged from 0.0264 to 0.0309, while the mean GSI for female walleye ranged

from 0.319 to 0.368 (Table 2.1). The mean GSI did not vary significantly between treatment groups, but females had a significantly ($p \leq 0.05$) higher GSI than males. When histological sections were examined from each treatment group, germ cells were observed in all individuals of both sexes (Figure 2.4). Germ cells in the testes consisted primarily of spermatogonia, while perinucleolar stage oocytes were observed in the ovaries. When viewed under light microscopy, spermatogonia could be recognized by their relatively large size, small volume of cytoplasm in relation to the volume of the nucleus, and well defined cytoplasmic and nuclear boundaries. Perinucleolar stage oocytes were many times larger than the surrounding somatic cells and had large nuclei with multiple nucleoli around the periphery (Patiño & Takashima, 1995). Despite differences in staining intensities between histological sections on slides from different fish, there were no observable pathologies.

Determination of the critical developmental interval

Significant mortalities occurred throughout this experiment, ranging from 26 to 98% among replicates (Table 2.2). The majority of this mortality occurred during the exposure intervals. Some mortality also occurred at the control temperature but declined steadily until the fish were 90 dph, after which mortalities were rare. No significant mortality occurred during the grow-out period following this experiment. The mean GSI for male walleye ranged from 0.0142 to 0.0147 (Table 2.3), and did not vary significantly among treatment groups. The GSI values for females were not analyzed due to a lack of data. Only six females were sampled from this experiment, and no females were sampled from intervals two and three. When histological sections were examined from each

treatment group, germ cells were observed in all individuals (Figure 2.5). Histological sections were similar in appearance to those shown in Fig. 2.4.

Discussion

The data presented here indicate that heat has no practical value for inducing sterility in juvenile walleye. These experiments provided no evidence for negative effects on germ cells in any of the fish that were examined. Furthermore, the treatments used involved temperatures that were very near lethal (34.1 ^{O}C), giving little scope to experiment with higher temperatures. Moreover, these temperatures are already higher than what would be practical for juvenile walleye culture for such a period of time. Even in the laboratory, working with small numbers of fish, it is difficult to keep juvenile walleye alive for very long at 33^{O}C. During the critical developmental interval experiment, survival during the exposure intervals was generally substantially less than survival of the control groups during the same interval. At these temperatures, growth is reduced and fish are more susceptible to diseases, such as columnaris. Therefore, even if an increase in the temperature or duration of the treatment could result in sterility, it still would not be practical to develop a production level method to sterilize juvenile walleye using elevated temperature.

There are two explanations for the lack of an effect of elevated temperature on walleye germ cells. The first is that the germ cells of juvenile walleye are extremely tolerant to heat and are not affected by the sub-lethal temperatures used in this study. Fish in both experiments were subjected to temperatures as high as 33^{O}C, which would be lethal to older juveniles (see Chapter 1), and most likely larvae as well (Hokanson, 1977). If the germ cells in these fish were indeed heat sensitive, one would expect such a high

temperature to lead to their destruction. The second possibility is that some, but not all, of the germ cells were destroyed in one or more of the treatments in either experiment. The remaining germ cells that survived then re-populated the gonad leading to a complete recovery during the interval after the treatment ceased but before sampling. Ito et al. (2003) found that any germ cells remaining in the gonads of pejerrey exposed to an elevated temperature would quickly proliferate once they were returned to a normal temperature. Therefore, all germ cells have to be destroyed for a given treatment to result in permanent sterility.

Table 2.1. Mean gonadosomatic index for groups of juvenile walleye exposed to a range of temperatures for 28 days, beginning 88 dph.

	Males		N	Females		N
Control	0.0309	±0.0127	10	0.323	±0.228	6
27^{O}C	0.0264	±0.0051	19	0.319	±0.029	10
30^{O}C	0.0266	±0.0062	20	0.322	±0.210	5
33^{O}C	0.0293	±0.0135	21	0.368	±0.252	9

Table 2.2. Survival of juvenile walleye exposed to 33^{O}C for 21 days over one of three overlapping developmental periods. Survival over the treatment period includes a warm up period of 7 days prior to reaching 33^{O}C, and a cool down period of 5 days after the exposure period. The survival of the corresponding control group during the same period is provided for comparison. The overall survival encompasses the period after stocking the experimental units and prior to sampling. There is no applicable value for cells with an N/A.

Treatment Group	Block	Treatment % Survival	Control % Survival	Overall % Survival
Control	A	N/A	N/A	48
	B	N/A	N/A	60
Period 1	A	17	57	18
	B	24	69	13
Period 2	A	74	59	50
	B	65	89	33
Period 3	A	2	90	1
	B	51	86	28

Table 2.3. Mean gonadosomatic index for groups of juvenile male walleye exposed to 33^{O}C for 21 days over one of three overlapping time periods. The time periods began at 54, 68, or 82 dph.

