EFFECTS OF TEMPERATURE, PHOTOPERIOD, AND SUBSTRATE ON THE MATURATION AND REPRODUCTIVE BEHAVIOR OF THE TOPEKA SHINER (*NOTROPIS TOPEKA*)

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by

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The undersigned, appointed by the dean of the Graduate School,
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OF THE TOPEKA SHINER (*NOTROPIS TOPEKA*)

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ABSTRACT

The Topeka shiner (*Notropis topeka*) is a federally-listed endangered cyprinid species native to small headwater prairie streams in the mid-west of the United States. It generally spawns over and around sunfish (*Lepomis* sp.) nests. The objectives of the present studies were to assess the effects that temperature, photoperiod, and substrate size have on the Topeka shiner's maturation and reproductive behavior.

A laboratory experiment demonstrated that maintenance under a longer photoperiod yielded greater male GSIs and female final weights and GSIs than the shorter photoperiod. The effects of temperature on weight and GSI varied, but the results generally indicated that 31 °C exceeded the optimum. Ovarian histology indicated that spawning had likely occurred in all treatments by the end of the experiment. The frequency of occurrence of reproductive behaviors did not differ between the temperature/photoperiod treatments, but was greater in the morning and decreased through the day. The details of these findings can be applied to propagation efforts and to studies of the species in its natural environment.

Substrate utilization by Topeka shiners in the absence of sunfish was tested to determine the shiner's fundamental preference. Fine substrates were chosen over Small Gravel, Large Gravel, Small Cobble, and the Bare Floor of the experimental tank. This preference may influence which sunfish nests are utilized, given that nest substrate characteristics differ both between sunfish species and within species across spawning site locations.

Life History of the Topeka Shiner

The goals of this research were to determine (i) how long-term exposures to different temperature and photoperiod regimes affect the reproductive maturation and behavior of the Topeka shiner (*Notropis topeka*), and (ii) whether substrate size is a factor in determining where males establish their spawning territories. This thesis was written in manuscript format to facilitate publication. I begin with a review of the species' ecology; this provides an ecological context within which my work can be considered.

Description

The Topeka shiner (*Notropis topeka;* Figure 1) is a rather slab-sided minnow with approximately 35 lateral line scales (Eddy & Underhill 1974). Adults reach a maximum total length of 75 mm (Harlan & Speaker 1987). Both males and females exhibit a dusky, dark stripe that extends along the midline from the tip of the snout to the end of the caudal peduncle. The stripe ends with a distinct wedge-shaped spot at the base of the caudal fin. The Topeka shiner's scales have prominent dark edges, especially those on the dorsal half of the body. Breeding males develop tubercles over much of their bodies, with their heads and especially the fins developing an orange hue (Pflieger 1997). The mouth is small and terminal (Cross & Collins 1995).

Distribution

The historic range of the Topeka shiner (Figure 2) extended south from southern Minnesota and South Dakota, through Nebraska, Iowa, Missouri, and

Kansas (Starrett 1950; Cleary 1953; Minckley & Cross 1959; Fisher 1962; Beckman & Elrod 1971). By comparing historical records with more recent collections, it is estimated that the species' range has contracted by about 80 %, with the remnant populations being highly fragmented (Tabor 1998).

Currently, the Topeka shiner is widely distributed in Minnesota throughout the Big Sioux and Rock river watersheds (Anderson et al. 1977; Dahle 2001; Ceas & Anderson 2004).

The Topeka shiner persists in South Dakota in highly fragmented populations (Blausey 2001) in the James, Vermillion, and Big Sioux river drainages (Bailey & Allum 1962; Tabor 1993; Braaten & Berry 1997).

Nebraska's historical populations were thought to be extirpated as of 1942, but two specimens were collected in 1989 from the Loup River drainage (Michl & Peters 1993).

Iowa has populations of Topeka shiners in the upper Des Moines and Raccoon river drainages; others are scattered in the upper northwest and east-central parts of the state (Harlan & Speaker 1987).

In Missouri, the species has been found over the past two decades in the Chariton, Grand, Lamine, and Des Moines river drainages, as well as the Bonne Femme and Moniteau Creek watersheds (Gelwicks & Bruenderman 1996; Hrabik 1996; Bonneau 1998). According to Gelwicks and Bruenderman (1996), only the Moniteau Creek population remains stable.

Once widely distributed throughout Kansas, Topeka shiners are now found only in small streams of the Kansas and Neosho river drainages located

within the Flint Hills, and in a few other streams in the state (Kerns & Leon 1982; Cross & Collins 1995; Eberle et al. 1997).

Nomenclature and Systematics

The Topeka shiner was first described by Girard in 1856 (Leidy et al. 1856), and was then given the name *Moniana tristis* (Mayden & Gilbert 1989). This collection was not designated as a holotype, and remained unacknowledged. Gilbert (1884) later named the species *Cliola topeka*. In 1978, Gilbert determined that this genus designation was incorrect, and that the more appropriate name should be *Notropis topeka* (Gilbert 1978). Years later, the Girard collections were positively identified as being the same species, after which Mayden and Gilbert (1989) argued that the specific name for the Topeka shiner should be *Notropis tristis*, given that this was earliest recorded description of the species. However, the suggested name change was declined by the International Commission on Zoological Nomenclature (Anonymous 1995).

Appearance-wise, Cross and Collins (1995) state that the Topeka shiner most resembles the sand shiner (*Notropis stramineus*), although the sand shiner lacks the dusky stripe along its sides and does not develop orange fins during the spawning season. A genetic analysis comparing the relatedness of Topeka shiners to seven other cyprinids also revealed that it was most closely related to the sand shiner (Schmidt & Gold 1995).

Further DNA analyses reveal that the vast majority (70 %) of the Topeka shiner's genetic variation is divided between three regional groups occurring in (i) the Kansas and lower Missouri river drainages, (ii) the Arkansas River drainage, and (iii) the Des Moines and upper Missouri river drainages (Michels 2000). The fact that so much of the genetic variation occurs between and not within these groups suggests that these metapopulations are reproductively isolated from one another. Changing environmental conditions or disease may pose a greater threat to populations with minimal genetic diversity because the fish may lack the variability to adapt or respond immunologically (Lacy 1987).

Decline and Status

The specific causes of the decline of the Topeka shiner are uncertain, but two general reasons have been proposed (Dahle & Hatch 2002). The first reason is that the species' required habitat has become less abundant, due mainly to human-induced changes in the landscape within the drainage basins of the streams they inhabit, resulting in degradation of water quality and increased siltation of the substrate (Pflieger 1997; Hatch 2001; Kerns & Bonneau 2002; Bayless et al. 2003). The second reason is that the species has suffered increased predation and competition from introduced species (Layher 1993; Mammoliti 1995). Winston (2002) found in Missouri that shrinkage of theTopeka shiner's range coincided with increases in the presence of a possible predator

(largemouth bass; *Micropterus salmoides*), of a potential competitor for food (blackstripe topminnow; *Fundulus notatus*), and of the bluegill sunfish (*Lepomis macrochirus*). Guy and Whiles (1999) found that largemouth bass and orangespotted sunfish (*Lepomis humilis*) were found in lesser numbers when Topeka shiners were present, and that green sunfish (*Lepomis cyanellus*) were more common at sites where Topeka shiners were present. Knight and Gido (2005) found that Topeka shiners were not more susceptible to predation from largemouth bass relative to several other minnow species, but noted that the presence of the predator greatly affected Topeka shiner behavior by forcing individuals into less suitable habitat.

The South Dakota State Management plan (Shearer 2003) lists the Topeka shiner's status:

> "The Topeka shiner is state-endangered in Missouri and Nebraska. Kansas and Iowa list the species as state-threatened, and Minnesota listed the Topeka shiner as a species of concern. The shiner is not state-threatened or endangered in South Dakota...A recent downgrade in the Topeka shiner's state rank from S2 (imperiled) to S3 (vulnerable) reflects new knowledge regarding distribution and abundance in South Dakota. The global rank of the Topeka shiner is G3 (vulnerable)"

The Nature Conservancy manages the Flint Hills Tallgrass Prairie Preserve (Butler and Greenwood counties, Kansas), on which the Topeka shiner is known to exist (Anonymous 2008). No management practices are specifically directed towards Topeka shiner populations there, but the goal is to maintain a functional tallgrass prairie ecosystem, which should benefit present populations.

Reintroduction of the Topeka shiner is planned for the Nature Conservancy's Dunn Ranch and Pawnee Prairie Preserve (Harrison County, Missouri).

Habitat

The Topeka shiner typically occurs in the headwaters of small, low-gradient prairie streams; these often cease flowing during the dry season, leaving only pools of water for the fish to occupy (Cross & Collins 1995). New water is usually supplied to these pools either by springs or by percolation through the gravel (Pflieger 1997; Berg et al. 2004). Kerns and Bonneau (2002) noted that juvenile Topeka shiners living in the evaporating pools of intermittent streams were one of the last fish species to survive. This tolerance of poor water quality, as Koehle (2006) found, may allow the species to out-survive competitors or predators having lesser tolerances (Mammoliti 2002).

Within these streams, Topeka shiners inhabit the slower moving waters of pools, runs, and off-channel oxbow and closed-basin ponds (Meek 1894; Hatch 2000; Kuitunen 2001). Blausey's (2001) physical habitat model predicts that the most suitable habitat for the Topeka shiner is runs, although pools contain suitable habitat conditions as well. Off-channel habitats (natural or man-made via channel re-routing or the abandonment of gravel pits) are important areas for this species in the north of its range (Clark 2000; Dahle 2001).

Clark (2000) suggests that the fish inhabiting suitable oxbows may

serve as local source populations within individual watersheds, at least in the Des Moines Lobe in Iowa. This may also apply to populations in Minnesota (Dahle 2001).

Tabor (1998) mentions that Topeka shiners seek the margins of flowing water during the summer, and often find refuge around debris or overhanging vegetation in the winter. Adams et al. (2000) found that Topeka shiners, especially mature adults, were able to sustain swimming at relatively high speeds for a prolonged amount of time; this may help prevent them from being washed out of certain locations or to recolonize the headwater streams which they evidently prefer.

In Missouri, a study of stream fish communities in Boone County, Missouri, found that the pools created by beaver dams served as important refuges for many species, including the Topeka shiner, and resulted in Topeka shiner densities that were higher than in the stream proper (Hanson & Campbell 1963). In Missouri, Topeka shiners have been found in stream locations that support higher species diversity and taxa richness than do comparable nearby sites; these findings have led to the Topeka shiner being considered a species indicative of high quality habitat (Gelwicks & Bruenderman 1996). In Minnesota, however, off-channel habitats support higher numbers of Topeka shiners but lower species richness than do nearby instream habitats (Dahle 2001). These contradictory outcomes, and the species' wide geographical range, suggest that Topeka shiners may be able to persist in a wide range of conditions and habitats.

This notion is supported by Kerns and Bonneau (2002), who found that juvenile Topeka shiners were the last fish to die in evaporating stream pools.

Wall et al. (2004) conducted a gap analysis in eastern South Dakota to investigate whether the presence of Topeka shiners was associated with particular environmental attributes. They determined that the probability of Topeka shiners being present was greater in locations having relatively high groundwater levels, low channel slopes, small streams, and where adjacent wetlands on uncultivated land were fewer.

Reproduction

In Missouri, Topeka shiners have been observed spawning from late May to mid-July (Pflieger 1997), and fish in breeding coloration have been observed until as late as August in Kansas (Kerns & Bonneau 2002).

Topeka shiners are reported to spawn at sites near the nests of spawning sunfish (*Lepomis* sp.). However, Katula (1998) was able to spawn Topeka shiners in captivity in the absence of sunfish while providing a substrate patch resembling a sunfish nest, suggesting that the nest may be the attraction, and not the sunfish. Further supporting this idea, Miller (1964) noted that *Hybopsis micropogon* nests seemed to be the object that attracted spawning aggregations of *Notropis cornutus*, and not the nest host.

If Topeka shiners can spawn in the absence of sunfish and/or their nests, then they must likely gain some benefit from electing to spawn near sunfish and/or their nests in natural settings. There are two proposed benefits.

First, male sunfish keep their eggs aerated and their nest substrates free of silt by constantly fanning the area with their fins: for spawned sunfish eggs to survive, they need sufficient oxygen and cannot become covered/coated with sediment. Topeka shiners that spawn over sunfish nests could benefit from the sunfish supplying oxygen to their eggs and keeping silt away: low oxygen levels and siltation are likely a significant concern for Topeka shiners, given that they spawn in pool habitats in small streams during the summer where stagnant conditions could occur. Second, territorial sunfish are larger than are Topeka shiners, and may provide better protection against egg predators than can the smaller minnows. For a sunfish male, providing protection to the added shiner eggs may be less costly than trying to keep the shiners away from its nest, leading to tolerance of the latter. The additional eggs deposited by the shiners may also benefit the sunfish by decreasing the probability of a predator feeding on the sunfish's own eggs (i.e., "the dilution effect"; see Alcock 2001).

Topeka shiner males establish and protect small territories on the periphery of sunfish nests (Pflieger 1997). Kerns and Bonneau (2002) described a territorial shiner male's behavior as swimming "continuously in circular and figure-eight patterns covering an area of less than 0.5 m in diameter". This behavior likely functions to deter other Topeka shiner males, but may also be a display to attract willing females. Katula (1998) has the only detailed account of spawning behavior in captivity. His description follows.

> "The male would swim alongside of the female, head to head, and vibrate. Several eggs would then fall to the substrate well below the midwater spawning Topekas. A female would repeat this process two to four times, and once disinterested in spawning,

> would resume swimming with the other females and non-spawning males. While spawning other species of minnows, I have often seen several males spawning with a single female, even in territorial species, but this was never evident in my observations of Topekas. Male Topekas were very adamant about spawning alone."

Katula's account differs slightly from Stark's (2002) as regards the orientation of the male to the female. Stark noted that, in a natural setting, the male would enter the territory, with the female, being

> "slightly behind and below the female. Near the center of the territory, the female slowed briefly and shuddered slightly as she released eggs. Simultaneously, the male rose at an angle, such that his head was slightly higher and his caudal fin slightly lower than those of the female in a vertical plane, but their anal fins were approximately parallel."

Pflieger (1997) supports Stark's description of the relative spawning positions of the male and female. In a study of congeners, Miller (1964) suggested that by being beneath a female, a spawning *Notropis cornutus* male could obtain a view of the female's distended abdomen, providing visual sexual identification. This orientation may also allow the male to detect the female's ovarian pheromones as a gauge of spawning readiness (Stacey et al. 2003)

Dahle (2001) inspected Topeka shiner ovaries and, using the ova developmental stages described by Heins and Rabito (1986), found that Topeka shiners are able to produce multiple clutches of offspring during a single spawning season. Topeka shiner ovaries contained eggs at several stages of development, which is common for *Notropis* species. Heins and Rabito's (1986) study of *Notropis leedsi* showed that having eggs at multiple stages of development allowed females to increase their overall reproductive effort,

in contrast to developing all their eggs at once. Because the breeding season of *N. leedsi* is extended, as is the case for many cyprinids, it is likely advantageous to be able to spawn multiple times when suitable conditions arise, in contrast to carrying and maintaining a full reproductive season's worth of mature eggs.

Gonadosomatic index values of Topeka shiners collected from May 16 to August 6 in 1998 by Dahle (2001) showed that 20 % of age 1, 86 % of age 2, and 100 % of age 3 males were mature. Females appeared to mature earlier in life, with 52 % of age 1, 93 % of age 2, and 100 % of age 3 individuals being gravid. Clutch sizes ranged from 157 to 839, with the average ova counts for each age class being 351 for age 1, 559 for age 2, and 478 for age 3 fish. Kerns and Bonneau (2002) found similar results, with 19 % of age 1, 80 % of age 2, 100 % of age 3 males, and 62 % of age 1, and 100 % of age 2 females being mature. Ova counts ranged from 140 to 1712, with the largest females producing the most eggs. The age 1 and age 2 females' average ova counts were 356 and 819, respectively (Kerns & Bonneau 2002).

Age and Growth

Dahle (2001) found that of the fish collected, 72 % were age 1 or younger, 26 % were age 2, and only 2 % of the fish reached an age of 3 years. Their respective back-calculated standard lengths at age were 29.3, 41.1, and 46.5 mm for females, and 29.6, 46.3, and 55.6 mm for males. Regarding population demographics, Kerns and Bonneau (2002) found that 90 % were age 1 year or younger, 9.8 % were age 1-2, and 0.2 % were greater than age 2.

Dahle (2001) found that this substantial decline in the numbers of older fish occurred just before young-of-the-year recruitment, suggesting the occurrence of post-spawning adult mortality due to the stress of spawning.

Species Associates

In Willow Creek, northwestern Kansas, Stark et al. (2002) reported collecting Topeka shiners together with the species listed in Table 1. Topeka shiners were observed near the nests of fathead minnows (*Pimephales promelas*) feeding on their eggs, and also near the nests of green and orangespotted sunfish where Topeka shiner males established territories and fed on the sunfish's eggs when the sunfish temporarily vacated their nests. Sunfish eggs could therefore serve as an additional source of food during the spawning season. Topeka shiners were also found feeding together in aggregations of central stonerollers (*Campostoma anomalum*), fathead minnows, orangethroat darters (*Etheostoma spectabile*), and sunfish that were less than 4 cm in length (Stark et al. 2002). Bayless et al.(2003) lists the species collected with Topeka shiners (Table 1) at sampling sites from the Moniteau Creek watershed (Cole, Cooper, and Moniteau counties, Missouri), but did not identify which species shared the specific habitat units that Topeka shiners occupied.

Winston (2002) compared population trends for the Topeka shiner to those of other species collected from the same areas. Of the species that were commonly captured at sites where Topeka shiners had been collected over

approximately 60 years, six other species besides the Topeka shiner also severely declined: black bullhead (*Ameiurus melas*), river carpsucker (*Carpiodes carpio*), plains minnow (*Hybognathus placitus*), orangespotted sunfish, suckermouth minnow (*Phenacobius mirabilis*), and fathead minnow. In contrast, four other species had markedly expanded their ranges: blackstripe topminnow (*Fundulus notatus*), bluegill sunfish, largemouth bass, and creek chub (*Semotilus atromaculatus*). Schrank et al. (2001) also found in Topeka shiner streams that the average number of largemouth bass was high in pools where Topeka shiners were absent. These largemouth bass were thought to have entered the studied streams from nearby impoundments; consequently, Topeka shiner numbers were also negatively related to the number of impoundments within each drainage.

Diet

While snorkeling in streams, Kerns and Bonneau (2002) observed Topeka shiners feeding and found them to be diurnal feeders, spending most of the day in the lower half of the water column feeding off of the substrate. The gut contents of Topeka shiner adults revealed a diet consisting primarily of insects (mostly chironomid larvae) and microcrustaceans, together with significant amounts of “miscellaneous” material (sand, silt, algae, and detritus). Hatch and Besaw (2001) concluded that Topeka shiners must be considered omnivorous, eating insects, microcrustacea, worms, fish larvae, filamentous green algae, vascular plant matter, and detritus. Chironomids were found most

consistently in the gut analyses of Hatch and Besaw (1998; 2001); however, when estimated, the volume of flowering plant seeds was at least twice the volume of the chironomids. Stark et al. (1999) noted that Topeka shiners can be opportunistic feeders, given that they consume food items suspended in the water column by other benthic feeding fish species, and because they raid the nests of other fish species to feed on their eggs.

Diseases / Parasites

Little has been published about the diseases or parasites that affect Topeka shiners. Koehle (2006) reported that some of the fish used in her laboratory experiments had been infected with an Asian tapeworm (*Bothriocephalus acheilognathi*). These infected fish were acquired from a stock maintained at the University of Kansas, where it is suspected this parasite was introduced from grass carp (*Ctenopharyngodon idella*) held in rearing ponds that were then occupied by the Topeka shiners. This tapeworm uses copepods as a secondary host, and these were the probable vectors by which the shiners were exposed. Koehle's (2006) results showed that tapeworm infection reduced growth rate and survival.

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Figure 1: Two male Topeka shiners in nuptial coloration engaged in territorial behavior while a female observes from above (aquarium heater in background).

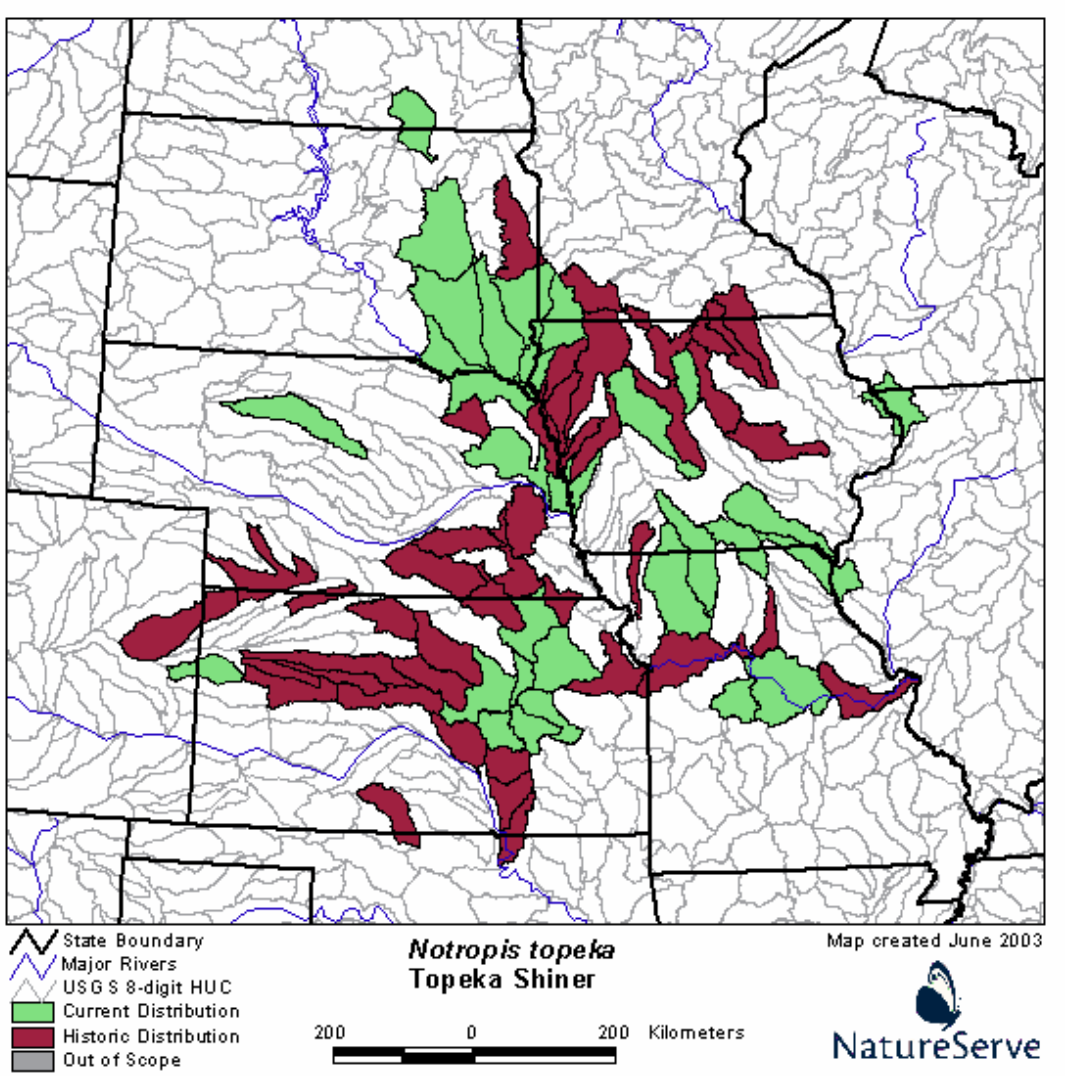

Figure 2: Current (in green) and historical (in red) range of the Topeka shiner (NatureServe 2007).