	Males		N
Control	0.0143	±0.0114	48
Period 1	0.0142	±0.0020	46
Period 2	0.0147	±0.0114	48
Period 3	0.0146	±0.0146	26

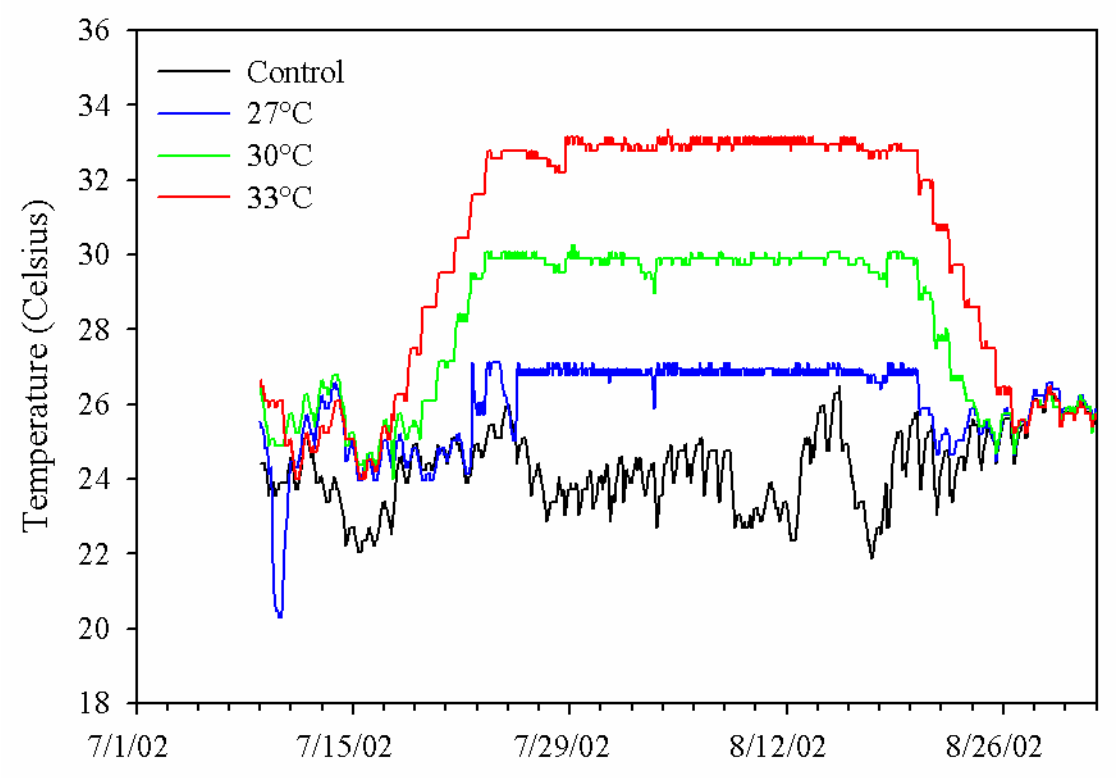

Figure 2.1. Temperature profiles constructed from data loggers placed in individual tanks during the critical temperature experiment.