Table 1: Alphabetic listing of species collected with Topeka shiners.

Species	Common Name	Stark (2002)	Bayless et al. (2003)
Ameiurus melas	black bullhead	X	X
Ameiurus natalis	yellow bullhead		X
Campostoma anomalum	central stoneroller	X	X
Catostomus commersoni	white sucker		X
Cyprinella lutrensis	red shiner	X	X
Etheostoma nigrum	johnny darter		X
Etheostoma spectabile	orangethroat darter	X	X
Fundulus notatus	blackstripe topminnow		X
Fundulus zebrinus	plains killifish	X	
Gambusia affinis	western mosquitofish	X	X
Lepomis cyanellus	green sunfish	X	X
Lepomis humilis	orangespotted sunfish	X	
Lepomis macrochirus	bluegill sunfish	X	X
Lepomis megalotis	longear sunfish		X
Luxilus cornutus	bleeding shiner		X
Lythrurus umbratilis	redfin shiner		X
Micropterus punctulatus	spotted bass		X
Micropterus salmoides	largemouth bass	X	X
Moxostoma erythrurum	golden redhorse		X
Notropis rubellus	rosyface shiner		X
Percina caprodes	logperch		X
Pimephales notatus	bluntnose minnow		X
Pimephales promelas	fathead minnow	X	
Semotilus atromaculatus	creek chub	X	X

Effects of Temperature and Photoperiod

on the Reproductive Maturation and Behavior

of the Topeka Shiner (*Notropis topeka*)

ABSTRACT

The Topeka shiner (*Notropis topeka*) is a federally-listed endangered cyprinid species native to small headwater prairie streams in the middle and upper mid-west of the United States. Little is known about their environmental requirements for spawning. Four temperatures (22, 25, 28, and 31 °C) and two photoperiods (12 and 15 hours of light/day) were tested to evaluate the effects of temperature and photoperiod on growth (length, weight) and reproductive development (GSI). The results of a two-month initial study showed that male GSIs, and that female final average weights and GSIs, were significantly greater in the 15 h photoperiod treatment. Although the effects of temperature varied depending on which growth or reproductive attribute was assessed, the results generally indicated that 31 °C exceeded the temperature optimum. After excluding the least effective treatment combinations (22 °C/12 h, 28 °C/12 h, 31 °C /12 h, and 31 °C/15 h), a follow-up study found average female GSI to be greatest in the 22 °C/15 h treatment. The ovarian histology of females from the second experiment revealed that empty follicles were present in those from each of the four tested treatments, evidence that spawning had occurred. The frequency of occurrence of reproductive behaviors by males and females did not differ between treatments in the second experiment. Activity was greater in the morning and decreased through the day. Overall, these results demonstrate how prolonged exposure to different environmental conditions affect growth and

reproductive development in Topeka shiners. These findings can be used to guide laboratory propagation procedures, and to further our understanding of the environmental cues that influence the timing of Topeka shiner reproduction in the wild.

INTRODUCTION

The environmental factors affecting gonadal maturation in fish vary with species, but in temperate climates the two most prevalent factors are temperature and photoperiod (Billard et al. 1981; Lam 1983). For temperate fishes, the highly dependable circannual photoperiod cycle (the seasonally varying amount of light exposure in a 24-h period) generally regulates such long-term processes as gametogenesis (the formation of sex cells). In contrast, shorter-term processes (such as gamete final maturation) tend to be initiated after water temperatures reach an acceptable level, especially for species which produce relatively small eggs that can mature rapidly (Billard et al. 1981). That photoperiod affects gonadal development in temperate fish species has been well established (e.g., Harrington 1950; Egami 1973; de Vlaming 1975; de Vlaming & Paquette 1977; Lam 1983; Jafri 1989).

When assessing gonadal maturation in fishes, one of the more common approaches is to measure gonadosomatic index (GSI), i.e., ratio of the weight of the gonads to the somatic weight of the fish, expressed as a percentage. Alternatively, one can obtain histological samples of the gonads and examine gametic development via microscopy. Finally (although a less commonly used approach), one can quantify reproductive behavior, given that fish nearing spawning readiness typically increase the exhibition of certain breeding

behaviors. The performance of reproductive behaviors is thus a good indicator that an individual has responded physiologically to the prevailing environmental cues and has undergone the gonadal maturation necessary to spawn (Baggerman 1980).

For the present study, we examine an endangered temperate freshwater fish species and, using the preceding three approaches for quantifying reproductive maturity, test for differences stemming from exposure over time to different temperature and photoperiod regime combinations. We also measure growth as an additional indicator of their well-being.

The species selected for this study is the Topeka shiner (*Notropis topeka*), a federally endangered headwater cyprinid species living in the north-central plains region of the United States. Its current range is about 20 % of its historic range (Tabor 1998), a drastic change given that the species was only recently given broad recognition (Gilbert 1978; Mayden & Gilbert 1989) after languishing in taxonomic obscurity following it's initial description (Leidy et al. 1856; Girard 1857) and subsequent re-description (Gilbert 1884).

Topeka shiners tend to be found in pools, backwaters, and other habitats having low water velocity (Hatch 2000; Kuitunen 2001). However, little is known about the environmental conditions under which Topeka shiners spawn, except that spawning occurs throughout the late spring and summer months (Pflieger 1997) when water temperatures can be quite variable. What constitutes the optimal temperature and photoperiod for breeding remains untested.

In this paper, we use GSI, gonadal histology, and reproductive behavior to assess the reproductive maturity of Topeka shiners exposed over time to different temperature and photoperiod regime combinations; we also measure growth as an additional indicator of the fish's well being. Our rationale for exposing Topeka shiners to several thermal conditions was that the species has been reported to spawn at a range of temperatures and over an extended period of time (several months) (Pflieger 1997; Katula 1998; Hatch 2001). Our objective in testing the two photoperiods we selected was to use values comparable to those experienced in nature just prior to and during the spawning season, allowing us to determine the approximate optimal photoperiod for maximizing gonadal growth in captivity. This differs from the approach taken in most previous studies of this issue in other species (see partial listing above) where two vastly different photoperiods were considered, one representing day lengths approximating summer (=spawning) conditions, and the other approximating winter (non-spawning) conditions.

Ultimately, knowledge of how and at what levels these environmental factors influence reproduction in Topeka shiners can be used to direct culturing/propagation procedures, to better understand the environmental cues that influence reproductive timing in the wild, and to predict the height of the spawning season in natural environments. For an endangered species, such information is especially applicable to the evaluation of conservation approaches and alternatives.

METHODS AND MATERIALS

Pre-Experiment Fish Rearing

Our fish were acquired as juveniles from the Missouri Department of Conservation's Lost Valley Fish Hatchery (Warsaw, Missouri) in December of 2003. Prior to the experiments (described below), the fish were reared indoors until adulthood (2+ years) at the U.S. Geological Survey's (USGS) Columbia Environmental Research Center (CERC).

During this time, the fish were cultured in three circular 900-L tanks, under a 8L:16D (hours of light: hours of dark) photoperiod. Tank water quality was maintained via continuous renewal from an on-site well that delivered potable non-chlorinated groundwater (yearly temperature range 16-19 °C). While in these holding tanks, males developed breeding coloration during the summer (at the upper limits of the annual water temperature range), but gravid females were never observed. Consequently, the fish were unlikely to have spawned prior to our experiments. During this time, fish were fed commercial flake food (Prime Tropical Flake Mixed; Ziegler Bros. Inc., Gardners, Pennsylvania) once daily until satiation was reached.

Fish Preparation

Two months preceding the start of each of the two experiments, those fish selected for use (healthy, sexable) were moved into a 720-L Living Stream system (Frigid Units, Toledo, Ohio) for exposure to a simulated “winter”. Males and females were confined to separate halves of the Living Stream by a plastic mesh barrier. Water quality was maintained therein via continuous renewal with groundwater from the on-site well. Continuous water circulation allowed any chemical cues critical to reproduction to traverse the plastic mesh barrier. This simulated “winter” exposure was performed in case Topeka shiners need to experience an annual temperature cycle to trigger spawning.

To achieve winter conditions, the Living Stream water temperatures were lowered from 17 °C to 10 °C at a rate of 1 °C every two days by means of a 3000-Watt water heater/chiller (Frigid Units, Toledo, Ohio). Photoperiod was simultaneously altered from 12L:12D to 8L:16D in half-hour decrements via a timer connected to the laboratory’s light switch. After reaching 10 °C and the 8L:16D photoperiod, the fish were held under these conditions for one month. Thereafter, the fish were warmed slowly (1 °C every two days) to 22 °C (the lowest treatment temperature; see below), accompanied by a simultaneous increment in photoperiod back to 12L:12D (half-hour increase in day length every two days). Fish were fed the commercial flake flood until satiation was reached.

Experimental Apparatus

Experiments were conducted at USGS-CERC in 24 glass aquaria (75-L; 61.0 cm wide X 30.5 cm deep X 40.6 cm high; Beldt's Aquarium, Hazelwood, Missouri). These were allocated to three side-by-side aquarium racks, each of which held eight aquaria. The aquaria were arranged four across on two shelves, and were individually enclosed in isolation cells that prohibited the influx of light and movement stimuli from other aquaria and the surrounding area. Barber (1986) determined that the water depth in optimum Topeka shiner habitat was 20-40 cm; consequently, we filled our test aquaria to depths of 34 cm.

To assure water quality, a water distribution system constantly replaced the water in each tank with well water at a rate of approximately 1.2 tank volumes/day. In-tank aquarium heaters warmed this to the temperatures specified for each tank (see below). Continuous aeration was supplied to each tank using two airstones (positioned on the bottom at opposite ends of the tank) attached by latex hoses to a pressurized airline.

Each tank's photoperiod and temperature regime were regulated individually by means of three programmable logic controllers (Direct Logic 205; Automation Direct, Cumming, Georgia), one for each aquarium rack.
Light for each individual tank was provided by two 61-cm, 20-Watt fluorescent lights ("cool white"; Osram Sylvania Products Inc., Versailles, Kentucky).

The water in each tank was warmed by a pair of 300-Watt submersible in-tank aquarium heaters (Visi-therm Deluxe; Marineland,

Moorpark, California) that, at maximum output, could maintain the highest experimental tank temperature (see below) without reducing the tank water exchange rate. Each tank's target water temperature was programmed into its respective programmable logic controller, which then maintained this temperature by turning on/off the aquarium heater as needed based on feedback from a continuously-monitoring 100-Ohm resistance temperature detector (Omega Engineering, Inc., Stamford Connecticut).

Each tank was equipped with a false floor. For the first experiment (see below), the floor insert was constructed of 3.2-mm thick opaque black plexiglass (Figure 1). To provide some semblance of a pebbled nest over which the fish could spawn, we took a rectangular glass dish, overlayed it with heavy-duty plastic mesh perforated with 6 mm square holes (U.S. Netting, Erie, Pennsylvania), and then attached bits of clean small gravel (longest axis average being approximately 3 cm) to the netting using indoor/outdoor silicone caulking (GE Sealants and Adhesives, Huntersville, North Carolina). A 19 X 14-cm hole cut into the center of the plexiglass floor panel at the tank end furthest away from the surveillance camera allowed the dish to be set into the false floor. The mesh size of the netting was small enough to prevent fish from accessing the collection dish interior, but large enough to allow eggs (average diameter 0.835 mm; Dahle 2001) to pass through and accumulate.

Because spawners never made use of these nests, an alternative design was used in the second experiment (Figure 2). Here, the floor insert was made entirely of the heavy-duty plastic mesh material we used previously (sans gravel),

and was positioned approximately 5 cm above the tank bottom, beneath which we placed a sheet of transparent plastic (3M, St. Paul, Minnesota). The purpose of this arrangement was to allow any spawned eggs to fall through the false floor and onto the plastic sheets, preventing potential conspecific egg consumption allowing spawnings to be detected/confirmed.

Both the glass dishes from the first experiment and the plastic sheets from the second experiment were replaced every third day and inspected for eggs using a magnifying glass. The tank walls were cleaned using an aquarium glass scraper every second time the plastic sheets were replaced (i.e., every sixth day).

Experiments

Two separate experiments were conducted, the second a refinement of the first.

Experiment 1: This test involved exposing fish to eight treatments (four temperatures; two photoperiods). Each temperature/photoperiod treatment was replicated in three randomly chosen aquaria from within the rack matrix, yielding 24 experimental units. This study began on August 10 and ended on October 5 of 2005.

The four temperatures we tested were 22, 25, 28, and 31 °C; these span the range of temperatures across which Topeka shiners have been reported to spawn in the wild (Hatch 2001).

Photoperiods of 12 and 15 hours of light exposure per day were chosen

to represent day lengths approximating the beginning and middle of the reported Topeka shiner spawning season in nature (late May to mid-July; Pflieger 1997), with fish in breeding coloration being observed until as late as August in Kansas (Kerns & Bonneau 2002). In other words, the shorter photoperiod (12L:12D) represented the approximate amount of daylight that Topeka shiners would be exposed to just prior to the spawning season, whereas the longer photoperiod (15L:9D) represented day lengths typical of the middle of the spawning season.

Each tank in this experiment was provided with one male in breeding coloration and seven females. A single male was used to avoid aggressive behavior between males that might detract from reproductive activity. In addition, in the only published account of Topeka shiners spawning in an aquarium, Katula (1998) suggested that males preferred to spawn in the absence of other males. More females than males were used to help assure a sufficient operational sex ratio for spawning, and to accommodate between-female differences in spawning preparedness. To stock each tank, “over-wintered” individuals from the Living Stream tank were randomly allocated to the experimental tanks until each contained the requisite number. At the time of stocking, all the tank water temperatures were 22 °C and the photoperiods 12L:12D. After stocking, the programmable logic controllers were set to increase temperature and photoperiod over seven days by 0.86 °C and 26 min each day until a 6 °C and 3 hr increment was attained. The fish were then allowed an additional seven days to acclimate to these new conditions before the experiment began.

Experimental fish were fed to satiation three times per day throughout this and the subsequent experiment using the commercial flake food listed above. This food has proven to be nutritionally adequate for Topeka shiners to reproduce (James Candrl, pers. comm.). The first feeding of the day occurred at 0730 h, 1 h after the lights turned on. The majority of feeding occurred within 5 min of providing the food; the amount of food they ate was carefully monitored to prevent over-feeding and water fouling. The second feeding occurred at 1230 h, which separated the "Morning" and "Mid-day" periods of the day (see below). The third feeding occurred at 1730 h, an hour before the end of the day for aquaria exposed to the short photoperiod (12L:12D) treatment.

Experiment 2: This experiment was intended to further test the most effective treatment combinations from the first experiment (see above). In addition, we used surveillance cameras and time-lapse video recording to record the fish's breeding behavior. This second experiment began on September 25 and ended on November 23, 2006. These fish came from the same stock as the fish used in the first experiment, and therefore were one year older.

Based on analysis of the GSI data obtained from our initial study (see Results), we determined that the 31 °C temperature and 12L:12D photoperiod treatments were least effective at eliciting reproductive maturation. Consequently, we eliminated these treatments. This left three temperatures (22, 25, and 28 °C) and the 15L:9D photoperiod, to which we added a

25 °C/12L:12D) treatment, the initial results from which were ambiguous. With four temperature/photoperiod combinations in all, we were able to increase replication in this experiment to six tanks per treatment, thereby using all 24 available aquaria. However, since this experiment was not a complete block design, the data were eventually analyzed using a single-factor (treatment) ANOVA, compared to the first experiment's two-factor (temperature and photoperiod) ANOVA.

The second experiment also differed from the first in that we increased the number of males in each aquarium to two, and decreased the number of females from seven to six. This change was made because observations during the first experiment suggested that male reproductive behavior in the first experiment was lacking with a single male per tank.

Fish Measurements

For both experiments, fish being stocked into the individual experiment tanks were anaesthetized (Tricaine-S™ MS222; Western Chemical Inc., Ferndale, Washington) so that their total lengths (TL; nearest mm) could be measured without causing undue stress or inadvertent injury. The total weight (TW; nearest 0.0001 g) of each fish was measured by gently placing it in a tared 250-ml beaker partially filled with water (AT261 Delta Range electronic balance; Mettler-Toledo, Columbus, Ohio). Additional fish were euthanized by anesthetic overdose in parallel with the stocking process to obtain a measure of the fish's pre-exposure gonadosomatic index (GSI; see below).

Midway through each experiment (after approximately four weeks), all the individuals in each tank were re-weighed. In addition, randomly selected females (two in the first experiment; one in the second) from each tank were euthanized via anesthetic overdose to determine their GSIs. Since the second experiment began with one less female, we decided to only sample a single female midway through the second experiment. This did not affect the sample size for statistical analysis, since the aquaria were the experimental units. The total weights of these euthanized fish were each measured by placing the fish in a tared plastic weigh boat. Their total lengths were also measured. We did not measure the total lengths of all fish mid-experiment because the necessary anesthetization might have altered their subsequent behavior.

Upon the conclusion of each experiment, TL, TW, and GSI data were collected on all remaining fish. Fish GSI (x 100 %) was calculated as the ratio of the gonad weight to eviscerated body weight (=somatic weight) as per Dahle (2001), to facilitate comparison. Dissections of the euthanized fish yielded gonads for weighing (nearest 0.0001 g; AT261 Delta Range electronic balance, Mettler-Toledo, Columbus, Ohio). Removing the remaining entrails from the body cavity yielded the individual's eviscerated body weight.

Gonad Histology

For the second experiment, the female gonads, after being weighed for the purpose of GSI calculation, were preserved in 95% ethanol. These were later processed using standard histological techniques. After embedding

in paraffin wax, tissue thin sections were made using a microtome (AO 820; Leica Microsystems Inc., Bannockburn, Illinois) and mounted on microscope slides. The cross-sections were then stained using hematoxylin and eosin to stain the basophilic structures (nucleus and ribosomes) purple and the eosinophilic structures (protein-based cytoplasm) pink, facilitating microscopic examination (Ellis 2007).

At least twelve cross-sections from each ovary were examined. These were collected by retaining three consecutive thin sections, discarding ten sections, and then repeating this pattern until the microscope slide was completely covered (a total of 9 or 12 sections). Each group of three consecutive sections helped insure there was a good-quality section to view after processing. Using several triplets allowed us to view different locations along the ovary. Our goals here were to find indirect evidence of spawning through the identification of empty ovarian follicles, and to determine whether the females' follicular development differed across the photoperiod/temperature treatments.

Each fish's ovary was categorized as previtellogenic, vitellogenic, atretic, or empty, based on the category into which "most" of its follicles fell, with "most" being defined as the follicular stage that in total comprised the largest area of the histological cross section (estimated by eye). Data were also collected regarding the presence/absence of each follicle stage within each ovary. If an ovary contained a follicle in a particular stage, that stage was marked as being present.

Previtellogenic follicles (Figure 3) exhibited oocytes that did not contain yolk (comparable to ovarian follicular stages 1-3; Groman 1982;

Takashima & Hibiya 1995). Vitellogenic follicles (Figure 4) held larger oocytes that contained yolk (follicular stages 4-6), and thus were ready or near ready for spawning. Atretic follicles (Figure 5) were ones where the follicle cells were reabsorbing their unshed mature oocytes via phagocytosis (Grizzle & Rogers 1976). Empty follicles (Figure 6) were ones that had released their mature oocytes, leaving empty follicles which had collapsed inwards on themselves.

Behavior

During the second experiment, two tanks from each of the four treatment groups (total of eight tanks) were randomly selected for behavior monitoring. To accomplish this, we used surveillance cameras (WV-BP140; Panasonic, Suzhou, China) and video-cassette recorders (Panasonic AG-RT850P; Matsushita Electrical Co., Ltd., Osaka, Japan). These recorded the fish's behaviors during daylight hours for the entire duration of this experiment (September 25 to November 23, 2006).

Afterwards, the 15L:9D experiment days were divided into three non-contiguous periods: "Morning" (0800 to 1100 h), "Mid-day" (1400 to 1700 h), and "Evening" (1800 to 2100 h). These periods avoided overlap with "dawn" and "dusk" (0630 and 2130 h, respectively) and with the morning and noon feedings (0730 and 1230 h, respectively). The shorter day lengths of the tanks with the 12L:12D photoperiod (0630 to 1830 h) prevented consideration of an evening period here.

After omitting the days during which disturbances occurred because the egg collection sheets were switched, 25% of each tank's remaining observation periods were randomly sub-sampled for behavioral analysis. Of these selected 3-h periods, 5-min intervals were randomly selected from each hour (a total of 15 min from each period) for behavioral data collection.

After constructing a behavioral ethogram (Table 1), we examined each selected 5 min observation interval and counted the occurrence of all Male, or Female to Male interactions using Noldus Observer® version 5.0 software (Noldus Information Technology, Wageningen, Netherlands). This restricted consideration to reproductive behaviors involving either of the two males; it also excluded acts performed by solo females or during female:female interactions, because recording these from the six females present was unmanageable given their lack of distinguishing features.

RESULTS

Total Length

Fish TL data were analyzed for both experiments separately. In addition, data from the treatments that were common to both experiments (i.e., the temperature/photoperiod treatment combinations included in Experiment 2) were combined and analyzed to increase statistical power. This "Combined" analysis required that an additional "study" factor be included in the statistical model to account for possible differences between the experiments.

Experiment 1: Because the aquarium was the experimental unit (thus avoiding pseudo-replication), our data were averages for each attribute measurement across all the fish of each sex in each tank. For males, a repeated-measures (time), two-factor ANOVA (temperature and photoperiod) revealed that average TL did not differ between treatments at any time during the experiment (overall model: n = 23, d.f. = 22; temperature: F = 0.23, d.f. = 3, p = 0.876; photoperiod: F = 0.70, d.f. = 1, p = 0.416). However, male TL did decrease significantly (F = 9.72, n = 23, d.f. = 22, p = 0.007) across the duration of the study (i.e., there was a significant within-treatment time effect; Figure 7). Beginning average TL for a male was 70.8 mm, and at the end of the experiment was 69.6 mm.

For the female TL analysis, we found evidence of a significant between-treatment photoperiod effect (repeated-measures, two-factor ANOVA;

F = 5.04, n = 23, d.f. = 22, p = 0.040); this resulted from females in the 15 h photoperiod treatments becoming longer than those in the 12 h treatments (Figure 8). A significant within-treatment time effect (F = 4.15, n = 23, d.f. = 22, p = 0.026) was also found; this resulted because females tended to increase in TL over time within each treatment. Beginning average TL for a female was 56.5 mm, and at the end of the experiment was 57.0 mm. The fact that female TL mid-study tended to vary more than at either the beginning or end is a consequence of the small sub-samples (two fish) we randomly obtained from each tank, these being the females from which our GSI values were derived.

Experiment 2: Again, male TL did not differ between treatments at any time during the experiment (repeated-measures, single-factor ANOVA; F = 0.06, n = 23, d.f. = 22, p = 0.980). However, male TL once more decreased significantly (F = 5.39, n = 23, d.f. = 23, p = 0.032) across the duration of the study (i.e., there was a significant within-treatment time effect; Figure 9), although this was less marked in the 25 °C/15 h photoperiod treatment.

For this female TL analysis, we found no evidence of a significant between-treatment effect (repeated-measures, single-factor ANOVA; F = 2.09, n = 22, d.f. = 21, p = 0.138), contrary to the Experiment 1 findings. However, female TL again tended to increase significantly over time within the treatments as in experiment 1 (repeated-measures one-factor ANOVA; F = 4.95, n = 22, d.f. = 44, p = 0.013), this being accompanied by a previously undetected significant time by treatment interaction effect (F = 2.42, n = 22,

d.f. = 44, p = 0.046). The latter may reflect the influence of the 25 °C/15 h photoperiod treatment, wherein female TL decreased from the beginning to middle of the study and then increased from the middle to end (Figure 10). However, this apparent change in growth trajectory might have arisen due to the small number of fish that we removed for the mid-study sample (i.e., one female from each tank).

Experiments Combined: For male TL, the results of the two experiments differed significantly (repeated-measures, two-factor ANOVA, F = 14.77, n = 35, d.f. = 34, p = 0.001), with the average starting TL being larger in the second experiment (73.9 mm) than in the first (70.2 mm). This was most likely due to the second experiment's males having had an additional year to grow. Like each individual experiment, no between-treatment effects were detected (F = 0.09, n = 35, d.f. = 34, p = 0.966), but the significant decrease in male TL within treatments over time was still evident (F = 6.72, n = 35, d.f. = 35, p = 0.015, Figure 11).

For female TL, the results of the two experiments also differed significantly (repeated-measures, two-factor ANOVA, F = 9.62, n = 34, d.f. = 33, p = 0.005), with the average starting TL being larger in the second experiment (58.9 mm) than in the first (57.0 mm). Again, this was most likely due to the second experiment's females having had an additional year to grow. There was no difference in TL between treatments (F = 2.17, n = 34, d.f. = 33, p = 0.116). However, because female TL generally increased from the beginning to the mid-point of

the experiments (Figure 12), time was again a significant within-treatment factor ($F = 5.25$, $n = 34$, d.f. = 68, $p = 0.008$)

Within each sex, the fact that males diminished in TL whereas females gained across time in both experiments demonstrates the repeatability of the outcome, even though the second experiment's fish were older and larger

Total Weight

Fish TW data were analyzed for both experiments separately. In addition, data from the treatments that were common to both experiments (i.e., the four treatments included in Experiment 2) were combined and analyzed to increase statistical power. This "Combined" analysis required that an additional "study" factor be included in the statistical model, to account for possible differences between the experiments.

Experiment 1: Neither temperature nor photoperiod affected male TW differently between treatments (repeated-measures, two-factor ANOVA; $n = 23$, d.f. = 22, temperature: $F = 0.68$, $p = 0.576$, photoperiod: $F = 0.25$, $p = 0.623$). As was the case with TL, male TW decreased over time within each treatment ($F = 25.97$, $n = 23$, d.f. = 46, $p < 0.001$). This decline was greater between the beginning and mid-point of the study than between the mid-point and end (Figure 13), yielding a significant time by photoperiod interaction effect ($F = 4.99$, $n = 23$, d.f. = 46, $p = 0.014$).

The response of female TW to photoperiod was significant (repeated-measures, two-factor ANOVA; $F = 17.37$, $n = 23$, d.f. = 22, $p < 0.001$),

with the 15 h treatment yielding greater weights (Figure 14). There were significant within-treatment time ($F = 25.19$, $n = 23$, d.f. = 46, $p < 0.001$), time by temperature interaction ($F = 3.31$, $p = 0.013$), and time by photoperiod interaction ($F= 12.61$, $n = 23$, d.f. = 46, $p < 0.001$) effects. To elaborate, the time effect reflects the fact that female TW tended to increase over time within each treatment. The time by temperature interaction arose because female TW increased between the beginning and mid-point and then decreased between the midpoint and end of the 25 and 31 °C temperature treatments, whereas the increase in TW was continuous through the entire experiment for the 28 °C treatment. The time by photoperiod interaction arose because female TW tended to increase during the first half of the experiment for both photoperiod regimes, tended to decrease slightly in the second half of the 12 h photoperiod treatment (with the exception of the 28° C treatment), but remained relatively unchanged in the 15 h treatments.

Experiment 2: All treatments influenced male TW similarly (repeated-measures, one-factor ANOVA; $F = 0.11$, $n = 23$, d.f. = 22, $p = 0.953$). Male TW decreased over time, with the majority of the loss occurring in the first half of the experiment (Figure 15). These differences manifested themselves as a significant time effect within treatments ($F = 60.29$, $n = 23$, d.f. = 46, $p < 0.001$) and as a time by treatment interaction effect ($F = 4.31$, $p = 0.002$), respectively.

In contrast, female TW responded differently across the treatments (repeated-measures, one-factor ANOVA; $F = 3.82$, $n = 23$, d.f. = 22, $p = 0.027$),

with time ($F = 47.33$, $n = 23$, d.f. = 46, $p < 0.001$), and a time by treatment interaction ($F = 5.49$, $p < 0.001$). The treatment effect likely stems from the lower TWs that occurred in the 25 °C/12 h treatment (the only 12 h photoperiod exposure; Figure 16). The time effect arose because female TW tended to increase through the experiment across nearly all the treatments. This gain was not seen in the 25 °C/12 h treatment, which likely gave rise to the significant time by treatment effect.

Experiments Combined: Male TW differed between the two experiments (repeated-measures, two-factor ANOVA, $F = 8.35$, $n = 35$, d.f. = 34, $p = 0.008$), but not across the treatments. Starting average male TW in the second experiment (4.745 g) was larger than in the first (4.044 g) when they were one year younger. Within treatments, male TW decreased with time ($F = 46.18$, $n = 35$, d.f. = 70, $p < 0.001$), with the majority of the loss occurring in the first half of each experiment (Figure 17).

Female TW also differed between the two experiments (repeated-measures, two-factor ANOVA, $F = 17.00$, $n = 35$, d.f. = 34, $p < 0.001$). Starting average female TW in the second experiment (1.977 g) was larger than in the first (1.697 g) when they were one year younger. Differences between the treatments were also apparent ($F = 5.84$, $p = 0.003$), because the 25 °C /12 h treatment group exhibited lower TWs than did any of the 15 h photoperiod treatment groups. Within treatments, the effects of time and of the time by treatment interaction were significant ($F = 74.43$, $n = 35$, d.f. = 70, $p < 0.001$,

and F = 11.00, n = 35, d.f. = 70, $p < 0.001$, respectively). These resulted from the general increase in female TW over time, the exception being the 25 °C/12 h treatment group (Figure 18).

The general trends over time of the male and female TW analyses were similar among experiments. As for the TL data, male TW tended to decline whereas females tended to gain TW in both experiments. This demonstrates the repeatability of the results, since different sized and aged fish were used in the two experiments.

Gonadosomatic Index

Fish GSI data were analyzed for both experiments separately. In addition, data from the treatments that were common to both experiments (i.e., the treatments included in Experiment 2) were combined to test for differences between studies and to increase statistical power. This "Combined" analysis required that an additional "study" factor be included in the statistical model, to account for possible differences between the experiments.

Experiment 1: Given that the single male used in each tank was euthanized at experiment's end, there were no repeated measures of male GSI. For males, their final GSIs varied with both temperature and photoperiod (two-factor ANOVA; F = 5.76, n = 23, d.f. = 22, $p = 0.008$ and F = 8.45, n = 23, d.f. = 22, $p = 0.011$, respectively). The results of a Tukey's studentized range test showed that average male GSI was greater for both the 22 and 25 °C than

for the 31 °C treatments (Figure 19), and that greater GSI values were recorded from fish in the 15 h than in the 12 h photoperiod treatment.

For females, their GSIs failed to differ in response to differences in either the temperature (repeated-measures, two-factor ANOVA; F = 2.04, n = 23, d.f. = 22, p = 0.152; Figure 20), photoperiod (F = 3.26, n = 23, d.f. = 22, p = 0.091), or over time within treatments (F = 0.01, n = 23, d.f. = 23, p = 0.905). There were no differences detected in the repeated measures design. However, visual inspection of female GSI over time (Figure 20) led us to suspect that there might have been differences at the end of the study. Therefore, we analyzed the female GSI data collected at the middle and end of the study separately. No treatment effects were detected at the middle of the study. However, the end of the study analysis found highly significant effects of temperature (F = 7.91, n = 23, d.f. = 22, p = 0.002), photoperiod (F = 18.20, n = 23, d.f. = 22, p = 0.001), and of a temperature by photoperiod interaction (F = 5.50, n = 23, d.f. = 22, p = 0.010). Here, the temperature effect resulted from female GSIs being greater at 22 and 28 °C than at 31 °C, whereas the photoperiod effect resulted from female GSIs being higher under the 15 h photoperiod. The most likely reason for the significant interaction was that the differences between the two 31 °C treatments were less than those between the two 22 °C treatments (i.e., the photoperiod effect varied among temperatures).

Experiment 2: For males, there were no detected treatment-related differences in GSI at the end of the experiment (one-factor ANOVA; F = 0.37,

n = 23, d.f. = 22, p = 0.777; Figure 21). Average male GSI at the end of the experiment for all individuals was 1.14 %, which was similar to the overall final average male GSI of the first experiment (1.24 %).

Female GSI did differ between treatments (repeated-measures, one-factor ANOVA; n = 22, d.f. = 21, F = 10.94, p < 0.001). For females at the end of the test, a Tukey's studentized range test indicated that fish in the 22 °C/15 h photoperiod treatment yielded comparatively larger GSIs (13.1 %; Figure 22) relative to the remaining treatments. In addition, female GSIs differed between the 25 °C/15 h and 25 °C/12 h treatments, with the former yielding appreciably greater values (12.7 versus 8.4 %). In comparison, the average GSI from the 28 °C/15 h photoperiod treatment was 11.7 %. No significant time effects were detected in this repeated measures analysis.

Sets of 20 individuals from both sexes were randomly selected from the stock of fish at the onset of Experiment 2 to estimate pre-exposure GSI levels. These fish were not subject to the repeated measures assessments of the experiment proper. The GSIs for these fish were compared to those collected from each sex at the middle (females only) and end of the experiment (both sexes) by means of a two-sample t-test. Averaged male GSIs from every treatment were significantly lower at the end of the study when compared to the group sampled at the beginning of the study (Figure 21). The cause of the decrease in GSI is unknown, but could either be a result of spawning or another indication of loss of body condition through gonad reabsorbtion. None of the female GSIs collected from any of the treatments at the middle of the experiment

differed from the group sampled at the beginning of the study (Figure 22). However, female GSIs from the 22 °C/15 h treatment were greater at the end of the study than for the group sampled at the beginning.

Experiments Combined: For males, there were no detectable effects on GSI of either experiment, treatment, or their interactions (two-factor ANOVA; overall model F = 0.76, n = 35, d.f. = 34, p = 0.628; Figure 23). The similarity in responses to the treatments included in both experiments (Figures 19 and 21) provide visual confirmation of this outcome. Overall, the final average male GSIs were slightly lower in the second experiment than in the first, but this difference was not statistically significant.

For females, their GSI values responded similarly in both experiments (repeated-measures, two-factor ANOVA; F = 0.46, n = 34, d.f. = 33, p = 0.503), with significant between-treatment effects being detected (F = 7.60, n = 34, d.f. = 33, p = 0.001). Within the treatments, a significant time by treatment interaction effect was detected (F = 4.09, n = 34, d.f. = 34, p = 0.017), which reflects differences in the degree of change in GSI value between the two halves of the experiment (Figure 24).

Ovary Stage Classification

Logistic regression analyses of the ovary data we collected from the middle of the second experiment detected no significant differences between treatments for the presence or absence of the previtellogenic, vitellogenic, or atretic stage follicles (Wald Chi-Square = 0.05, n = 22,

clustered by 21 tanks, p = 0.832, count pseudo-R^2 = 0.727). (NB: count pseudo-R^2 measures the goodness of fit of the model by calculating the ratio of correctly predicted observations to the total number of observations.) All ovaries contained previtellogenic and vitellogenic follicles, and therefore no treatment differences were detected for the presence or absence of these stages. However, there were treatment differences in the presence of empty follicles, which were found in 20% of the 22 °C/15 h treatment versus 100% of the 28 °C/15 h treatment fish (Wald Chi-Square = 6.54, n = 22, clustered by 21 tanks, p = 0.011, count pseudo-R^2 = 0.818; Figure 25). For comparison, empty follicles were found in 60% of the 25 °C/12 h treatment versus 83% of the 25 °C/15 h treatment fish.

Previtellogenic follicles were present in every female, regardless of the time sampled. By experiment's end, all ovaries contained empty follicles (Figure 25), and there were no apparent differences between the treatment groups as regards the presence/absence of any single follicular stage: vitellogenic (Wald Chi-Square = 0.33, n = 112, clustered by 23 tanks, p = 0.566, count pseudo-R^2 = 0.946), atretic (Wald Chi-Square = 0.39, n = 112, clustered by 23 tanks, p = 0.534, count pseudo-R^2 = 0.554).

The percentages of ovaries classified into each follicular category at the middle and end of the study are illustrated in Figure 26. The percentage of previtellogenic ovaries generally increased (corresponding with a decrease in vitellogenic ovaries) from the middle to end of the study. Examination of the ovaries revealed that this increase resulted from the development of replacement

primary oocytes following the release of mature eggs. Many of these previtellogenic oocytes formed near the remnants of older empty follicles (Figure 27).

A single-factor ANOVA comparing GSI values for the differently categorized ovaries yielded a significant result ($F = 15.77$, $n = 154$, d.f. = 153, $p < 0.001$): fish with vitellogenic ovaries exhibited greater GSIs (averaging 13.9%) than did ones classified as previtellogenic (9.9%) or as empty (8.0%), although the differences between vitellogenic and empty ovaries were not significant because only two ovaries were classified as empty. These results further support the idea that GSI increased as female ovaries developed more mature ova.

Behavior

The values of the frequencies of occurrence for individual behavioral acts were not normally distributed (due to behaviors not occurring in many observations). Consequently, we summed acts within two new behavioral categories: “Male” behaviors (acts initiated by a male, which could be directed at either a male or female) and “Female to Male” behaviors (acts initiated by a female and directed toward a male). This revised approach allowed the observer to concentrate on just the two males, and how they interacted with each other and with the females. Occurrences of female to female interactions were not recorded because there were too many females in each tank to monitor at once.

Under the amended data collection regime, we tallied occurrences of the Male and the Female to Male acts for each of the three five-minute intervals taken within a period, and then averaged these to get a single value for each period. Consequently, each datum represented the average number of behavioral acts performed in three 5-min intervals within the period. We then tested for treatment and time period effects on these using a one-factor ANOVA incorporating time period as a repeated measure.

For the Male behaviors (Figure 28), we found no differences in act frequencies between treatments ($F = 0.12$, $n = 7$, d.f. = 6, $p = 0.892$) or within treatments for the within-day time period effect ($F = 3.06$, $n = 7$, d.f. = 14, $p = 0.103$). For this analysis, we excluded the 12 h photoperiod treatment group because the shorter photoperiod precluded making an evening data collection. Also, the mid-day period of the 12 h photoperiod treatment was unlike the other treatments' mid-day or evening periods, due to the fact that the lights went off and feeding occurred shortly after the period ended (unlike other treatments' mid-days and evenings, respectively).

Since no significant differences were found in the examination of male behavior, a *post hoc* power analysis (PROC POWER, Base SAS version 9.1, Cary, North Carolina) indicated that, given the demonstrated treatment means and standard deviations, the detection of a significant treatment effect ($\alpha = 0.05$, with $\beta = 0.7$) would have required a total sample size of approximately 160 tanks.

For the Female to Male behaviors (Figure 28), we also found no differences in act frequencies between treatments ($F = 0.35$, $n = 7$, d.f. = 6,

p = 0.727). However, this analysis did detect a significant within-treatment effect of within-day time period (F = 5.00, n = 7, d.f. = 14, p = 0.040), with act frequencies being greatest in the morning and fewer as the day progressed. A *post hoc* power analysis revealed that a total sample size of approximately 70 tanks would have been necessary to detect a significant difference (α = 0.05, with β = 0.7) for the between-treatment effect.

To analyze the 12 h and 15 h photoperiod treatment data together, we also tested for differences in act frequencies across all four treatments using only the data collected during the first period of the day (i.e., the one period the timing of which was common across all four treatments). A one-factor ANOVA yielded no significant differences between the treatments for either the Male (F = 0.10, n = 9, d.f. = 8, p = 0.957) or the Female to Male behaviors (F = 0.61, n = 9, d.f. = 8, p = 0.639).

DISCUSSION

Experiment 1 was designed to explore if and how different temperature and photoperiod regimes affected Topeka shiner TL, TW, and GSI. We included temperatures representing the full range (22 to 31 °C) across which Topeka shiners reportedly spawn (Hatch 2001), and photoperiods approximating day lengths at the onset and middle of the spawning season (12 and 15 h, respectively). Experiment 2 provided further insights by focusing on those temperature and photoperiod regimes that elicited the most positive responses, and by considering histological and behavioral evidence of effect. Therefore, we believe that our results provide a reasonable test of how temperature and photoperiod affect Topeka shiner reproductive maturation and behavior.

Total Length and Total Weight

Male average TL and TW declined over time in both experiments. This outcome suggests that males were not feeding sufficiently to maintain body size. In fishes, losses in total length can result from the fin erosion that can accompany reduced levels of feeding (Kindschi 1987). Although we did not notice such erosion during the taking of our final TL measurements, a subtle amount would have been sufficient to yield the decrease in average male TL we detected across both experiments (averaging 1.3 mm and 0.5 mm in Experiments 1 and 2, respectively).

As in our Topeka shiners, breeding-related weight losses resulting from

a reduction in feeding have also been documented in male smallmouth bass (*Micropterus dolomieui;* Wiegmann & Baylis 1995) and bluegill sunfish (*Lepomis macrochirus;* Gross & MacMillan 1981) during their week-long periods of nest-guarding. The Eurasian minnow (*Phoxinus phoxinus*), a closer relative of the Topeka shiner, also exhibits weight loss during the spawning season (Mills 1987). As Heins and Rabito (1986) have shown, our finding of follicles at several developmental stages within an ovary is evidence that Topeka shiners will spawn successively during the summer months; thus, how rapidly a male can replace his weight losses via feeding may relate to how many times he spawns and the length of time between the spawnings.

In contrast to the males, the TL and TW of female Topeka shiners increased under longer photoperiod exposures (although temperature had no significant effect). These gains suggest that females were feeding adequately throughout the experiments. Female activity levels (and therefore energy expended) were generally much lower than those of males, which might lessen their weight losses relative to males.

For females, the frequent occurrence of empty follicles in their ovaries might lead one to expect an appreciable loss in weight over time (due to egg releases). However, ovaries classified as containing primarily empty follicles were uncommon (only two of 137 examined females), meaning that most ovaries were dominated by previtellogenic and vitellogenic follicles, and that eggs were shed a few at a time. Our failure to detect spawned eggs on the egg collection

sheets is consistent with the latter, given the greater likelihood of detecting the deposition of a larger number of eggs.

Koehle (2006) concluded that temperatures near 27 °C are optimal for Topeka shiner growth when activity levels are low, but that high levels of reproductive activity occurring at temperatures above 23 °C would most likely slow their growth. Our findings support these assertions, given that the increases in female TW were greatest under the 28 °C/15 h photoperiod treatment, and that male losses in TL/TW while exhibiting high levels of reproductive activity occurred across the temperatures we considered. Koehle (2006) also reported that reproductive activity decreased estimates of the species' optimal temperature for growth by at least 9 °C. This could explain the loss of male TW over time, since a 9 °C decrease in optimal temperature for growth from 27 °C would drop the optimal temperature below the minimum we used in the current study.

Gonadosomatic Index

For males, the various temperature/photoperiod treatments we tested resulted in differing GSI responses: longer photoperiods resulted in greater GSIs, but heightened temperatures resulted in GSI decreases. Using GSI as an indicator of optimal spawning conditions (but see discussion of histology below), our results suggest an optimal temperature for reproduction in males near or perhaps below the 22 °C minimum we tested. Supporting this, during

our pre-test exposure of these animals to a simulated winter (see Methods section), we noticed that males began to develop nuptial coloration as water temperatures rose to *ca.* 17 °C. Therefore, it is possible that the optimal temperature for obtaining the greatest male GSI is below the range tested in this study.

For females, the greatest positive influence on GSI occurred in the 22 °C/15 h treatment. Thus, it was the longer of the photoperiods we tested, in combination with cooler temperatures, that yielded the greatest GSIs for females (as was the case for the males). Consistent with this, females did not appear to become gravid during our pre-test exposure to the simulated winter conditions, but became so only after holding them at 22 °C/12 h for the ensuing month. This further supports the notion that photoperiod has a "priming" effect on female reproductive maturation (Billard 1981).

Together, these results suggest that Topeka shiners which inhabit streams that stay cooler for a longer period of time in the Spring may have more time to develop greater numbers of mature gametes before temperatures acceptable for spawning are attained. Whether this would result in greater numbers of eggs being released during a breeding season's first spawning by an individual female is uncertain. Also uncertain are the relative merits of spawning these eggs with a single male versus spawning fewer eggs with many males, given issues of differing male quality, potential male sperm limitation, and the "bet-hedging" associated with placing eggs in multiple "baskets" (i.e., nests, any one of which risks possible failure).

Ovary Stage Classification

The use of GSI as a measure of reproductive activity has been deemed problematic by de Vlaming and Paquette (1977), given that oocyte atresia can sometimes occur without accompanying changes in female GSI. The results (Figures 25 and 26) obtained using our ovary developmental stage categorization scheme suggest that it may provide information superior to that gained by measuring GSI, since GSI does not give any insight as to whether or not multiple-clutch spawners, who continually release and develop new ova throughout the spawning season (i.e. the Topeka shiner), have spawned (evident as empty follicles).

de Vlaming and Paquette's (1977) findings suggest that there may be environmental conditions that are good for gametogenesis (i.e., that yield high GSI values), but which are less optimal for eventual spawning (egg deposition). Consistent with this notion, the female GSI data from the second experiment indicate that the 22 °C/15 h treatment was likely "best" for oogenesis. In contrast, the histological data show that as temperatures increased further, so did the mid-study presence of empty follicles, with empty follicles being considered evidence of spawning (Macewicz & Hunter 1993; Selvakumaraswamy & Byrne 1995; Maddock & Burton 1998).