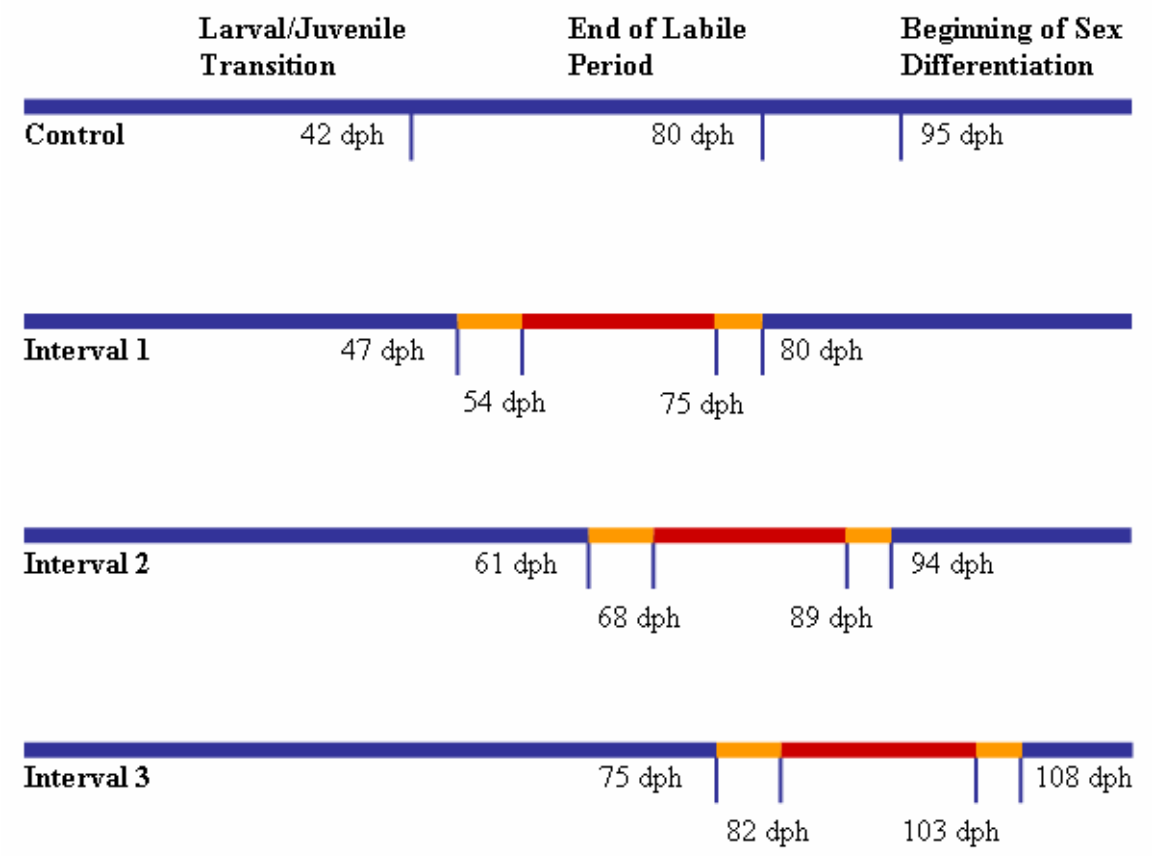

Figure 2.2. Experimental timeline for the critical developmental interval experiment showing the timing (dph) of each exposure interval in relation to one another. Control intervals, during which the temperature was held between 19 and 22^{O}C, are depicted in blue. Treatment intervals, during which the temperature was held at a constant 33^{O}C, are depicted in red. The "warm-up" and "cool-down" periods are depicted in orange. The approximate timing of three key developmental events is shown along the timeline for the control group.

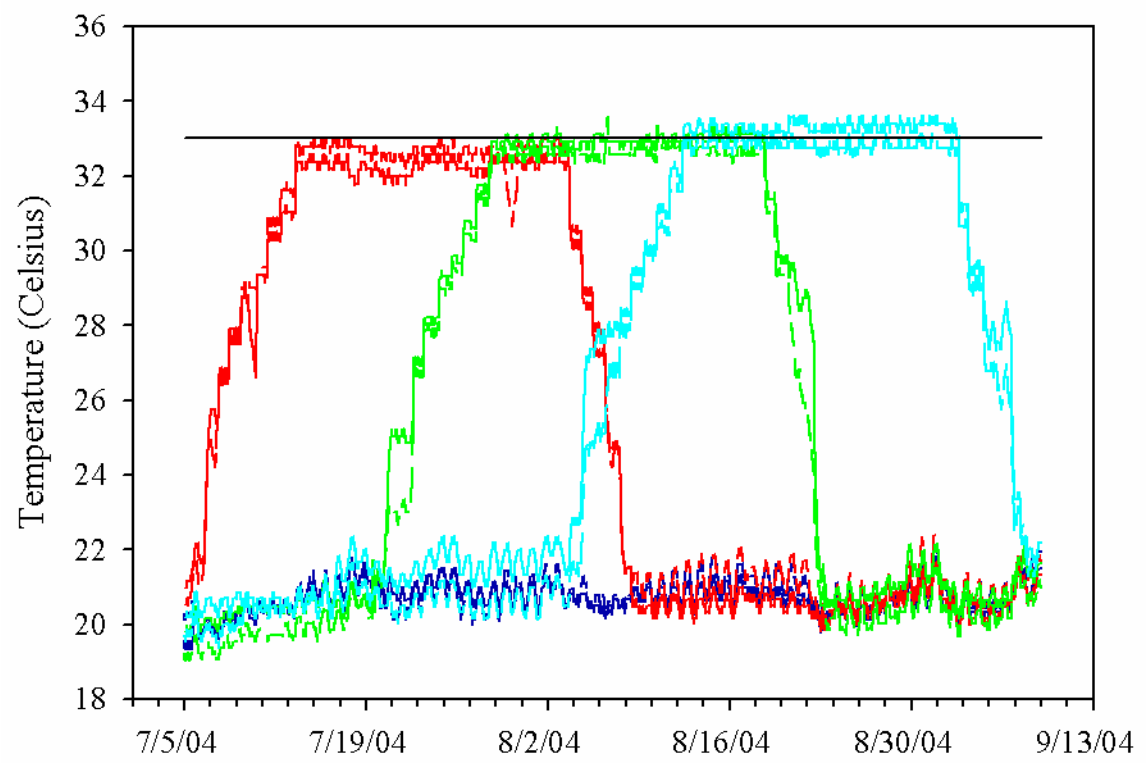

Figure 2.3. Temperature profiles constructed from data loggers placed in individual tanks during the critical developmental interval experiment. The solid black line across the upper portion of the graph represents the target treatment temperature (33^{O}C). The blue lines represent the control tanks, the red lines period 1, the green lines period 2, and the cyan lines period three.