Over the duration of our experiment, the percentage of ovaries classified as predominantly vitellogenic increased in most treatment groups from the beginning to middle of the study (the exception being the 25 °C/12 h treatment),

whereas the percentage of ovaries classified as predominantly previtellogenic increased from mid-study to the end. In addition, the percentages of ovaries containing atretic and empty follicles generally increased from mid-study to the end. That ovaries became increasingly previtellogenic likely reflects the loss of vitellogenic follicles [either by the release of mature ova (yielding empty follicles) or by reabsorption (yielding atretic follicles)], in conjunction with the recruitment of additional previtellogenic follicles for likely use in subsequent spawnings. The minimal presence of empty follicles (coupled with the highest GSI values) in mid-study females from the 22 °C/15 h treatment group also suggests that it was photoperiod that was working to "prime" reproductive maturation, since mature gametes were being developed but not released. The fact that higher temperatures were accompanied by an increase in the occurrence of empty follicles indicates that it was higher temperatures that triggered the occurrence of actual spawnings in females.

Fish with previtellogenic ovaries averaged GSIs of 9.9 %, whereas fish with vitellogenic ovaries averaged 13.9 %. These GSIs are comparable to Dahle's (2001) results where the GSIs for early maturing (EM) and late maturing (LM) females were 5.1 % and 14.9 %, respectively. Dahle's EM and LM classifications were adopted from Heins and Rabito (1986), with the changes required for transformation between them being similar to those required for transformation between the previtellogenic and vitellogenic stages of the present study. This difference between previtellogenic and vitellogenic ovaries further

supports the notion that GSI can be a good indicator of the “primedness” of an individual for spawning.

Behavior

We counted Topeka shiner reproductive acts as a means of determining which temperature and photoperiod treatment was optimal, our assumption being that an increase in the occurrence of reproductive behaviors would correspond to increased spawning preparedness. However, we detected no differences between our treatments in the frequencies of occurrence of either the Male or the Female to Male behaviors. This outcome suggests that all the treatments we considered were comparable for eliciting the performance of reproductive behaviors. This implies that the intensity of reproductive activity does not change appreciably once conditions are acceptable for spawning.

The fact that we observed males performing territorial behavior at temperatures below those we tested (i.e., during pre-experiment holding) raises the possibility that differences in behavior between experimental treatments might be more pronounced under cooler conditions akin to those earliest in the spawning season. That activity might be high at temperatures lower than we tested is consistent with observations of male dominance and territories being established prior to spawning (Stark et al. 2002).

The lack of difference in activity between our treatments may also be partially attributable to our experimental design, having only two tanks per

treatment. However, our *post hoc* power analyses revealed that detecting significant treatment differences would have required sample sizes that far exceeded our resources (i.e., 160 tanks for the Male and 70 tanks for the Female to Male behaviors, respectively). The magnitude of these estimates suggests that any differences in activity level were minimal and thus of potentially little biological significance. We conclude that the sample size we used in the present study was sufficient for detecting biologically relevant activity level differences, and therefore that activity levels do not change once temperature and photoperiod conditions acceptable for spawning are reached.

Both the Male and the Female to Male behaviors occurred most frequently in the morning following feeding, after which the intensity decreased as the day progressed. Other cyprinid species similarly exhibit their greatest spawning activity in the morning (Harrington 1947; Gale 1986; Albanese 2000). Therefore, the Topeka shiner is not unique in this regard. This heightened activity in the morning may be a result of males re-establishing their dominance hierarchy or territory, since Topeka shiners are not active at night (Noel Burkhead, pers. comm.).

The current study analyzed behavior based solely on their frequencies of occurrence. Due to the experimental design, the collection of other information (e.g., behavioral sequence data) was not feasible. Also, since individual behaviors were of short duration, we were unable to record the total amount of time spent performing specific behaviors. In the future, collection of

the aforementioned data may provide further insights regarding Topeka shiner reproductive behavior.

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Figure 1: Still image of experimental tank design used in Experiment 1 (8 treatments; 4 temperatures and 2 photoperiods). Shown are the plexiglas false floor and the egg-collection dish in the rear-middle of the tank.

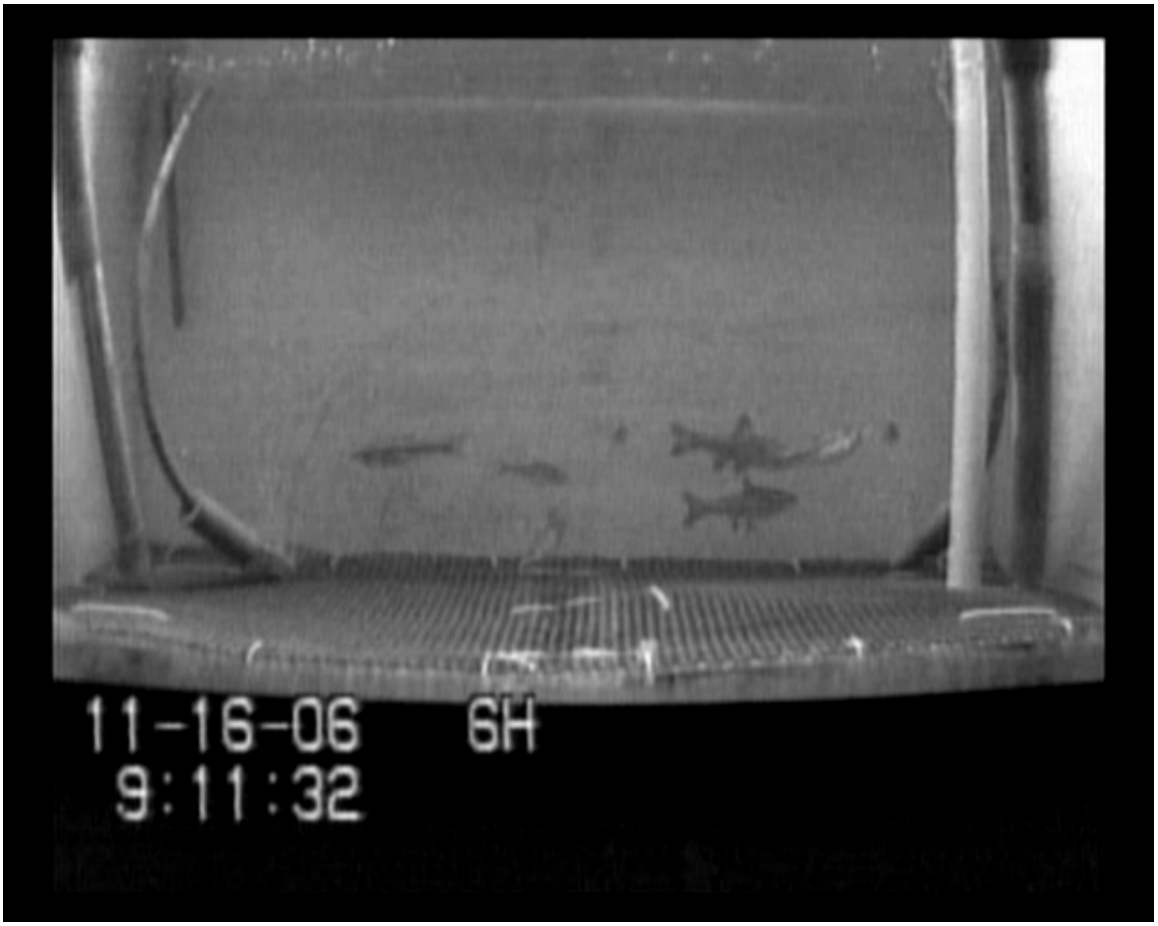

Figure 2: Still image captured from time-lapse surveillance videography displaying the tank design in Experiment 2 (4 temperature/photoperiod treatments). Shown is the plastic mesh false floor design.

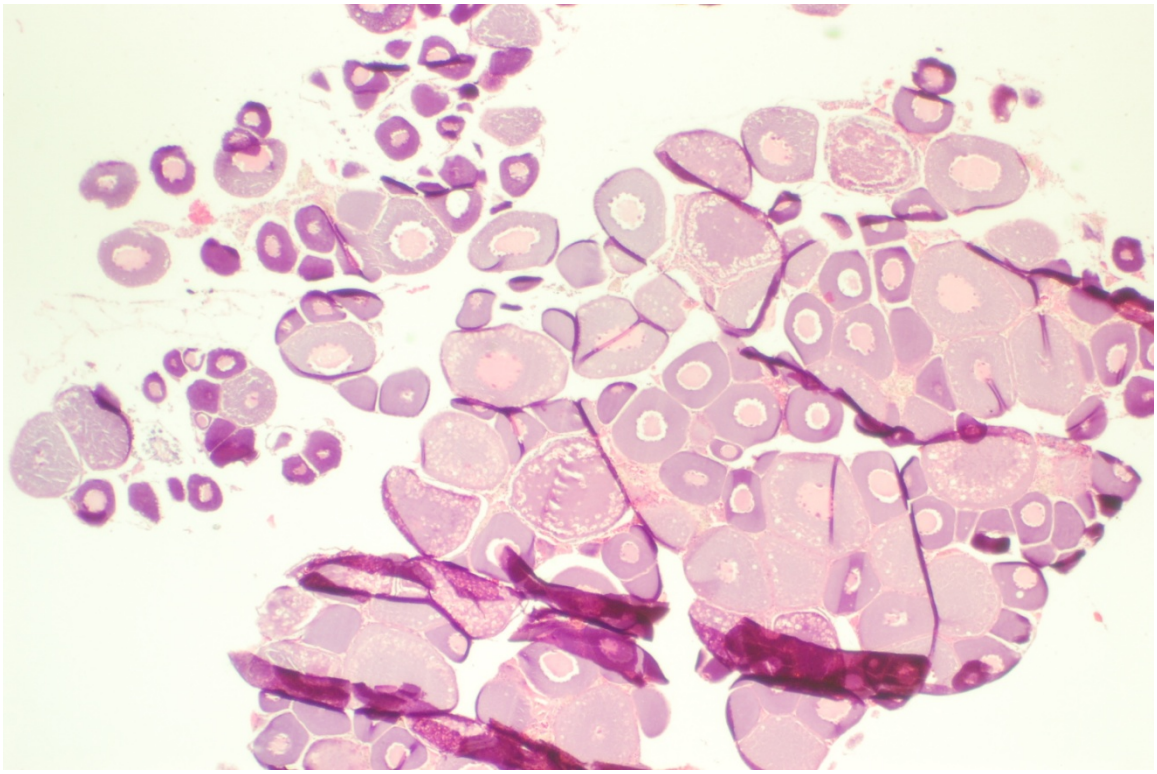

Figure 3: Previtellogenic ovary from a Topeka shiner female from Experiment 2. The relatively small previtellogenic ova lack large yolky globules and take up the vast majority of the area in this cross section.

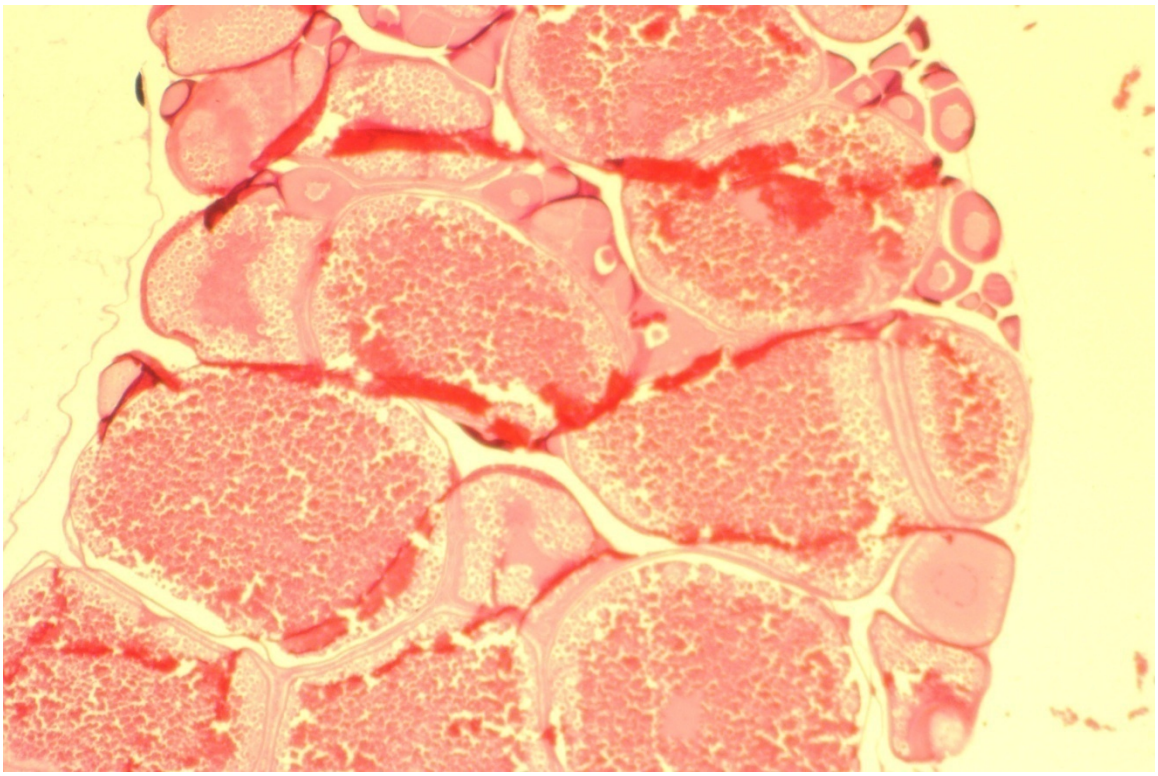

Figure 4: Vitellogenic ovary from a Topeka shiner from Experiment 2. The majority of follicles present in this cross section contain vitellogenic ova, which are identified by the presence of yolky granular globules within the cell.

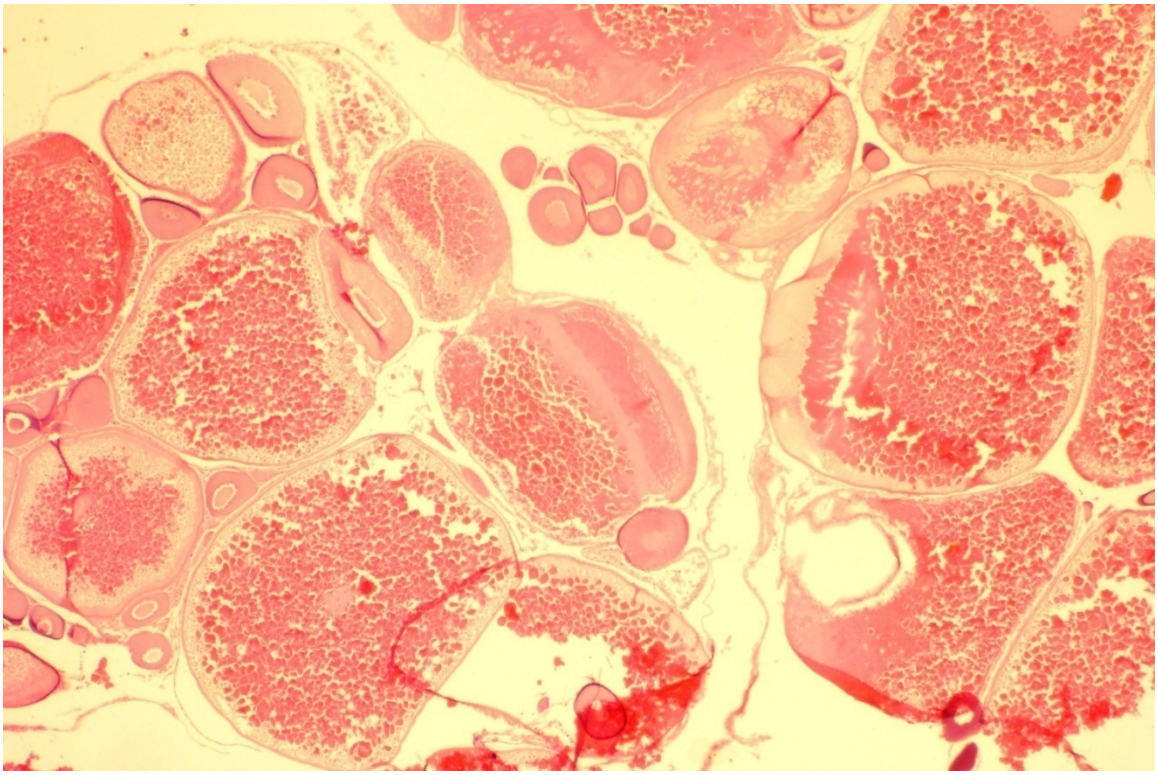

Figure 5: An example of atretic follicles from a Topeka shiner from Experiment 2. Atresia is occurring in the follicles at the center, right of center, and top of the photo. Atresia involves reabsorption; consequently, the yolk globules within the ova appear to be “melting”.

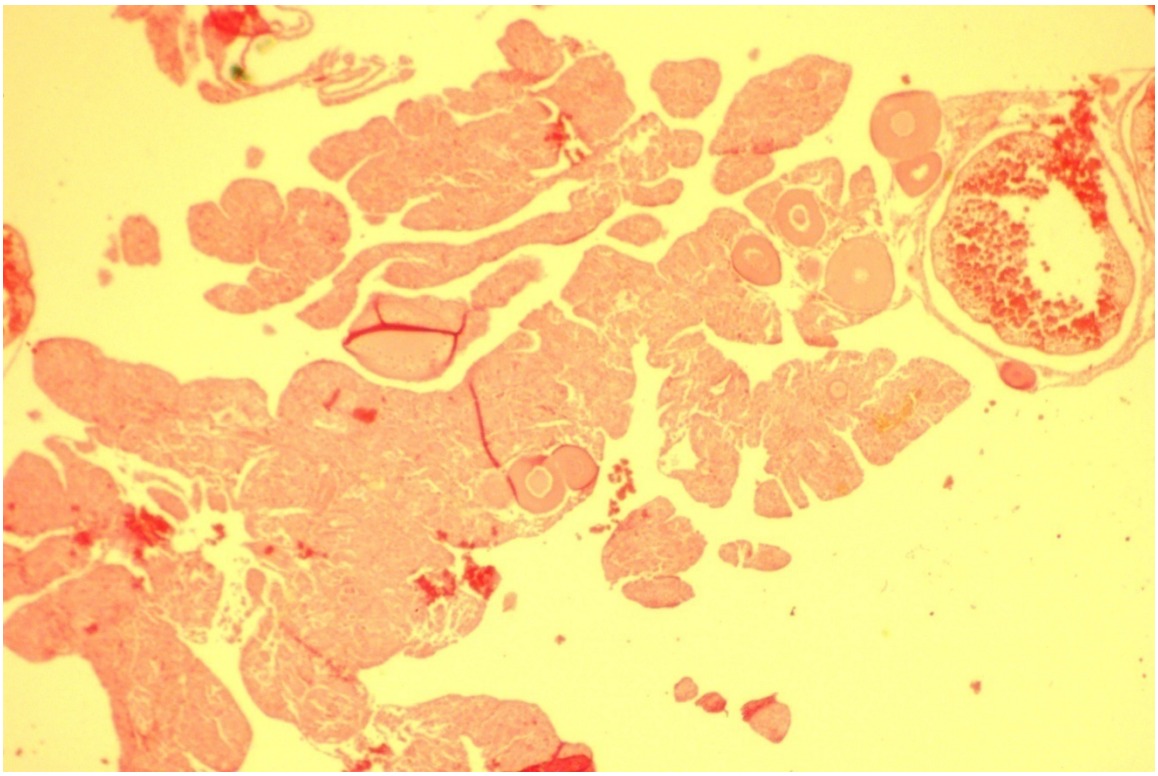

Figure 6: Ovary classified as empty from a Topeka shiner from Experiment 2, containing a few interspersed previtellogenic oocytes, and one vitellogenic oocyte (far right). An empty follicle is identifiable by the presence of collapsed groups of follicular cells that no longer surround an ovum.

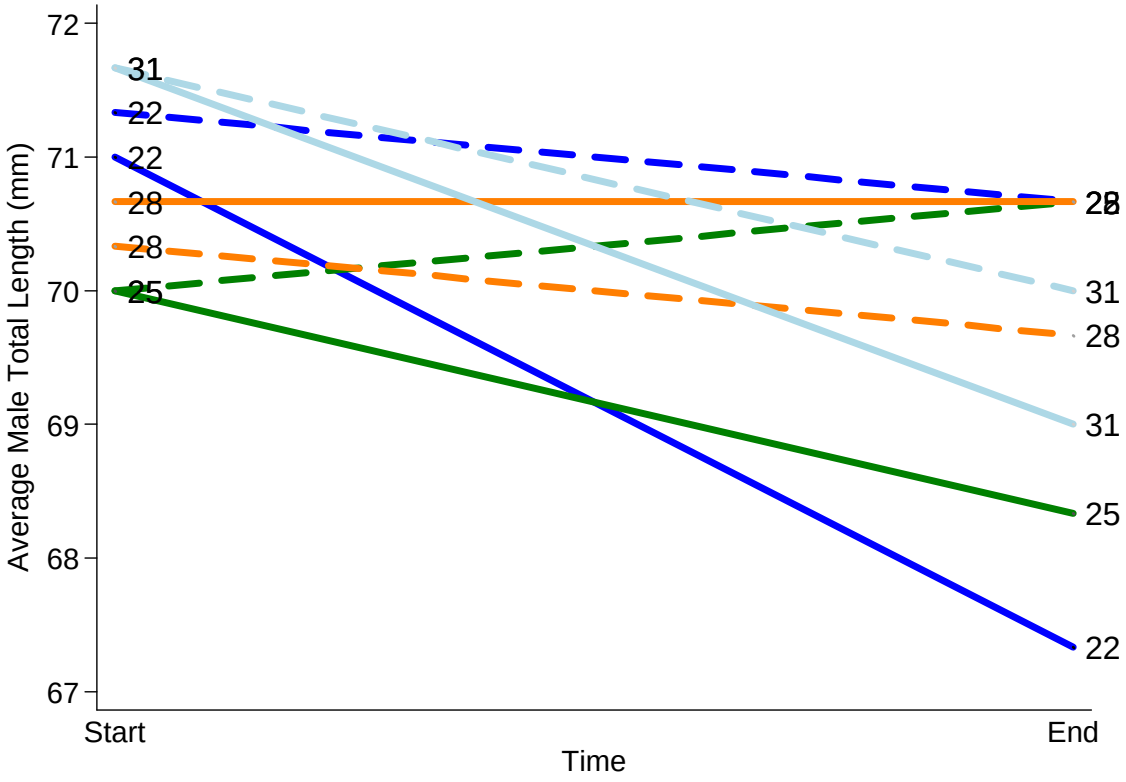

Figure 7: Illustration of the male total length results from Experiment 1. Numbers next to lines represent treatment temperatures. Solid lines represent the long photoperiod treatment (15 h), and dashed lines represent the short photoperiod (12 h). Total length generally decreased over time.