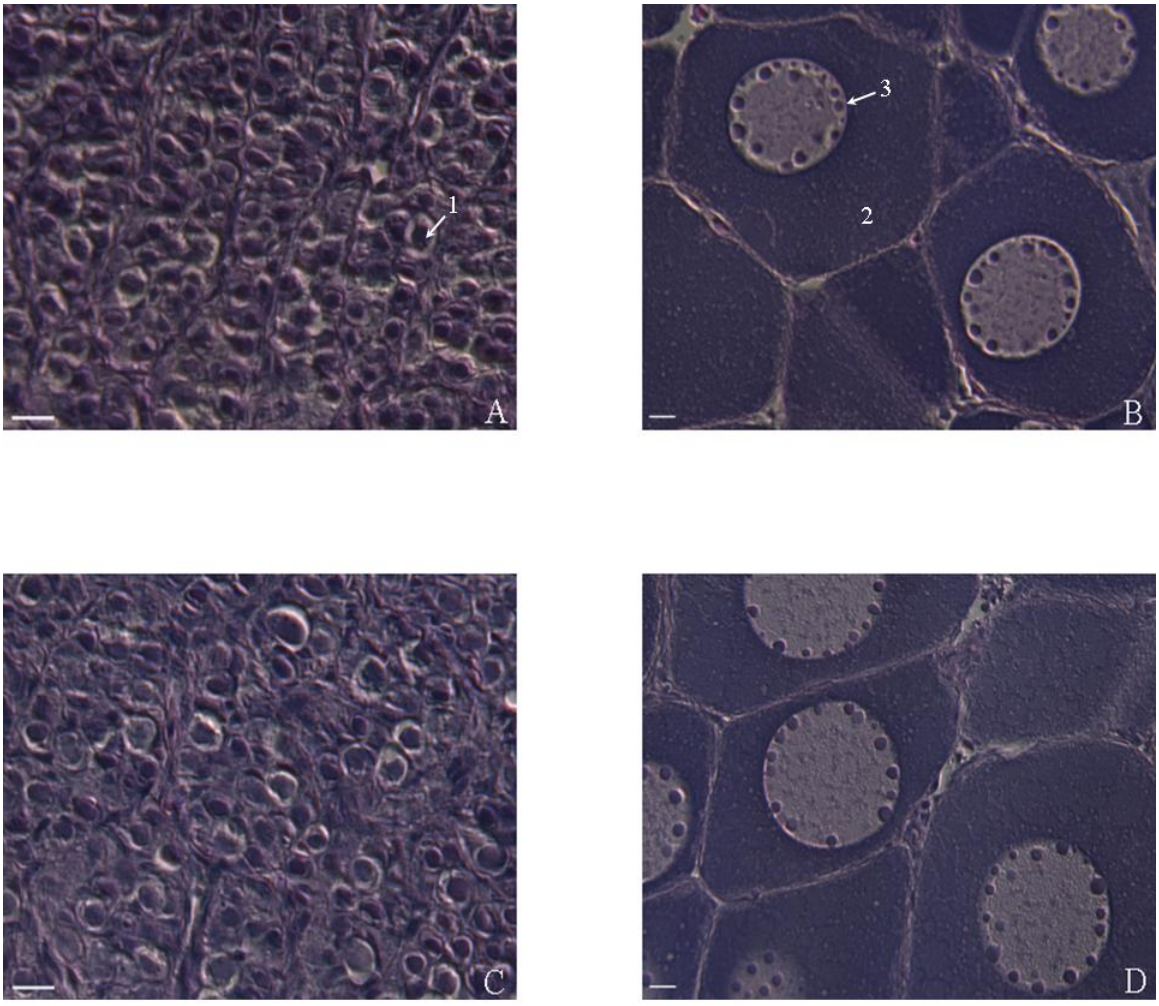

Figure 2.4. Histological sections of the gonads from juvenile male and female walleye in the critical temperature experiment. Samples were taken from the highest temperature treatment group (33^{O}C) and the control group (24 ^{O}C). A - control testis showing spermatogonia (1, arrow). B - control ovary showing perinucleolar stage oocyte (2) and nucleolus (3, arrow). C – treated testis. D – treated ovary. Scale bars represent 0.1 microns.

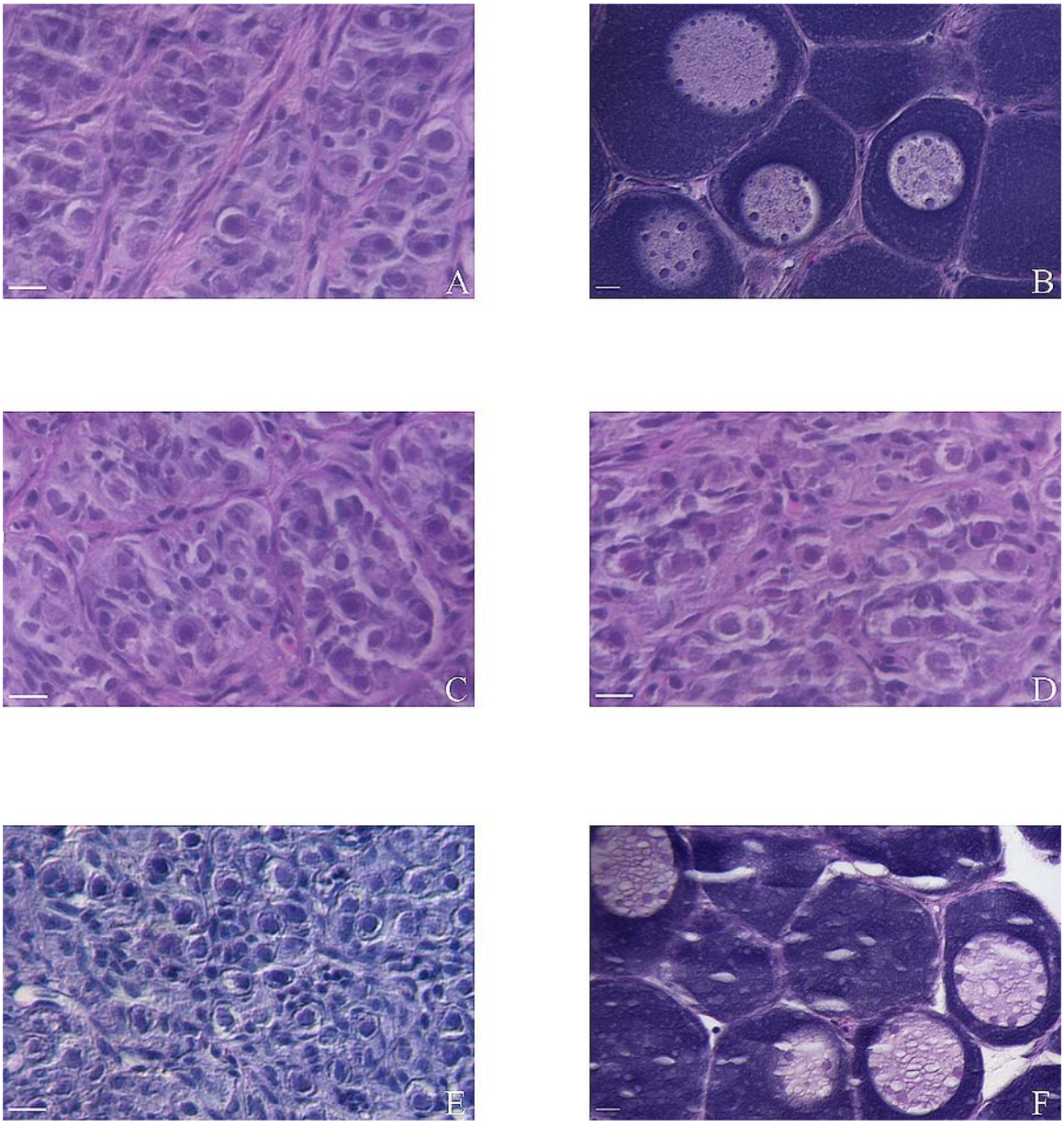

Figure 2.5. Histological sections of the gonads from juvenile male and female walleye in the critical developmental interval experiment. Samples were taken from each treatment, however, no females were encountered in intervals 1 and 2. A - control testis. B - control ovary. C - testis treated at 33^{O}C during the first interval. D - testis treated at 33^{O}C during the second interval. E - testis treated at 33^{O}C during the third interval. F - ovary treated at 33^{O}C during the third interval. Scale bars represent 0.1 microns.

Chapter 3. Sex ratios among groups of juvenile walleye used in this study

Introduction

The walleye is a gonochoristic teleost in which the male is the heterogametic sex, as evidenced by studies involving the indirect use of androgens to create monosex female populations (Malison et al., 1998). With this type of genetic sex determination, random matings should result in a 1:1 sex ratio at fertilization. However, while genotypic sex is fixed at fertilization, evidence of phenotypic sex does not become apparent until the larval or early juvenile period, depending on the species. In the walleye, sex differentiation cannot be influenced by exogenous steroid hormones after the juveniles are approximately 60 mm total length (TL) (Malison et al., 1998). Histological evidence of sex differentiation first becomes apparent when the juveniles are approximately 75 mm TL (Malison et al., 1990). Furthermore, sexual differentiation becomes apparent in the walleye ovary before it does in the presumptive testes (Malison et al., 1990).

Controlling sex in fishes may be useful for both commercial aquaculture and wild fisheries management. For example, in many species of percids, females grow faster, mature later, and reach a larger overall size than males (Craig, 1987; Colby et al., 1979). Therefore, commercial aquaculture operations would benefit from raising all female populations of fish such as the yellow perch (*Perca flavescens*) or walleye (Malison & Garcia-Abiado, 1996). Furthermore, monosex populations may have applications in fisheries management. For example, stocking all female walleye would be advantageous for developing a trophy fishery, since males are unlikely to reach a trophy size (Malison et al., 1998). Additionally, since one male can potentially spawn with multiple females, stocking all females may be useful when supplementing a naturally spawning population

with hatchery fish in an attempt to increase the reproductive potential of that population. Alternatively, monosex populations may be useful in controlling unwanted reproduction. For example, an all male population would not be able to reproduce, and stocking all males would not likely increase the reproductive potential of a naturally reproducing population. Stocking all males could be particularly useful when managing species that tend to overpopulate, such as centrarchids.

Although it may often be desirable to control sex in fishes, unintentional sex reversals can also occur that may be detrimental to a naturally reproducing population. In particular, a variety of estrogenic compounds have the potential to skew sex ratios in the wild (Afonso et al., 2002). While sex ratios that are skewed toward females could potentially increase the reproductive potential of a population, the offspring of feminized genetic males (i.e. XY) should be 75% male. Furthermore, 25% of these broods should be supermales, possessing an YY genotype. Supermales would in turn only sire genotypically male progeny, thus highly skewing the sex ratio in favor of the male and potentially reducing the reproductive potential of the overall population.

In addition to estrogenic compounds, numerous other environmental factors, namely temperature, have been demonstrated to effect sex differentiation in fishes (reviews by Strüssmann & Patiño, 1999; Baroiller et al., 1999). In nature the most notable case is that of the Atlantic silverside (*Menidia menidia*), in which temperature dependent sex determination (TSD) is apparent in certain populations. In southern populations of this species, larvae exposed to cooler temperatures become predominantly female, whereas larvae exposed to warmer temperatures become predominantly male. Therefore, the sex ratios of fish that hatch earlier, when the water is cooler, are skewed towards females,

while those that hatch out later, when the water is warmer, are skewed toward males (Conover and Kynard, 1981). Since body size has a stronger effect on reproductive fitness in females than it does in males, TSD may be an adaptive advantage in these populations (Conover, 1984). However, TSD does not occur naturally in northern populations where the shortened breeding and growing season does not offer a distinct growth advantage, suggesting that this trait is selected for at southern latitudes (Conover & Heins, 1987).