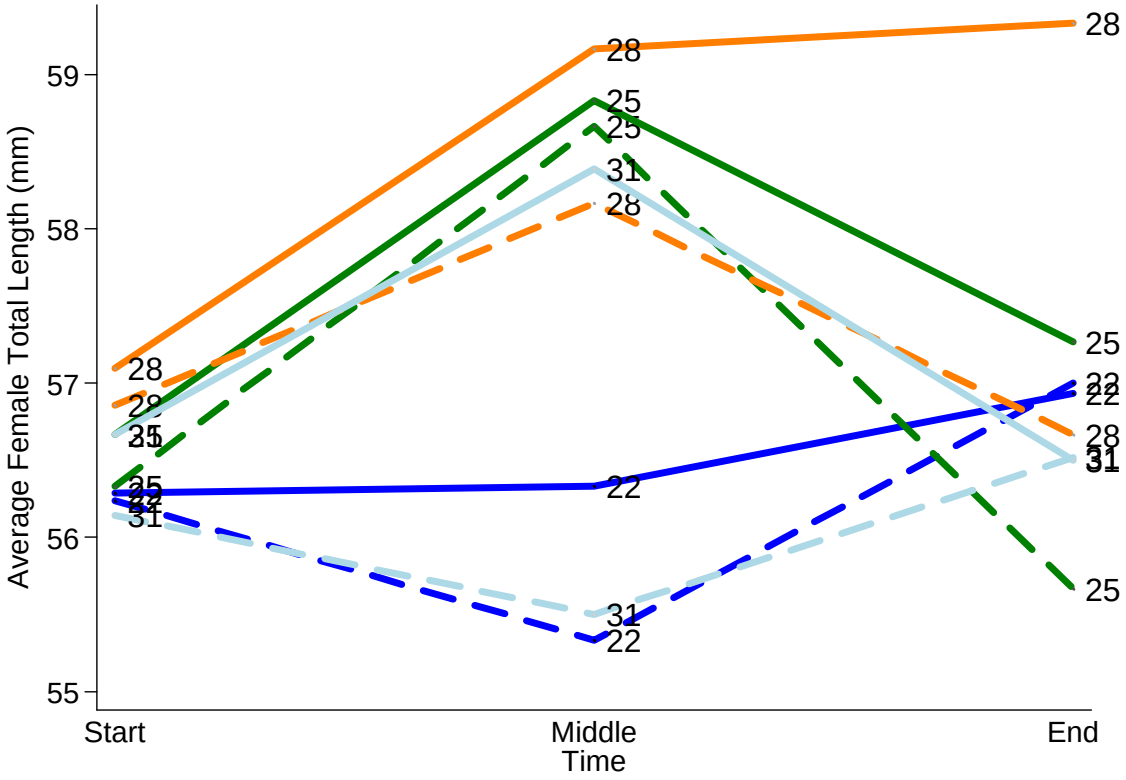

Figure 8: Illustration of the female total length results from Experiment 1. Numbers next to lines represent treatment temperatures. Solid lines represent the long photoperiod treatment (15 h), and dashed lines represent the short photoperiod (12 h). The longer photoperiod treatment yielded greater female total length. Total length generally increased by experiment's end.

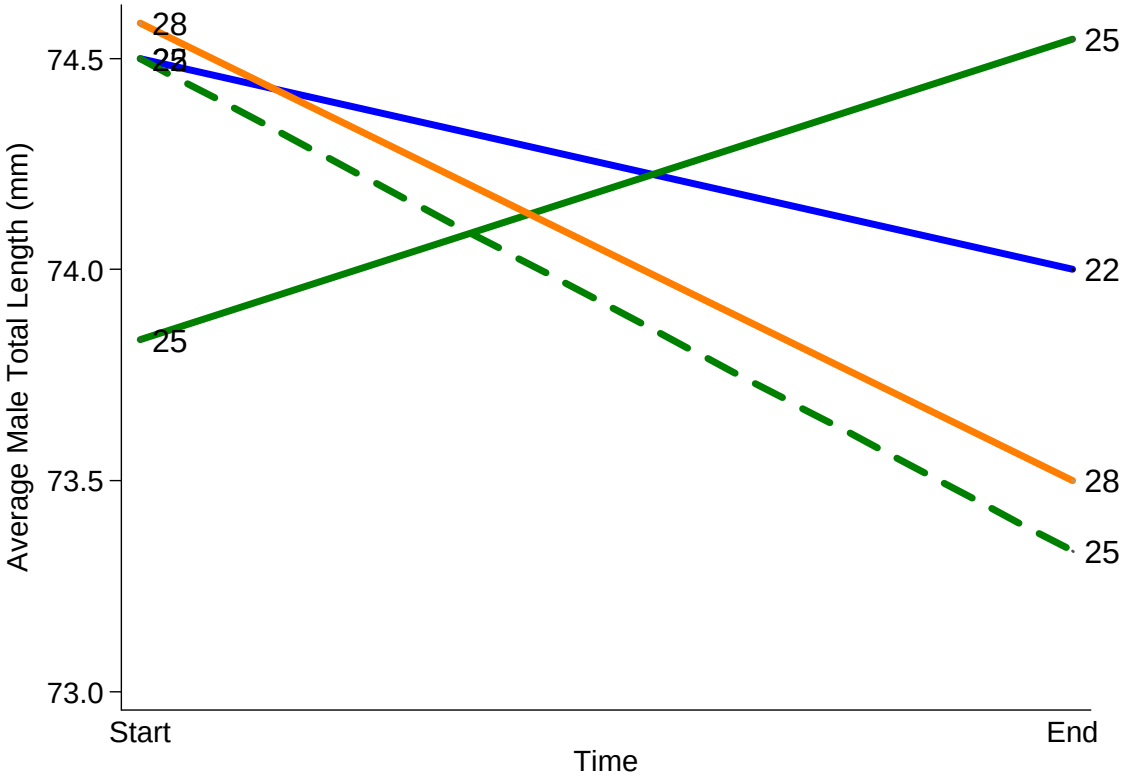

Figure 9: Illustration of the male total length results from Experiment 2. Numbers next to lines represent treatment temperatures. Solid lines represent the long photoperiod treatment (15 h), and dashed lines represent the short photoperiod (12 h). Total length generally decreased over time.

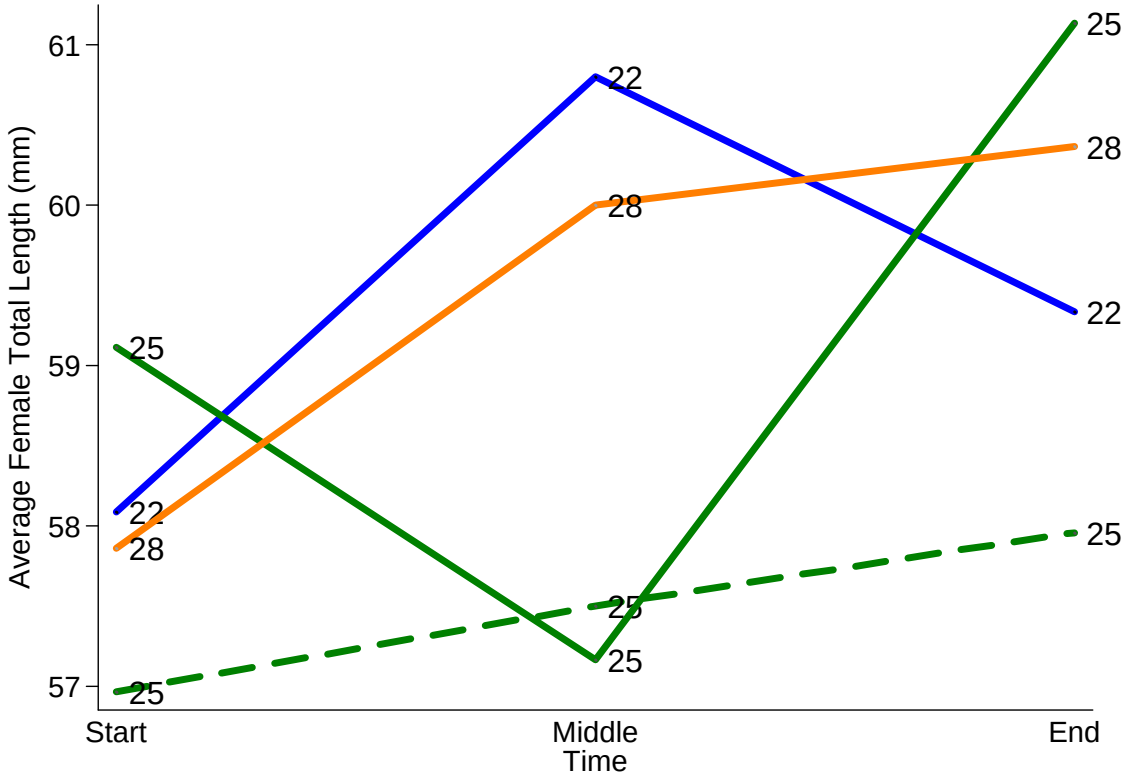

Figure 10: Illustration of the female total length results from Experiment 2. Numbers next to lines represent treatment temperatures. Solid lines represent the long photoperiod treatment (15 h), and dashed lines represent the short photoperiod (12 h). Total length generally increased over time.

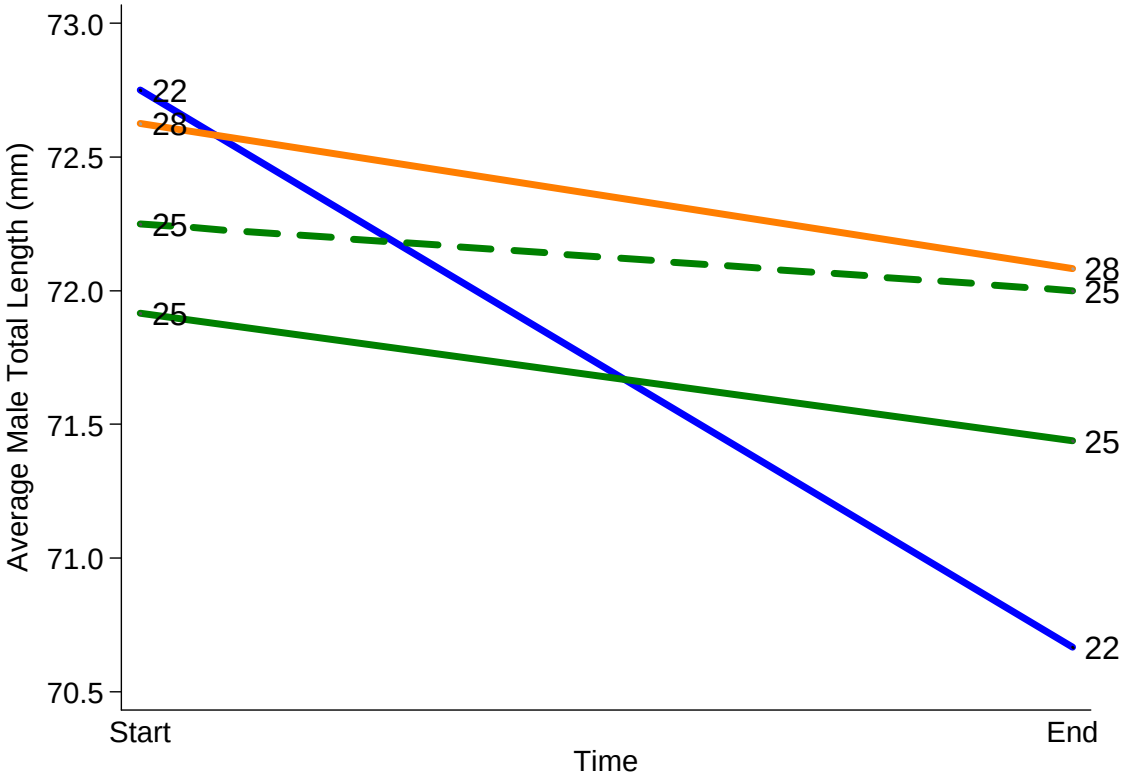

Figure 11: Illustration of the male total length results from the combined experiment analysis. Numbers next to lines represent treatment temperatures. Solid lines represent the long photoperiod treatment (15 h), and dashed lines represent the short photoperiod (12 h). Total length generally decreased over time.

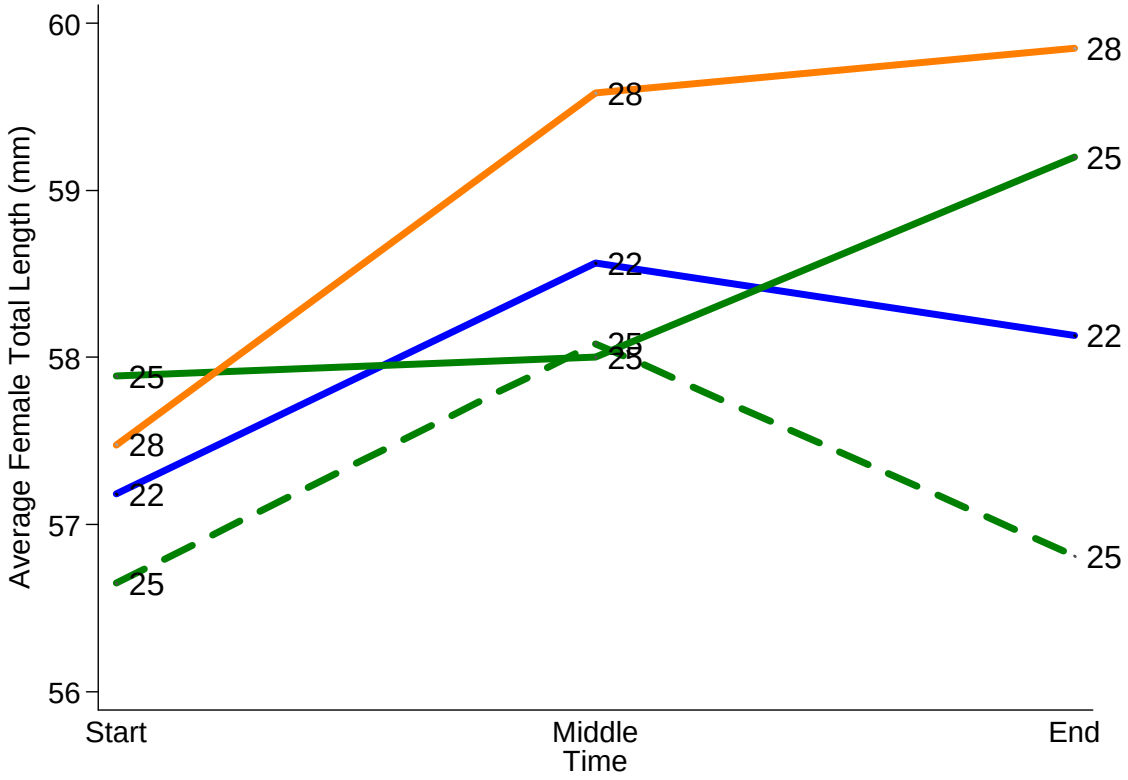

Figure 12: Illustration of the female total length results from the combined experiment analysis. Numbers next to lines represent treatment temperatures. Solid lines represent the long photoperiod treatment (15 h), and dashed lines represent the short photoperiod (12 h). Total length generally increased over time.

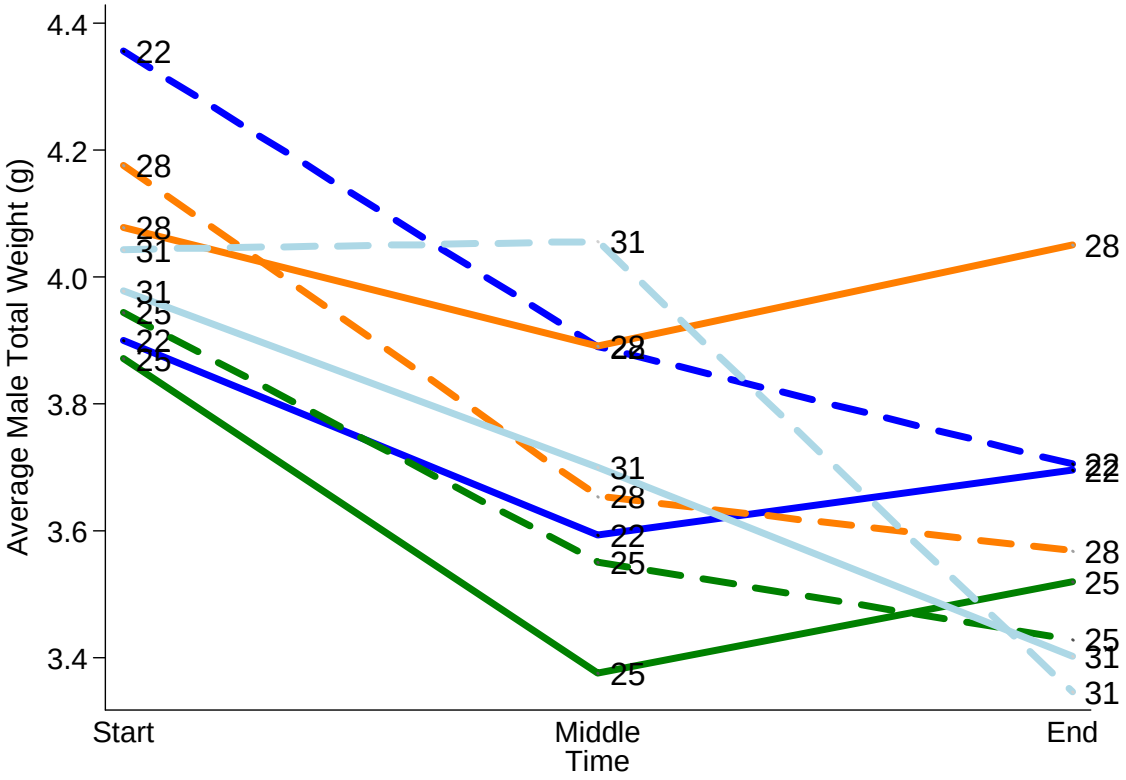

Figure 13: Illustration of the male total weight results from Experiment 1. Numbers next to lines represent treatment temperatures. Solid lines represent the long photoperiod treatment (15 h), and dashed lines represent the short photoperiod (12 h). Total weight generally decreased over time.

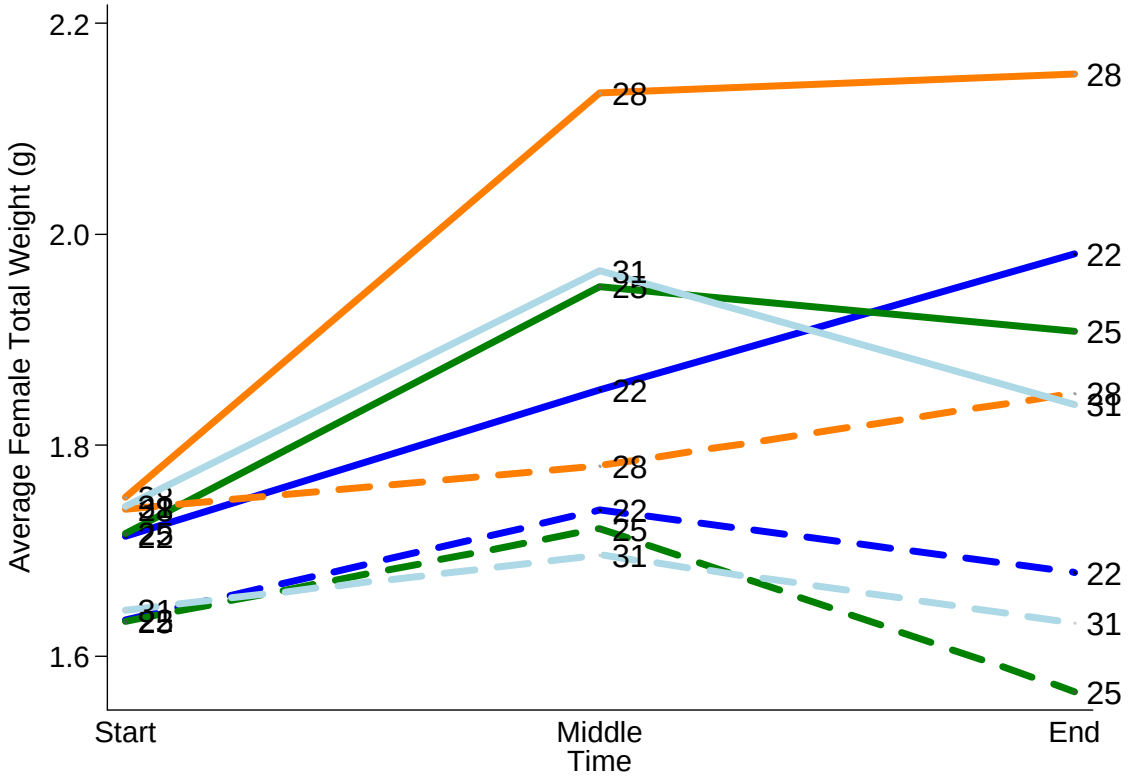

Figure 14: Illustration of the female total weight results from Experiment 1. Numbers next to lines represent treatment temperatures. Solid lines represent the long photoperiod treatment (15 h), and dashed lines represent the short photoperiod (12 h). Long photoperiod treatments tended to yield greater total weight, and total weight generally increased over time.

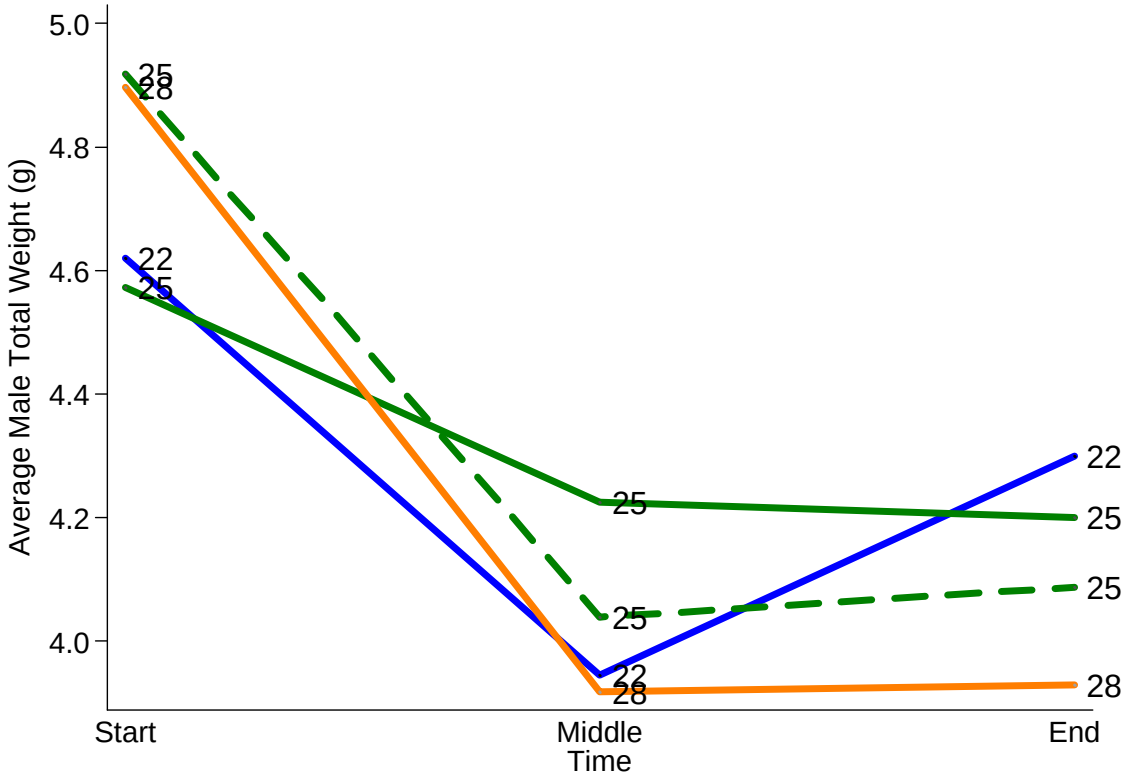

Figure 15: Illustration of the male total weight results from Experiment 2. Numbers next to lines represent treatment temperatures. Solid lines represent the long photoperiod treatment (15 h), and dashed lines represent the short photoperiod (12 h). Total weight generally decreased over time, with the majority of the loss occurring in the first half of the experiment.

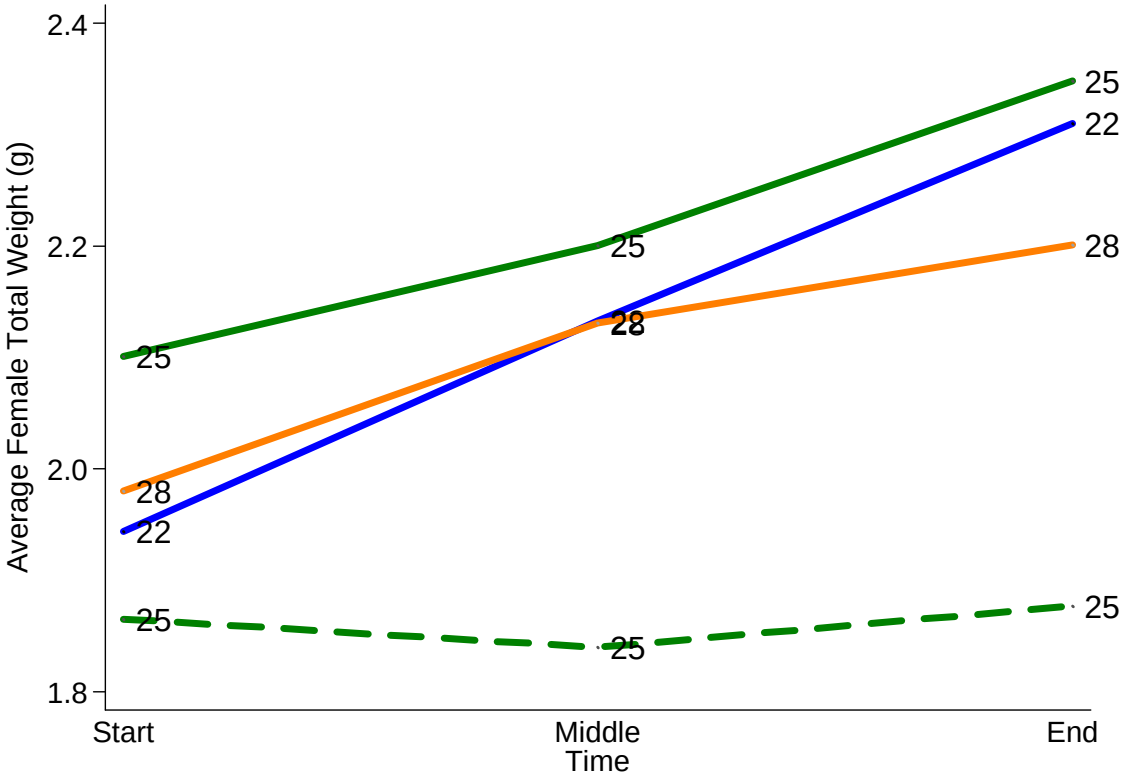

Figure 16: Illustration of the female total weight results from Experiment 2. Numbers next to lines represent treatment temperatures. Solid lines represent the long photoperiod treatment (15 h), and dashed lines represent the short photoperiod (12 h). Females exposed to the 25 °C/12 h treatment averaged significantly lower total weight, and total weight generally increased over time.

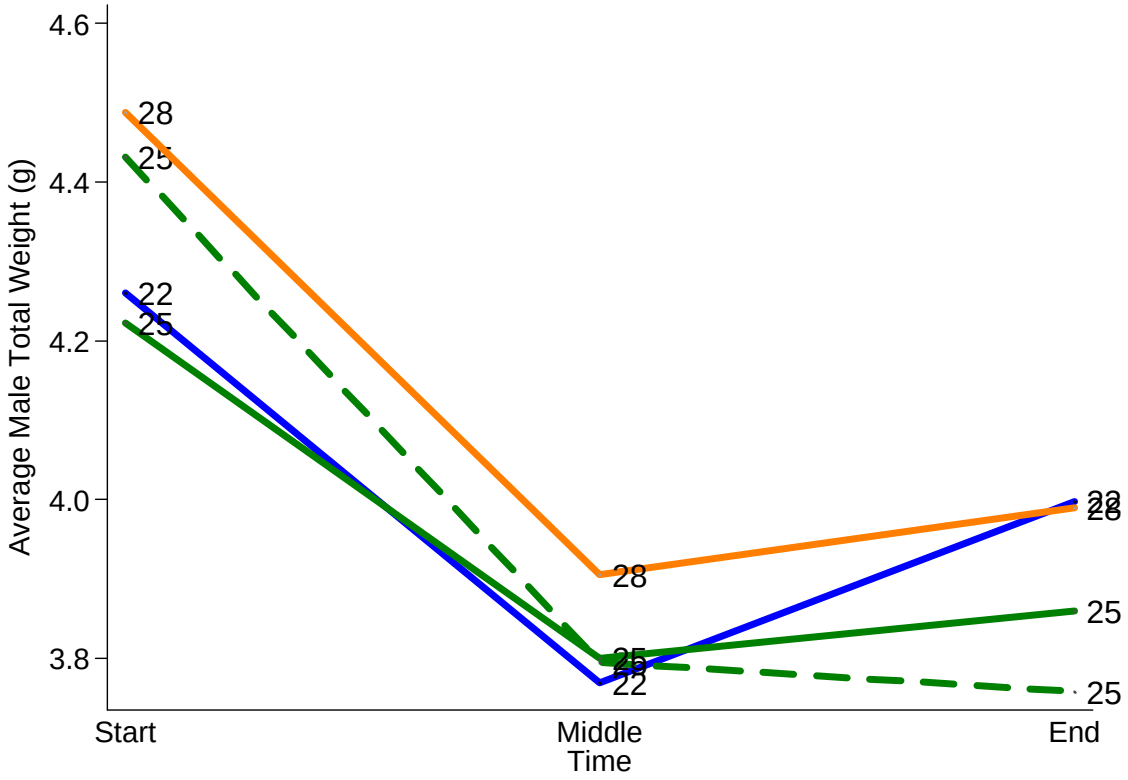

Figure 17: Illustration of the male total weight results from the combined experiment analysis. Numbers next to lines represent treatment temperatures. Solid lines represent the long photoperiod treatment (15 h), and dashed lines represent short the photoperiod (12 h). Total weight generally decreased over time, with the majority of the loss occurring in the first half of each experiment.

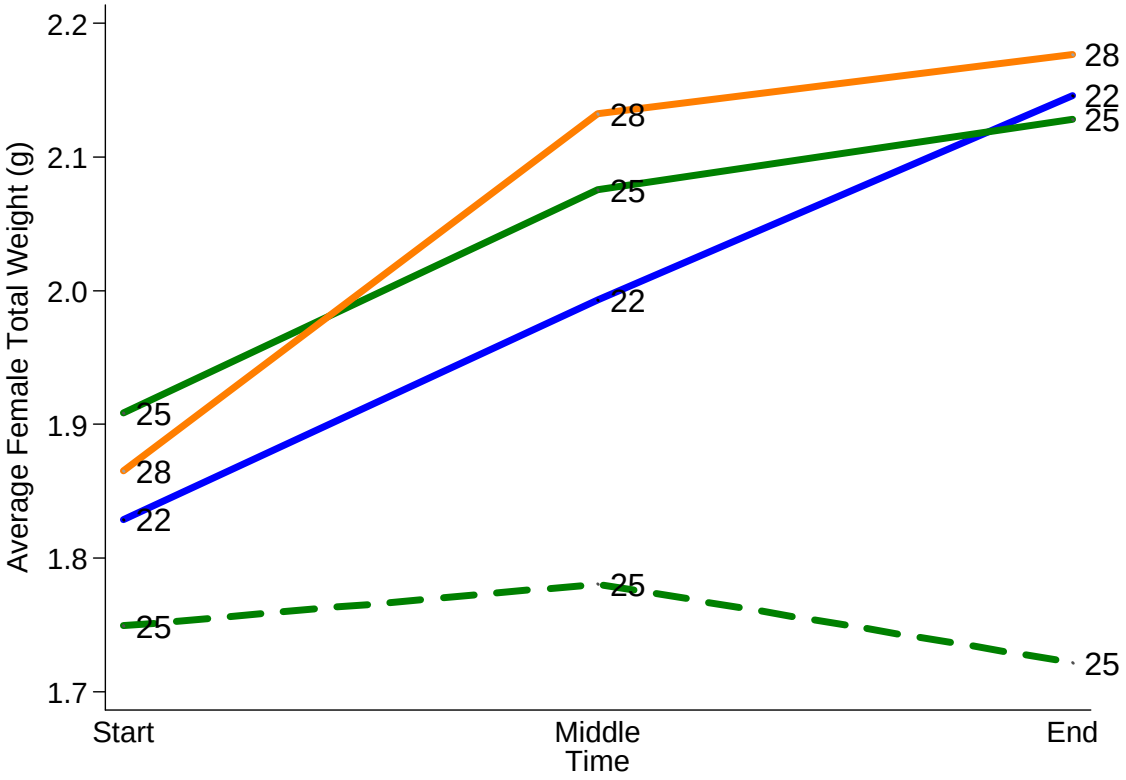

Figure 18: Illustration of the female total weight results from the combined experiment analysis. Numbers next to lines represent treatment temperatures. Solid lines represent the long photoperiod treatment (15 h), and dashed lines represent the short photoperiod (12 h). Differences between the treatments were apparent because the 25 °C /12 h treatment group exhibited lower total weights than did any of the 15 h photoperiod treatment groups.

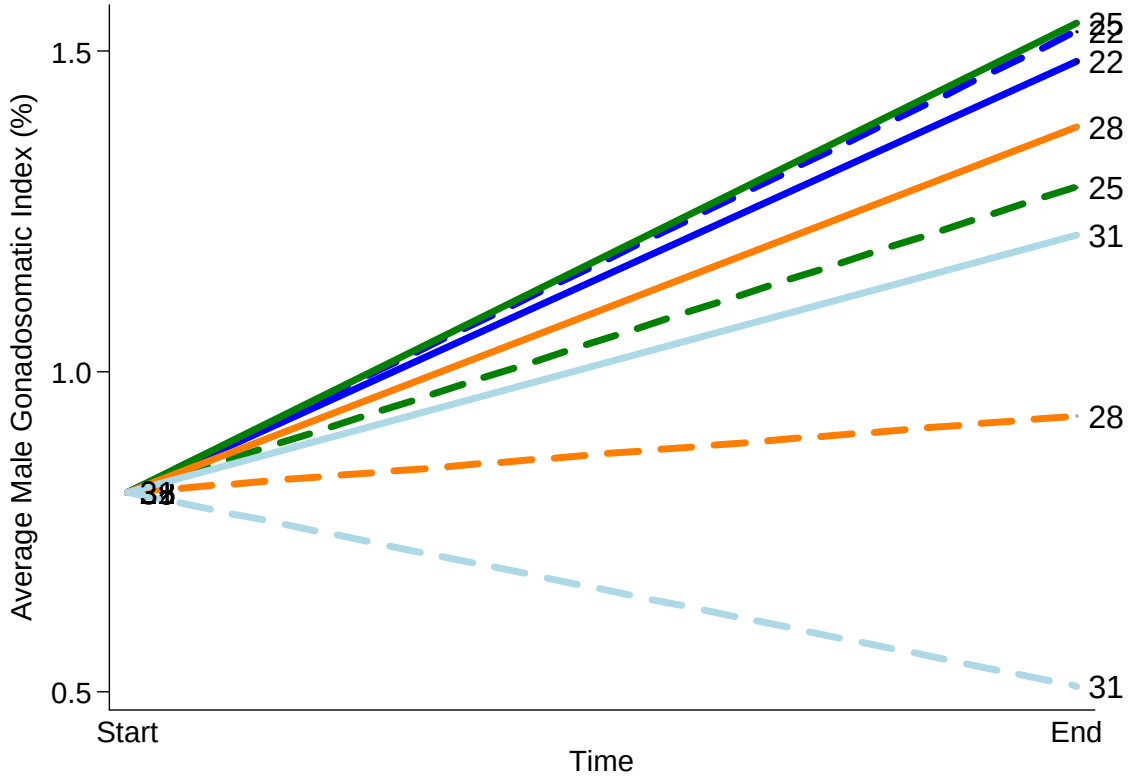

Figure 19: Illustration of the male gonadosomatic index (GSI) results from Experiment 1. Numbers next to lines represent treatment temperatures. Solid lines represent the long photoperiod treatment (15 h), and dashed lines represent the short photoperiod (12 h). Average male GSI was greater for both the 22 and 25 °C than for the 31 °C treatments, and greater GSI values were recorded from fish in the 15 h than in the 12 h photoperiod treatments.

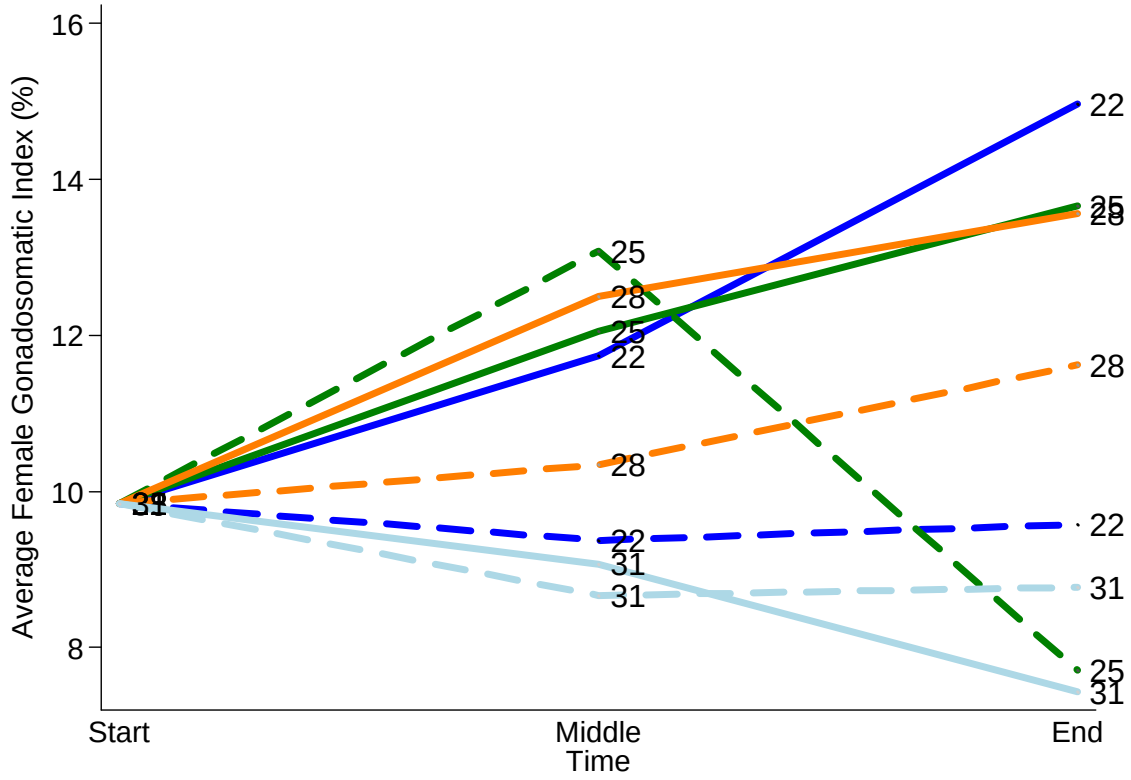

Figure 20: Illustration of the female gonadosomatic index (GSI) results from Experiment 1. Numbers next to lines represent treatment temperatures. Solid lines represent the long photoperiod treatment (15 h), and dashed lines represent the short photoperiod (12 h). Female GSIs were greater at 22 and 28 °C than at 31 °C, and greater under the 15 h photoperiod.

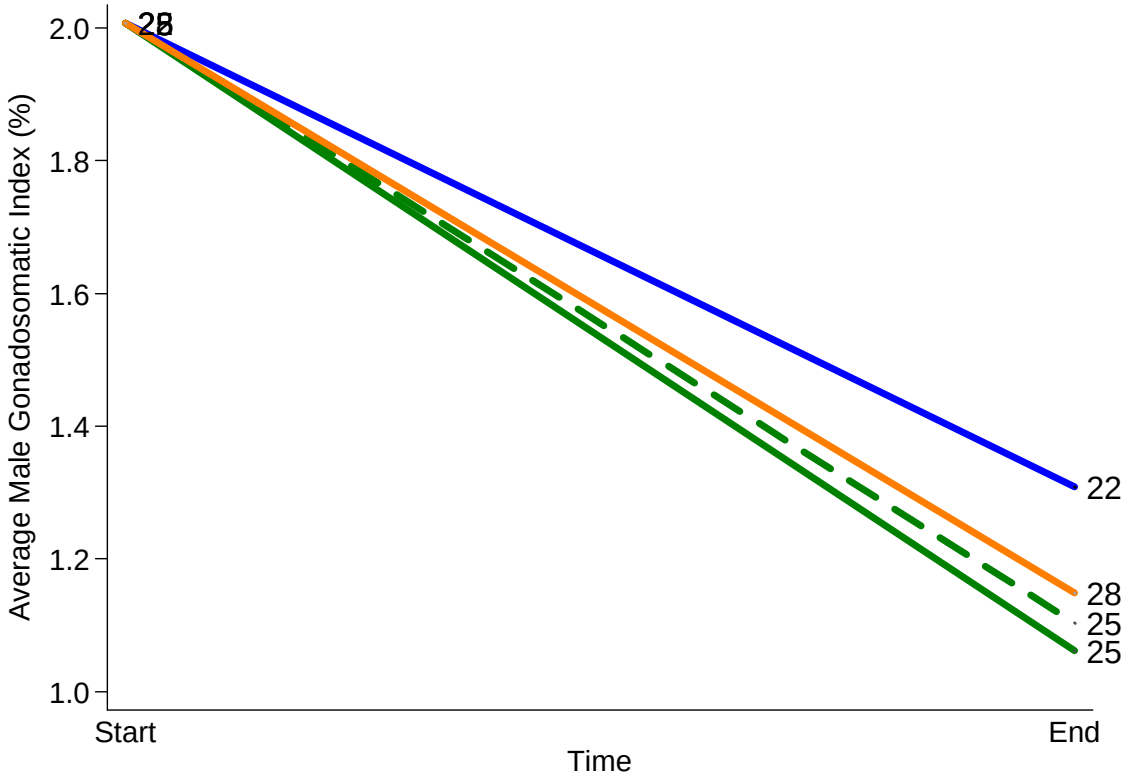

Figure 21: Illustration of the male gonadosomatic index (GSI) results from Experiment 2. Numbers next to lines represent treatment temperatures. Solid lines represent the long photoperiod treatment (15 h), and dashed lines represent the short photoperiod (12 h). No treatment effects were detected.

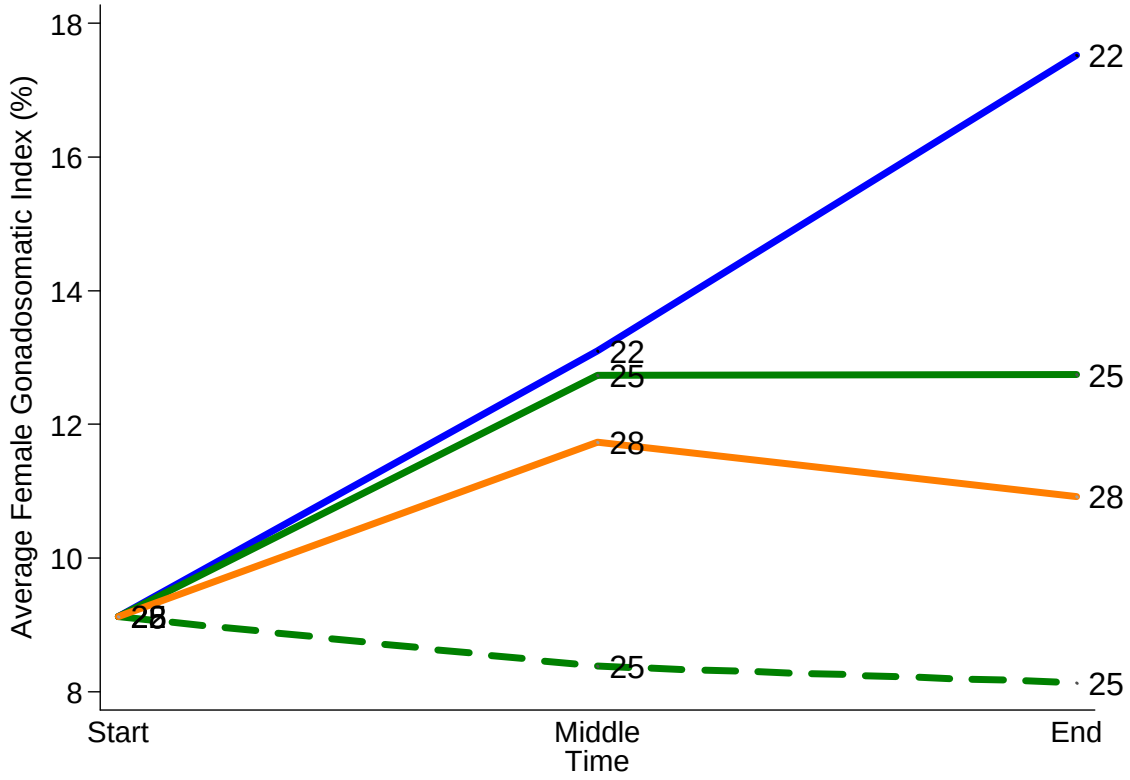

Figure 22: Illustration of the female gonadosomatic index (GSI) results from Experiment 2. Numbers next to lines represent treatment temperatures. Solid lines represent the long photoperiod treatment (15 h), and dashed lines represent the short photoperiod (12 h). The 22 °C/15 h photoperiod treatment yielded comparatively larger GSIs relative to the remaining treatments. In addition, female GSIs differed between the 25 °C/15 h and 25 °C/12 h treatments, with the former yielding appreciably greater values.