Throughout this study the sex ratios of different walleye year classes appeared to be biased toward males. For example, in the critical developmental interval experiment the lack of females precluded them from the analysis (see Chapter 2). While the treatments themselves may have had an effect on the sex ratio, lower than expected numbers of females in the control groups were also noted. The purpose of this component of the overall study was to first assess the effects of elevated temperature by analyzing the data from the previous experiments with respect to sex ratios, and secondly to survey several groups of walleye to determine if diet (i.e., feed manufacturer) may have influenced the sex ratios.

Methods

Effects of elevated temperature on sex ratios

To assess the effects of temperature, sex ratios were determined for each treatment in both experiments reported in Chapter 2. This data was then organized into two separate contingency tables for analysis. A chi-square analysis was considered inappropriate due to the high proportion of cells with expected values less than 5 for the critical time interval experiment (Zar, 1984). For consistency, data from both experiments were

analyzed using a Fisher's exact test (Zar, 1984), and a p value ≤ 0.05 was considered significant.

An additional analysis was performed to test the possibility that the samples from intervals one and two of the critical time interval experiment contained no females purely by chance. For this analysis, a total of eight samples were collected from the general population used to stock the experiment and compared with the four samples (2 replicates of interval 1 and 2) obtained from the experiment itself. These samples consisted of 24 fish, with the exception of 1 sample from the experiment and 1 from the general population that contained only 22 fish, due to insufficient numbers left in the tanks. This data was then arranged into a 2x2 contingency table, in which samples with and without females were enumerated for each source. This table was then analyzed using a Fisher's exact test (Zar, 1984), with a p-value ≤ 0.05 considered significant. All analyses were performed using SAS 8.02 software (SAS Institute, Inc., Cray, NC).

Effects of diet on sex ratios

Biokyowa FFK series feeds have been the standard diet for larval walleye culture since its development (Summerfelt, 1996). However, these feeds were no longer available for import into the United States after the first year of this study. Lansy CW series feeds were among those being considered as a replacement for the Biokyowa feeds (Alan Johnson, Iowa Department of Natural Resources, personal communication), and therefore were used in subsequent years. To assess the effect of diet, sex ratios were determined for several groups of fish that were reared over the course of the study using Biokyowa, Lansy, or natural feed (i.e. zooplankton) during the larval and early juvenile periods. Of these groups, those fed the commercial diets were reared indoors under

similar conditions. Those fed natural diets were reared outdoors, either in static tanks or earthen ponds, during the larval and early juvenile periods.

Five groups were included in the survey. Group 1 consisted of the fish used in the critical temperature experiment from Chapter 2. These fish were fed Biokyowa FFK B series diet during the larval and early juvenile periods (see Chapter 2). Group 2 consisted of surplus fish from the general population that were reared for, but not used in the UUILT experiment in Chapter 1. These were fed Lansy CW diet during the larval and early juvenile periods (see Chapter 1). Group 3 consisted of surplus fish sampled from the general population that was reared for, but not used in the critical developmental interval experiment (see Chapter 2). These fish were also fed Lansy CW diet during the larval and early juvenile periods. Group 4 was derived from the same group of embryos as Group 2, but was instead raised in static outdoor tanks and fed native zooplankton. These fish were moved indoors and transitioned to commercial diets during the early juvenile period. This group was briefly fed the Lansy CW diet, but was switched to an extruded salmon grower diet (Nelson and Sons, Inc., Murray, UT) about the same time as the previous groups. Group 5 consisted of fish obtained from Montana Fish, Wildlife and Parks. These fish were of the same genetic stock as the fish obtained from Garrison Dam National Fish Hatchery, but were reared in earthen ponds located at Miles City, MT. These fish consumed natural feeds (native zooplankton followed by juvenile suckers) until being moved into indoor tanks and converted to the extruded salmon diet. Once the data was collected, it was organized into a contingency table for analysis. The sex ratios of all groups were then compared using a chi-square analysis (Zar, 1984), which was

performed using SAS 8.02 software (SAS Institute, Inc., Cray, NC), with a p value ≤ 0.05 considered significant.

Determination of sex

Fish were euthanized and the gonads sampled as previously described (see Chapter 2). Fish sampled from Groups 1 and 3 were sampled as sub-yearlings, whereas the other groups were sampled as yearlings. For sub-yearlings, sex was determined by the individual GSI values because the gonads were too small for visual inspection to be used. To justify this, individual GSI values from a subset of 148 fish were compared with their sex determined by examination of histological sections from each fish. The GSI values for females were found to be an order of magnitude higher than that of males in every case (Figure 3.1). Based on this data, all sub-yearling walleye used in this analysis with a GSI value ≥ 0.1 g were considered female, while all those below this value were considered male.

For yearling walleye in Groups 2, 4 and 5, visual inspection of the gonads upon dissection was used to determine the sex. The outward appearance of the gonads from both sexes was similar to that reported by Eschmeyer (1950). Testes were opaque, and typically white in color, although the color varied between some individuals (Figure 3.2). Ovaries, in contrast, were translucent in color (Figure 3.2).

Results

Effects of elevated temperature on sex ratios

The sex ratios from each treatment group within the critical temperature experiment ranged from 20 to 37.5% female and did not vary significantly between treatment groups

(Table 3.1). The overall sex ratio was 30% female, which was significantly different ($p < 0.05$) from 50%.

The sex ratios from each treatment group within the critical timing experiment ranged from 0 to 10.4% female (Table 3.1). No females were observed in treatment intervals 1 and 2 of the critical timing experiment. The percentage of females in these two groups was found to be significantly different ($p<0.05$) from the controls and the stock population they were originally derived from. In addition, the percentage of females in all groups, as well as the stock population was significantly different ($p<0.05$) from 50%.

Effects of diet on sex ratios

Groups raised outdoors and fed natural feeds through the larval and early juvenile life history periods did not differ significantly from the expected ratio of 1:1 (Table 3.2). Groups reared indoors and fed only commercial diets had significantly ($p < 0.05$) fewer than 50% females. Groups that were fed the Lansy CW diet through the larval and early juvenile life history periods had significantly ($p < 0.05$) fewer females than those fed Biokyowa during this same time interval.

Discussion

Effects of elevated temperature on sex ratios

Sex ratios did not differ between treatments in the experiment to determine the critical temperature. However, since the fish used in this experiment were presumably past the labile stage (i.e. > 60 mm TL) at which the gonad in walleye is susceptible to sex alteration (Malison et al., 1998), these treatments would not be expected to influence the sex ratio. Conversely, no females were sampled from periods one and two in the critical developmental interval experiment, which is significantly different from the control

group. These treatments occurred prior to sex differentiation (Malison et al., 1990) at a size for which phenotypic sex is expected to be labile (Malison et al, 1998). Unfortunately, due to the high mortality incurred during this experiment, and the paucity of females in the general population, it is not known whether this difference was due to sex reversal or differential mortality.

While still few, the number of fish species in which temperature is known to influence sex differentiation has increased with the amount of research in this field that has been carried out (reviews by Strüssmann & Patiño, 1999; Baroiller et al., 1999; Devlin & Nagahama, 2002). To date, the most commonly observed pattern of TSD is for males to become more prevalent at high temperatures, whereas females become more prevalent at lower temperatures (Baroiller et al., 1999). Another pattern of TSD in fishes is for one sex to become more prevalent at high temperatures, whereas sex ratios are unbiased at lower temperatures. The data in this study could potentially fit either of these patterns.

In some species, the mechanism of TSD may involve an inhibition of aromatase expression at high temperatures. This is in turn coupled with a decline in the production of estrogens, which are thought to be essential for ovarian development (Kitano et al., 1999; D'Cotta et al., 2001). However, the effect of temperature on other mechanisms involved in sex differentiation cannot be ruled out (Strüssmann & Nakamura, 2002).

If a practical method of using temperature to sex reverse walleye were desired, it would be useful to conduct a similar set of experiments using an all female population, or in conjunction with the development of a sex specific genetic marker. Malison et al. (1998) have already developed all female lines of walleye using sperm obtained from intersex fish that had been treated with 17α-methyltestosterone. By using genetically all

female fish, any males found in a given treatment group would be masculinized genetic females. Alternatively, a reliable sex specific genetic marker could be used to confirm the genotypic sex of each fish, which could be compared with the phenotypic sex.

Effects of diet on sex ratios

Several patterns emerge that suggest that the choice of diet for intensive walleye culture needs to be investigated. First, only groups of fish that were reared outdoors and fed natural feeds (i.e., zooplankton) exhibited a sex ratio that was not different from the expected ratio of 1:1. Unfortunately, these two factors are confounded, since there were no groups reared outdoors on a commercial diet, and vice versa. Second, although sex ratios in all the groups reared indoors were significantly different from 1:1, there was a significant difference in sex ratios between groups based on the diet. The group fed Biokyowa had sex ratios that were much closer to 1:1 than those fed Lansy CW. In fact, females were very rare in groups that were fed Lansy CW. Furthermore, both of these groups were reared indoors under similar culture conditions. Therefore, the feed effect noted in these groups was not confounded with any other variables. The most plausible explanation for this is that the Lansy CW feed contains an unknown androgenic compound. The groups of walleye fed Lansy CW diet exhibited sex ratios similar to those noted in other studies in which juvenile fish have been fed androgens (Donaldson and Hunter, 1982), with the exception that intersex individuals were not observed.

The Biokyowa fry feeds have been the standard larval diet for intensive larval walleye culture since its inception (Summerfelt, 1996). However, these feeds are no longer available for import into the United States, due to regulations to prevent the spread of bovine spongiform encephalopathy. Lansy CW diets, developed for Atlantic cod culture,

have been considered as alternative to Biokyowa, and feed trials have already been conducted (Johnson & Rudacille, 2005). However, these trials have focused on aspects such as survival and growth. In light of the evidence presented here, any effects on reproduction should also be examined as a part of these trials. Therefore, the possible effects of both diet and culture conditions need to be understood before further research is conducted on the reproductive physiology of this species.