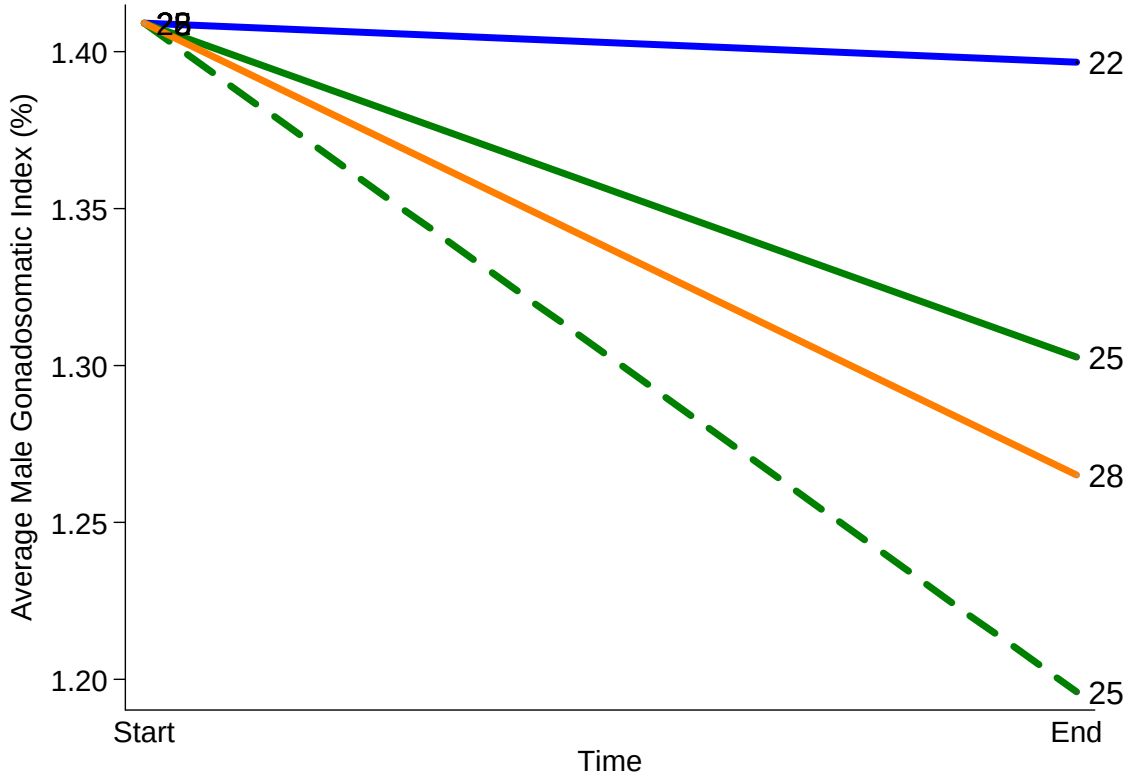

Figure 23: Illustration of the male gonadosomatic index (GSI) results from the combined experiment analysis. Numbers next to lines represent treatment temperatures. Solid lines represent the long photoperiod treatment (15 h), and dashed lines represent the short photoperiod (12 h). No treatment effects were detected.

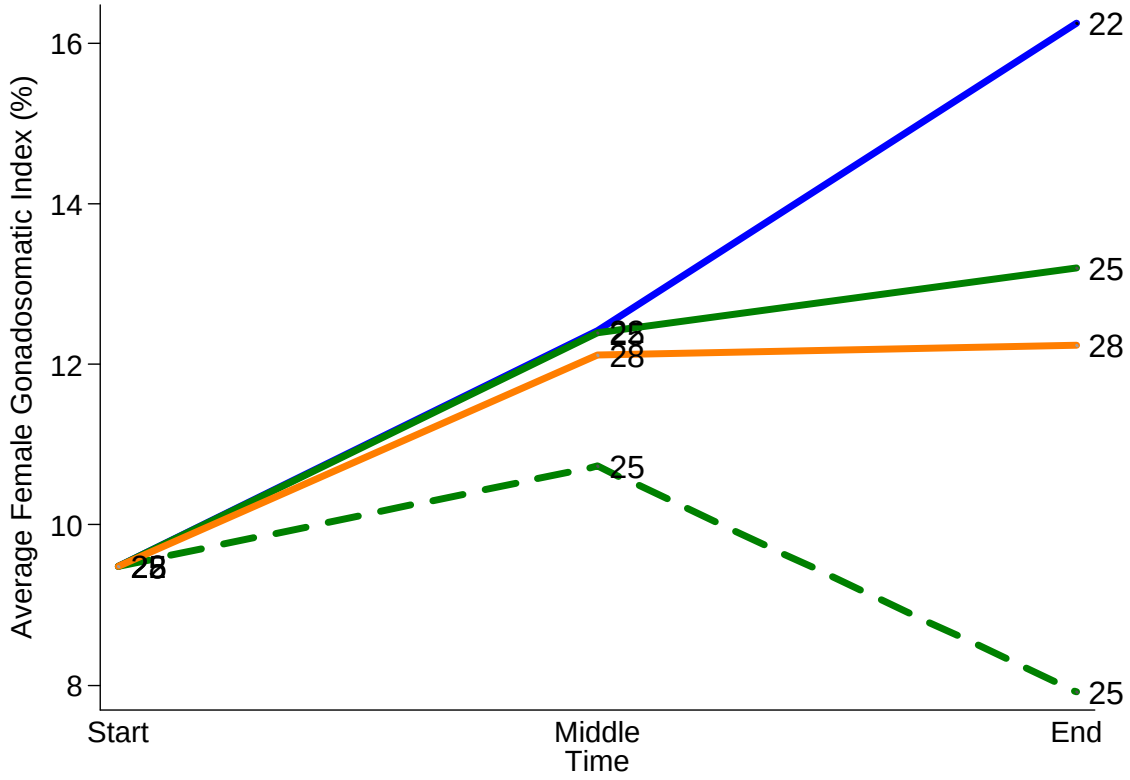

Figure 24: Illustration of the female gonadosomatic index (GSI) results from combined experiment analysis. Numbers next to lines represent treatment temperatures. Solid lines represent the long photoperiod treatment (15 h), and dashed lines represent the short photoperiod (12 h). By the end of the experiment, GSIs were significantly greater in the 22 °C/15 h photoperiod treatment than all other treatments, and lower in the 25 °C/12 h photoperiod treatment than all others. The 25 °C/15 h and 28 °C/15 h treatment results were not significantly different from one another.

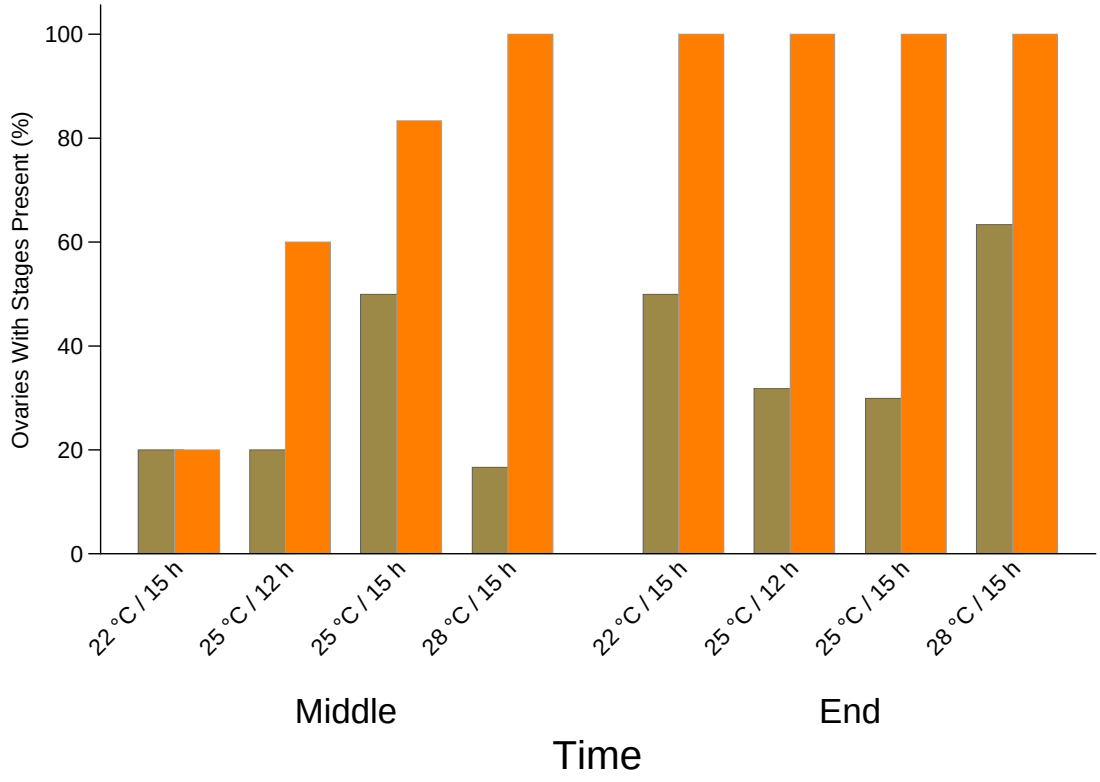

Figure 25: Average percentage of Topeka shiner ovaries containing atretic (brown) and empty (orange) follicles in the middle (left) and end (right) of Experiment 2.

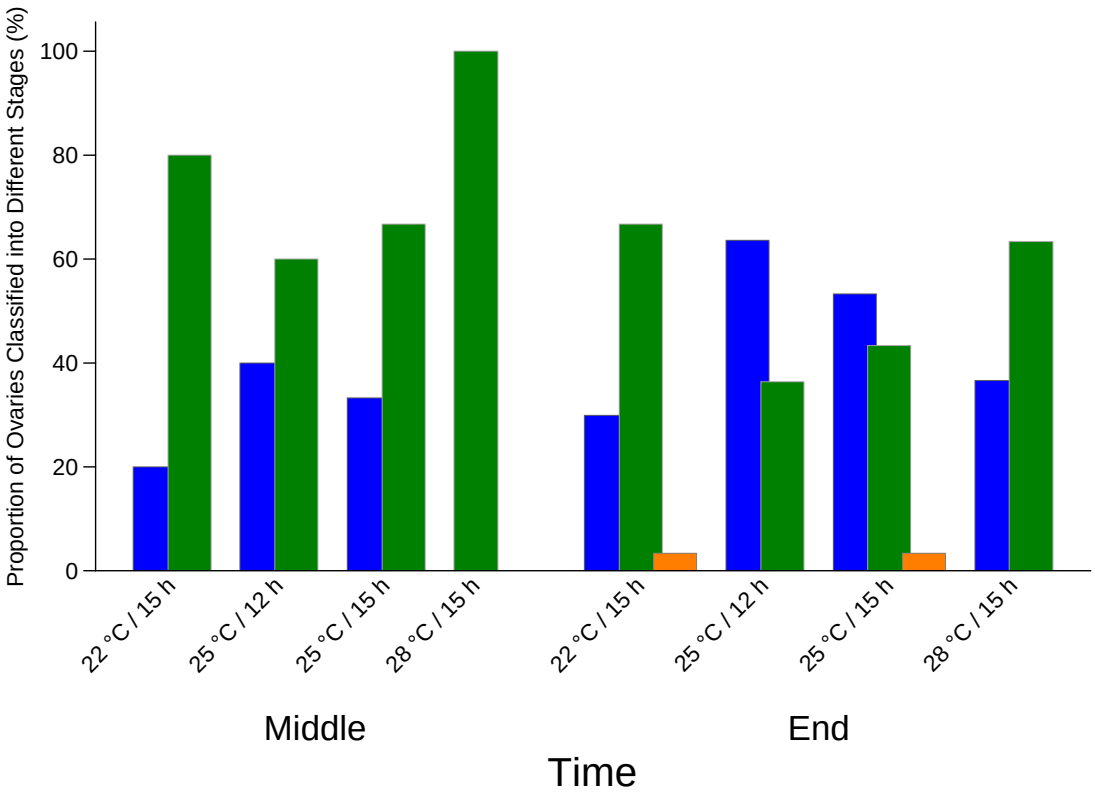

Figure 26: Percentages of ovaries classified into previtellogenic (blue), vitellogenic (green), and empty (orange) developmental categories at the middle (left) and end (right) of Experiment 2. The criterion for classification was that the majority of follicles were of the designated stage.

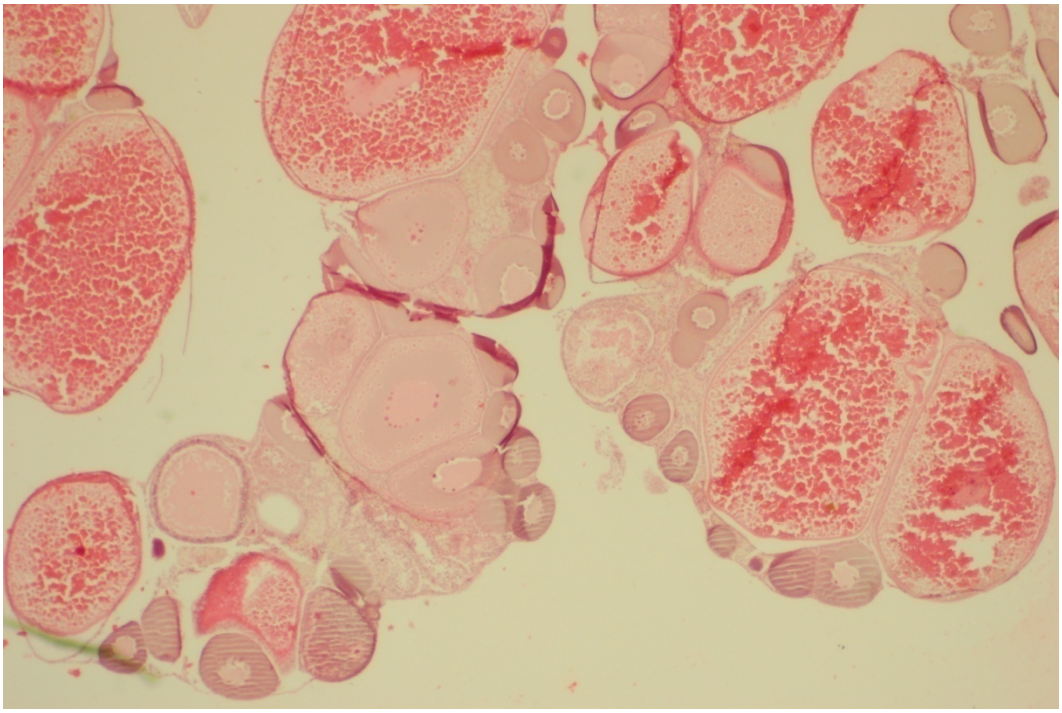

Figure 27: Ovary from a Topeka shiner from Experiment 2 where previtellogenic oocytes are replacing empty follicles with previtellogenic oocytes. Shown here are previtellogenic oocytes forming in locations where follicular cells have coalesced after having released mature ova, as evidenced by the close proximity of the previtellogenic oocytes to the empty follicles.

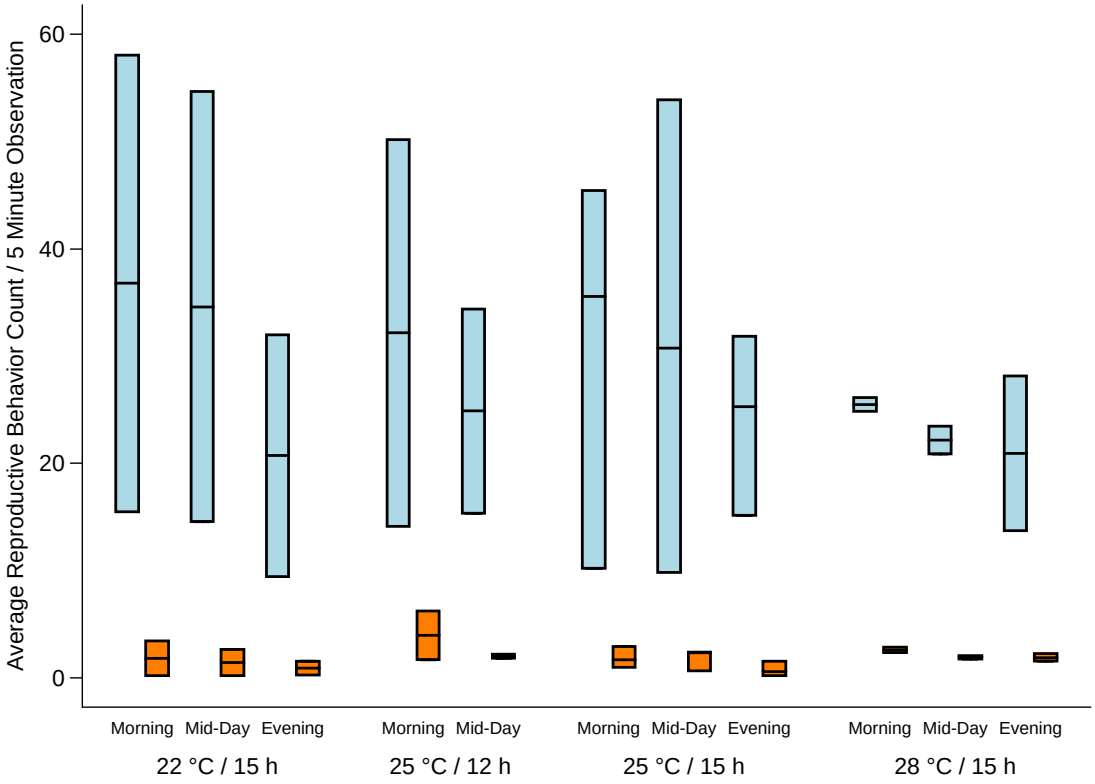

Figure 28: Frequencies of occurrence of the Male (blue) and the Female to Male (orange) behaviors of Topeka shiners by treatment and period of day for the 15 h photoperiod treatments. Boxes span the 75th and 25th percentiles; center line depicts the median. There were no significant differences between treatments, but significant within-treatment period effects were detected. Female to Male behavior occurrences tended to decrease in frequency as the day progressed.

Table 1: Ethogram of Topeka shiner behaviors observed during this study. For analysis purposes, the Specific Behaviors were pooled into General Behavior categories based on the gender of the fish initiating the Specific Behavior.

General Behavior	Specific Behavior	Description
Male, or Female to Male	Charge	Fast swim towards an individual conspecific.
Male, or Female to Male	Abdominal Nudge	Fish nudges conspecific's underside with head.
Male, or Female to Male	Incomplete Charge	Fish moves, and conspecific moves away. Often appears to be an aborted Charge.
Male, or Female to Male	Slow Approach	Fish slowly swims toward or follows a conspecific. Resembles Charge, but approach velocity is much slower.
Male, or Female to Male	Wrestle	Conspecifics butt heads, sometimes resulting in a head-to-tail circular swimming/chasing motion.
Male	Circle Swim	Fish swims in a complete continuous circle or figure-eight pattern, in a horizontal plane.

Substrate Preference

of Territorial Male Topeka Shiners (*Notropis topeka*)

in the Absence of Sunfish (*Lepomis* sp.)

ABSTRACT

Topeka shiners (*Notropis topeka*), an endangered minnow species, typically spawn on or around the nests of breeding *Lepomis* sunfish (Centrarchidae). Why spawning Topeka shiners are attracted to these nests is unclear, but having the nesting sunfish provide shiner eggs with improved aeration, relief from siltation, and protection from egg predators are possibilities. We tested the substrate preferences of Topeka shiners in outdoor tanks in the absence of sunfish to determine the shiner's fundamental preference. Shiners were provided with patches of cleaned Fines, Small Gravel, Large Gravel, and Small Cobble substrate, and the Bare Floor of the tank. The substrate above which a male shiner established his territory was used as evidence of preference. A statistically significant preference for Fine substrates was demonstrated. This fundamental preference may influence which sunfish nests Topeka shiners use, given that nest substrate characteristics differ both between sunfish species and within species by spawning site location.

INTRODUCTION

Some species of the genus *Notropis*, commonly referred to as shiners, are attracted to and establish spawning territories over, or on the periphery of, sunfish nests (Noltie & Smith 1988; Pflieger 1997). Fish that behave in this manner, including Topeka shiners (*Notropis topeka*), are termed “nest associates” (Johnston & Page 1992). There are at least three hypothesized benefits of nest association.

First, with respect to habitat, the fanning and nest-building activities of the host sunfish sweep away most of the smallest substrate particles, rendering the in-nest substrates more coarse, less embedded, and oftentimes of a contrasting color, when compared to those adjacent. Johnston (1994) notes that these substrate patches may constitute the best available spawning habitat in a location, in that the risk of deoxygenation is minimized, interstitial spaces between substrate particles become available for shelter against predation, and the risks of burial due to silt deposition are reduced. As such, Miller (1964) noted that the nests of river chub (*Nocomis micropogon*) seemed to be what attracted spawning aggregations of common shiners (*Luxilis cornutus*), and not the nest host itself. Reinforcing the importance of substrate, Topeka shiners (*Notropis topeka*) have successfully spawned in an aquarium that lacked sunfish but contained several spawning substrates, one of which was a “simulated pebbled sunfish nest” of unspecified particle size. The dominant male’s territory

was established over this pebbled nest, and most of the deposited eggs were discovered therein (Katula 1998).

The second benefit of nest association is that when shiner eggs are deposited in a *Lepomis* nest, the parental male sunfish protects the nest's contents from potential egg predators and aerates any eggs within by the fanning motions of his fins (Johnston 1991). The quality of care provided by the host thus becomes an issue; correspondingly, both Hunter (1961) and Johnston (1994) found that redfin shiners (*Lythrurus umbratilis*) were more attracted to the host sunfish than to its nest.

Third, the nest associate and host may simultaneously benefit from having their eggs co-occupy a nest through the dilution effect (Alcock 2001), as the risk of egg predation is subsequently spread across more total prey items. This assumes that neither the sunfish host nor its progeny are appreciably disadvantaged by having additional heterospecific eggs co-occupy the nest.

To reproduce, male Topeka shiners establish spawning territories from late May to mid-July over and around the periphery of the nests of spawning sunfishes (Pflieger 1997). Topeka shiner females enter these territories, are courted, and then release eggs into the water column alongside the male (Stark et al. 2002). The fertilized ova then settle to the bottom where they become adhesive (James Candrl, pers. comm.). As such, they belong to a subset of Balon's (1975; 1990) lithophil guild of non-guarding, open substratum egg scatterers, that are rock and gravel spawners with benthic larvae. They are also termed "broadcast spawners" (Johnston & Page 1992): no direct

intraspecific parental care is provided, although some interspecific care may accrue indirectly through the efforts of the nesting sunfish. An interesting suggestion (Hatch 2001; Kerns & Bonneau 2002; Mammoliti 2002) is that this nest association could be an evolutionarily recent behavioral adaptation in response to increased siltation caused by post-settlement changes in human land use practices and the resulting search by Topeka shiners for the suitable substrates (i.e., those exposed by the nesting sunfish.