Table 3.1. Sex ratios of juvenile walleye exposed to a range of temperatures for 28 days over the same developmental time interval (Critical Temperature Experiment), or to 33^{O}C for 21 days over one of three overlapping developmental intervals (Critical Developmental Interval Experiment). A single asterisk (*) denotes values that are significantly different ($p < 0.05$) from 50%, whereas a double asterisk (**) denotes values that are different from the corresponding controls.

Chapter 2 Experiment		Percent Female	Upper 95% CL	Lower 95% CL	N
Critical Temperature Experiment	Control	37.5	64.5	15.2	16
	27^{O}C	34.5	54.8	17.9	29
	30^{O}C	20.0*	40.1	6.8	25
	33^{O}C	30.0*	49.6	14.7	30
	overall	30.0*	40.1	21.2	100
Critical Timing Experiment	Control	10.4*	22.6	3.5	48
	Period 1	0.0**	7.7	0.0	46
	Period 2	0.0**	7.4	0.0	48
	Period 3	3.9*	19.6	0.1	26
	Stock Tanks	4.7*	8.8	2.2	190

Table 3.2. Sex ratios of juvenile walleye reared either indoors or outdoors, and fed one of three different diets (one of two commercial varieties, or live feeds the fish would encounter naturally) during the larval and early juvenile period. An asterisk (*) denotes values that are significantly different ($p < 0.05$) from 50%. Walleye originated from either Garrison Dam National Fish Hatchery (GDNFH) or Miles City, MT (MCMT), however, both locations use the same genetic stock.

Group	Brood Year	Origin	Rearing	Diet	Percent Female	Upper 95% CL	Lower 95% CL	N
1	2002	GDNFH	Indoors	Biokyowa	30.0*	40.1	21.2	100
2	2003	GDNFH	Indoors	Lansy	0.0*	21.8	0.0	15
3	2004	GDNFH	Indoors	Lansy	4.7*	8.8	2.2	190
4	2003	GDNFH	Outdoors	Natural	31.6	56.6	12.6	19
5	2003	MCMT	Outdoors	Natural	43.3	63.3	25.1	30

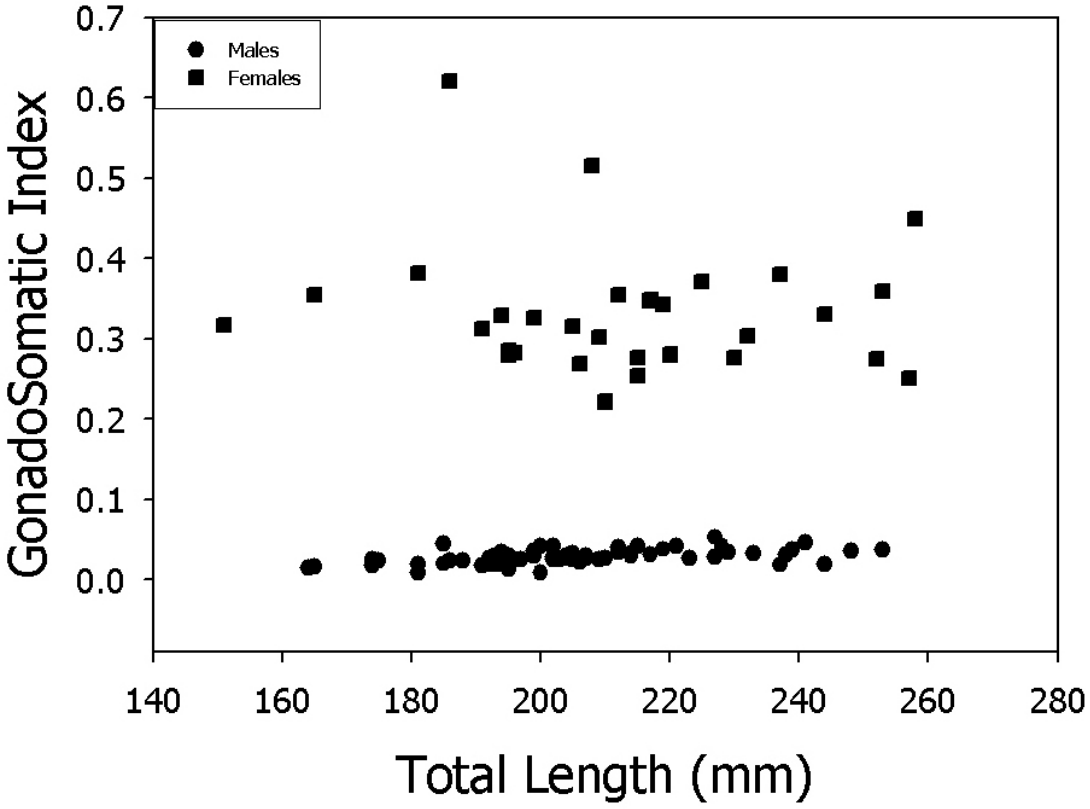

Figure 3.1. The relationship between sex and gonadosomatic index in sub-yearling walleye, demonstrating the difference in magnitude of GSI values between males and females.

Figure 3.2. Yearling walleye with ventral incisions exposing the gonads showing the testis in a male (A, arrow) and the ovary in a female (B, arrow) .

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