Originally, the Topeka shiner's historic range spanned the plains of the upper midwestern United States within the borders of South Dakota, Minnesota, Iowa, Nebraska, Kansas, and Missouri (Tabor 1998). The species' current range is estimated to be 80 % smaller; consequently, Topeka shiners have been designated as federally endangered (Tabor 1998). In Missouri, their occurrence may be more closely linked to the physical properties of streams (i.e., steeper gradients and vegetated banks) than to chemical or biological characteristics such as water quality or fish community structure (Bayless et al. 2003). Knowing which substrates Topeka shiners prefer to spawn over will help guide conservation efforts to protect or create spawning habitat, or to identify suitable sites for reintroduction.

In the present study, we experimentally test the substrate preferences of Topeka shiners during the breeding season using groups of adult males and females in large outdoor experimental tanks containing patches of cleaned Fines, Small Gravel, Large Gravel, Small Cobble, and Bare Floor substrate. The substrate above which a male established territory was used as evidence of

preference. Given the possibility that the tendency to associate with nesting sunfish is a recent development (see above), our tests here were completed in the absence of sunfish to determine the shiner's fundamental substrate preference.

MATERIALS AND METHODS

Fish Maintenance

Given the status of wild populations of the species, we obtained our experimental animals as young-of-the-year on December 17, 2003, from the Missouri Department of Conservation's Lost Valley Fish Hatchery, Warsaw, Missouri. These were maintained indoors at the U.S. Geological Survey's Columbia Environmental Research Center (CERC), Columbia, Missouri, in three 910-L indoor holding tanks until the individuals reached maturity.

Preceding the experiment, approximately 275 adults (75 males, 200 females) were selected from our stock population, after which they were maintained indoors for 8 months in a 720-L Living Stream System (Frigid Units; Toledo, Ohio). Continuous water circulation and temperature regulation (20 to 22 °C) were provided by a 3000-Watt water heater/chiller (Frigid Units; Toledo, Ohio). Aerated well water provided continuous fractional water changes and the replacement of evaporative losses. A 12L:12D (hours of light: hours of dark) photoperiod was maintained during this time by means of overhead fluorescent lighting. The males and females were segregated in the holding tank using a plastic mesh divider that allowed water and chemical cues to circulate throughout.

On May 13, 2006, approximately two weeks before the start of the substrate preference experiment, all of the males and females were moved outdoors and held separately in two large holding tanks identical to those in

which the experiment would be run. Each tank's floor (PolyTuff Water Tanks, Columbus, Nebraska) measured 150 cm in diameter, and held approximately 1000 L when filled to a depth of 38 cm. A submersible magnetic-drive pump (Model MD-24; Drs. Foster and Smith, Rhinelander, Wisconsin), placed near the side within each holding tank, circulated water through an external vertical degasser to release ammonia and provide aeration. The degasser was self-constructed using 75 cm-tall X 11 cm-diameter PVC pipe, and was filled with 3.8 cm-diameter polypropylene degassing rings (Jaeger Products Inc., Houston, Texas). A fine mesh cover was placed over each tank, which provided protection from potential avian predators and prevented fish escape but still allowed a clear view of the fish's behavior.

When moved outdoors, some males were already showing breeding coloration and tubercles atop their heads (Pflieger 1997). After approximately one week, warming "outdoor" weather increased tank water temperatures to near 25 °C, after which the fish were allowed an additional week's conditioning before beginning the experiment. The best account of captive Topeka shiner spawning indicates that 25 °C is the optimal spawning temperature (Katula 1998).

Substrates

All substrates for the preference experiment were collected from a stream previously occupied by Topeka shiners (Gans Creek, near Columbia, Missouri; see Gelwicks and Bruenderman 1996) with the exception of

the Small Gravel which was already on hand and clean. Hamilton's (1984) substrate size classification was used to delineate Fines (F; < 2 mm), Small Gravel (SG; 4-25 mm), Large Gravel (LG; 50-75 mm), and Small Cobble (SC; 76-150 mm). Sieving was used to isolate each of the smaller size classes, whereas the Small Cobble was graded by measuring each particle's medial dimension, or b-axis (i.e., neither the longest nor the shortest axis). For cleaning, the separated substrates were soaked in water for a day, scrubbed with a brush, and then rinsed. During cleaning, many of the smaller-sized particles in the Fines substrate were washed away; consequently, the remaining particles were predominantly those at the upper end of the size distribution.

Our circular substrate patches (diameter 30 cm) were placed on 12" Plant Pallets (Plastec, Delray Beach, Florida). We selected this patch size because Becker (1983) noted that green sunfish (*Lepomis cyanellus*) nests, which Topeka shiners have been reported to spawn over and around (Pflieger 1997), can range from 15 to 38 cm in diameter. The four substrate patch locations (one of each substrate size) were spaced evenly throughout each tank (Figure 2), with each being assigned a location at random. From its perimeter, each substrate patch was 20 cm away from the tank wall, 25 cm away from the adjacent substrate patches, and 50 cm from the substrate patch across the tank center from it.

Experiment Set-Up

Our objective was to stock each of the 10 experimental tanks (Figure 1) with six females and two males randomly selected from the outdoor holding tanks.

However, a few of the individuals initially thought to be females proved to be immature males, which later required us to include sex ratio as a variable in our analyses. This 6 female:2 male target sex ratio allowed malesto vie for territory, allowed each male to be tracked for behavioral data collection without overwhelming the observer, and maximized use of the fish available for the experiment.

Each experimental tank was filled to a depth of 38 cm with well water the day prior to stocking to allow the water temperatures to rise to ambient. Fish were then introduced into each tank, allowing a full day for them to acclimate to their new surroundings. Once in the experimental tanks, the fish were fed daily (Prime Tropical Flake Mixed; Ziegler Bros. Inc., Gardners, Pennsylvania) following every mid-morning and mid-afternoon observation period (see below).

Tank dissolved oxygen levels were measured after each feeding at mid-depth, approximately half the distance from the tank wall to the center of the tank, using an oxygen meter (YSI Model 95; Yellow Springs Instruments, Yellow Springs, Ohio) that we calibrated daily. Dissolved oxygen was measured just after feeding to confirm that adequate levels were maintained (concentrations varied from 6.69 to 11.78 mg/L across the tanks and runs).

Water temperatures were continuously monitored at the center of each tank, from the day of filling, using temperature loggers (Hydrolab Hobo Water Temp Pro; Onset Computer Corporation, Bourne, Massachusetts) positioned mid-depth (Figure 2). Temperatures were logged every 10 min, and the one

recorded nearest to the time each observation period began in a tank was used in subsequent analyses.

The complete experiment was executed in two runs using 10 tanks each (first and second runs began on May 29, 2006 and June 3, 2006, respectively). Each run lasted for three observation days (see below); two days separated the two runs. Before the second run, all the tanks were drained, scrubbed, and refilled with well water as before. Each patch of substrate was also re-cleaned and was randomly assigned a new position in its tank, irrespective of its position during the previous run. One day was again allowed for the temperature of the replacement water to equilibrate to ambient before the second run's fish were added to each tank. The fish used in the second run had been brought outside at the same time as the first run's fish, and therefore had experienced an additional five days of outdoor exposure.

Fish Observations

Fish observations commenced following the acclimation day. Observations were made five times daily at 0600 h (sunrise), 0925 h (mid-morning), 1250 h (midday), 1615 h (mid-afternoon), and 1935 h (sunset) on each of three consecutive days (May 29-31 and June 3-5 for the first and second runs, respectively). These times were chosen to account for possible fluctuations in fish activity throughout the day. Fish in each tank were observed for 5 min during each observation period, from behind and through small slits in

a 1.5 m-high black plastic blind that encircled the outer perimeter of all of the tanks (Figure 1). The start of every observation period began by randomly selecting the first tank to observe. Subsequent observations progressed from tank to tank in a counter-clockwise direction around the perimeter of the tank array.

To begin each observation of a tank, we recorded the time and percent cloud cover (estimated by eye from the visible sky) in an effort to assess whether these potential factors might also influence the fish's behavior. The substrate patch over which each male held its territory was then recorded, this determination being based on whether an individual male (i) remained over or within 10 cm of a single substrate for three or more minutes of the 5-min observation period, and (ii) did not spend the remaining time in another specific location. If the territory occupied was not over one of the substrate patches we provided, a "Bare Floor" (BF) association was recorded. Where the males in a tank failed to meet the criteria above during a 5-min observation period, a "No" (N) substrate association was recorded (i.e., males showed no fealty to a particular location).

Territorial males also exhibited one or more of three behaviors, "Figure 8", "Charge", and "Return", the frequencies of occurrence of which we recorded. Behaviorally,

i) the Figure 8 behavior involved a territory-holding male swimming rapidly outwards from its territory along a looping path that

frequently formed a figure 8 or circle, such forays not being directed toward any particular conspecific;

ii) the Charge behavior involved a rapid velocity, short-duration, direct swim toward a particular conspecific, the distance traveled being longer if a fleeing conspecific was followed;

iii) the Return behavior involved the territorial male leaving its territory and then returning without aggressively pursuing conspecifics, its velocity being more leisurely and the path being more variable than for the Figure 8 and/or Charge.

After each run of three observation days, all the fish from each tank were removed and euthanized by means of Tricaine-S™ MS222 (Western Chemical Inc., Ferndale, Washington) overdose. Each fish's Total Length (TL; mm), Total Weight (TW; mg), Gonad Weight (GW; mg), and Eviscerated Body Weight (EBW; mg) were measured respectively by ruler (mm) or an electronic balance (AT261 Delta Range; Mettler-Toledo, Columbus, Ohio) accurate to 0.0001 g. A gonadosomatic index (GSI; %) for each fish was calculated by dividing the gonad weight by the eviscerated body weight and multiplying the quotient by 100 %, following Dahle (2001). Our measures of tank sex ratio (female:male) were based on these gonadal sex determinations. Finally, an average TW, TL, and GSI was calculated across the fish of each sex within each tank.

Data Analysis

Between-Run Comparisons: To maximize our sample size, we pooled the data from the two experimental runs. Before doing so, a t-test was performed to compare the mean TL, TW, and GSI values for each of the ten tanks between the two runs. Only average male GSI differed significantly, being lower in the first run (1.07 %) than in the second (1.57 %) ($t = 2.98$, d.f. = 18, $p = 0.008$). To negate this difference, we transformed the male GSI data by calculating the differences between each experimental tank's average male GSI value and the corresponding run's average male GSI value. Subsequent analyses were carried out using these deviation values. Discussion of analyses conducted on the "centered" GSI data will use the GSI* designation.

Substrate Use: We used Base SAS version 9.1 software (Cary, North Carolina) to test whether territories were held over particular patches more or less often than would be expected if all were occupied with equal frequency. A proportion of usage of each of the five substrates (the four substrate patches and Bare Floor) was calculated for the fish in each tank by dividing the number of times each substrate was chosen by the total number of observations recorded for that tank. This resulted in 20 proportion of usage values (one from each experimental tanks) for each of the six outcomes. Because the mean of these proportional usage values did not differ significantly between the runs for any of the substrates (t-tests, all $p < 0.05$), the data for both runs were pooled. The SAS UNIVARIATE procedure then allowed us to test each of the sets of proportional usage for normality using the Shapiro-Wilks method, as well as

perform a one-sample t-test to determine whether the set of proportion values differed significantly from an expected value (using the MU0 = *expected value* option). The expected value was calculated by subtracting the number of "N" outcomes from the total number of observations made during the experiment, and dividing the difference by five. If the data were normal, the Student's t-test statistic was used. If the data were not normally distributed, the Sign test statistic was used.

Factors Associated With Substrate Use: Substrate occupation (the outcome variable) was categorical in nature; consequently, we used multinomial logistic regression modeling for analysis purposes (Stata Statistical Software, Release 9.2; StataCorp, College Station, Texas). Our first model tested whether male substrate choice varied with respect to the fish's attributes (TL, TW, GSI, female:male sex ratio) or the prevailing environmental factors (water temperature, cloud cover). The second model tested whether the frequency of behaviors associated with male territoriality (Figure 8, Charge, Return) differed across the respective substrate types.

Analytically, multinomial logistic regression is an extension of simple logistic regression, where the outcome is membership in one of three or more categories, instead of the two in ordinary logistic regression (Hosmer & Lemeshow 2000). Specifically, we used the multinomial logistic regression approach to contrast the data associated with the uses of each particular substrate against those associated with the "N" outcome (i.e., non-occupation of any substrate).

To address concerns regarding multi-collinearity among the predictor variables in the fish attributes-environmental measurements model, preliminary pairwise correlations between temperature, cloud cover, male and female TL, TW, GSI, and sex ratio were calculated. Significant correlations ($p \leq 0.05$) were remedied by excluding one of the correlated variables from the analysis (see Results). The multinomial logistic regression was then performed using Stata's "mlogit" command (StataCorp 2005), which fits a maximum likelihood model with categorical dependent variables. The "cluster" option grouped the observations by experimental tank, since observations taken from an individual tank constituted repeated (non-independent) measures. This analysis uses a robust estimator of variance, which adjusts for the number of observations and number of clusters within a finite sample (StataCorp 2005).

Ultimately, our final multinomial logistic regression model (see Results), comparing substrate occupation with the fish and environmental measurements, included only variables that were measured at the end of each experimental run (TL, GSI, sex ratio). However, the values of these measurements were applied to every substrate occupation observation made within the corresponding tank. Therefore, these variables were considered categorical in nature (i.e., the values did not change over the three days of observation). For example, an outcome showing male GSI being positively associated with use of a certain substrate would indicate that males with larger GSIs at the end of the three day test preferred that substrate.

Behavior Occurrences: Backward selection stepwise multiple regression (Stata's "stepwise" command) was used to test whether the frequency of occurrence of particular behaviors could be predicted by either the environmental or the fish measurement data, again using the "cluster" option to group the data collected within each tank. Variables were excluded from the regression when the significance of their contribution met a $p \geq 0.25$ criterion. Although this was a liberal value, the retained variables proved far more significant (see Results).

Daily Activity Patterns: To test whether the frequency of occurrence of the Male or the Male to Female behaviors differed between observation time periods, we used a repeated-measures ANOVA (Stata's "anova" command). For each behavior, the ANOVA compared the frequency of occurrence between the tanks and time periods, with the latter constituting the repeated measure.

RESULTS

Fish Attributes

The mean male and female TL, TW, and GSI values for the overall experiment (both runs combined) were 72.8 mm and 60.2 mm, 4.03 g and 2.00 g, and 1.34 % and 3.21 %, respectively. For females, their average GSI was low compared to Dahle's (2001) 15 % estimate for mature females. In addition, many of the ovaries of our females were small, watery, and a yellowish color, suggesting that reabsorption had begun. These female GSI values differed significantly (ANOVA, $n = 145$, d.f. = 144, $F = 38.09$, $p < 0.001$) from those of two other laboratory experiments (these authors, unpublished data) wherein mean GSI values of 9.84 % and 9.12 % were reached in females maintained indoors at 22 °C and on a 12L:12D photoperiod (same as current experiment prior to placement into the outdoor tanks). The only differences between the current and the two previous experiments was that fish from the latter were subjected to simulated winter conditions (10 °C, 8L:16D photoperiod) for a month approximately one month before their GSI measurements were obtained, and were always kept in the laboratory.

The average GSI for males from the current study (1.34 %) lies between the values obtained from these authors' unpublished previous experiments (0.81 and 2.01 %), and therefore we consider this value to be within the typical range for mature males. Additionally, Dahle (2001) determined that reproductive maturity in males generally began as their GSIs reached 0.5 - 0.6%.

Factors Associated With Substrate Use

General Usage: There were six possible outcomes for any observation of a male's substrate occupation: F, SG, LG, SC, BF, and N. On average, 44% of the observations from each tank were of males that occupied No territory (N outcome; proportion of usage = 0.442; Table 1). The remaining usage proportions (i.e., when territories were established) were tested to determine whether they exceeded expectation (1/5 of 56 % = 0.112). The results (Table 1) show that only use of the Fine substrate exceeded expectation, evidence that Topeka shiners preferred to establish territories above the Fine substrates. In contrast, use of the Large Gravel and Small Cobble substrates fell short of expectation, suggesting avoidance. Use of the Small Gravel and Bare Floor substrates did not differ from expectation. However, given that the Bare Floor substrate area was larger than the area of all the other patches combined, but was still only chosen to the degree expected for the remaining patches, indications are that this substrate was avoided as well.

Behavioral Responses and Substrate Use: Three modifications to the data set needed to be made before the multinomial logistic regression analysis could be performed.

First, when No territory was established (outcome of N), the Return behavior could not occur (i.e., a fish could not Return to a non-existent territory). Consequently, the Return behavior data were excluded from the model considering the association between behavior occurrences (Charge, Figure 8)

and substrate occupation. Additionally, the frequency of occurrence of the Return behavior was found to be positively correlated with that of both the Charge ($r = 0.757$, $n = 20$, $p < 0.001$) and Figure 8 ($r = 0.767$, $n = 20$, $p < 0.001$) behavior. Therefore, Charge was the only behavior used in subsequent analyses.

Second, several variable pairs proved to be significantly correlated, requiring elimination of one of the variables from the model. Because the frequencies of occurrence of the Figure 8 and Charge behavior were positively correlated ($r = 0.738$, $n = 20$, $p < 0.001$), we excluded the former; it also occurred less frequently than Charge (average of 14.0 Charges per 5-min observation, versus 1.8 Figure 8s). Cloud cover (removed) was found to be positively correlated with water temperature ($r = 0.960$, $n = 20$, $p < 0.001$). Because fish TW was positively correlated with TL within each sex (male $r = 0.720$, $n = 20$, $p < 0.001$; female $r = 0.897$, $n = 20$, $p < 0.001$), the TW data for both sexes were also removed from the model.

Third, due to constraints regarding too few degrees of freedom, some comparisons could not be performed in the multinomial logistic regression testing of whether environmental variables and fish attributes were associated with the occupation of particular substrates. To partially remedy the problem, we removed the LG and SC substrate use cases from the data set, as these occurred only 5 times out of 283 total substrate occupation observations across both runs of the experiment combined (2 for LG, and 3 for SC). Temperature

was also removed from the initial analysis to due to lack of degrees of freedom, leaving sex ratio and male and female TL and GSI in the model.

Table 2 presents the results of performing the multinomial logistic regression analysis (data set reduced as per above) to test whether environmental variables and fish attributes were associated with particular substrate occupations (model: n = 278, clustered by 20 tanks; Wald Chi-Square = 381.27, $p < 0.001$, count pseudo-$R^2 = 0.572$). These analyses demonstrate that there was an increased probability of F being chosen (relative to N) as male GSI and female:male sex ratio increased (i.e., that male territories were more likely to be established over the Fine substrate when their own GSI values were higher at experiment's end and when there were proportionally more females present than in other trials). In addition, as the probability of SG being chosen increased (relative to N), male and female TL also increased, but male GSI and sex ratio tended to decrease (i.e., male territories were more likely to be established over the Small Gravel substrate when the males and females present were larger than in other trials, when there were proportionally fewer females present than in other trials, and when the participating males ended these trials with low GSI values).

Since the effect of female GSI was not significant in the model, we substituted temperature (originally removed for lack of model degrees of freedom) in its place to see if it had a significant effect, but it did not. Finally, none of the fish/environmental attributes that we measured (sex ratio,

temperature, and male and female TL and GSI) significantly influenced the likelihood of territories being established over the Bare Floor substrate (Table 2).

The multinomial logistic regression analysis relating substrate occupation to fish behavior (model: n = 278, clustered by 20 tanks, Wald Chi-square = 11.15, p = 0.011, count pseudo-R^2 = 0.525) found that territories were more likely to be established over Fine and Small Gravel substrates (relative to No territory) when more occurrences of the Charge behavior were exhibited (p = 0.004 and 0.001, respectively). Figure 3 further illustrates how occurrences of the Charge behavior differed across the occupied substrates across the entire experiment (i.e., all observations pooled), with the highest activity occurring over the Fines and Small Gravel substrates. Given that the count pseudo-R^2 is simply the portion of observations the model correctly predicted (i.e., the model predicted the correct outcome 52.5 % of the time), this model predicted correct outcomes approximately twice as well as a random guess (25 % chance of choosing the correct outcome, since there were four substrates considered in the model). However, it is not quite as good a predictor as the 57 % success rate of the environmental variable/fish attribute model.

Behavior Occurrences

As stated above, the frequencies of occurrence of the Charge and Figure 8 behaviors were positively correlated. Likewise, the frequencies of the Charge and Return behaviors were positively correlated (r = 0.757, n = 20, p < 0.001). Consequently, only the Charge behavior data were considered in

the stepwise regression analysis that tested whether behavior occurrence frequencies varied with fish and environmental attributes.

From our stepwise regression analysis (model: n = 278 clustered by 20 tanks, $F = 8.92$, $p = 0.002$, $R^2 = 0.125$), Charge frequency proved to be positively related to female TL ($p < 0.001$) and negatively related to female GSI ($p = 0.011$). In other words, larger females and females with low ovarian weights for their body sizes elicited more male Charges.

The repeated-measures ANOVA, which tested for differences in the occurrences of behaviors between the observation periods throughout the day, yielded no significant differences ($F = 1.20$, d.f. = 4, $p = 0.318$; see Figure 4). Therefore, activity levels were not found to vary appreciably as the day progressed.

DISCUSSION

Substrate Use

We anticipated finding that Topeka shiner males would establish territories over the coarser substrates. As precedents, Barber (1986) found that Topeka shiner fry occurred predominantly over rubble substrate and suggested that this might reflect where spawning occurred. Kuitenen (2001) also considered rubble (>128-256 mm) to be the preferred substrate for spawning.

Contrary to these suggestions, the results of the present study show that male Topeka shiners most often established territories over our Fine substrates in the absence of sunfish. Consistent with this, Topeka shiners in the wild are collected more often in low-velocity off-channel/backwater areas during the spawning season (Minckley & Cross 1959; Michl & Peters 1993; Cross & Collins 1995; Clark 2000; Blausey 2001; Dahle 2001; Hatch 2001; Kuitunen 2001; Ceas & Anderson 2004) where fine-sized substrates predominate. Hatch (2000) found evidence of reproduction in such off-channel habitats and described these as having very thick muddy substrates. In addition, Topeka shiners spawn around/over green sunfish (*Lepomis cyanellus*) nests (Pflieger 1997), and Johnston (1994) noted that “green sunfish nests can be built in substrates ranging from clean gravel to mud and are usually constructed in shallow water where there is little flow”.

The discrepancies between our results and those of other published studies may reflect several possibilities: (i) the fundamental substrate preference

of Topeka shiners differs from that of their sunfish hosts; (ii) that our experimental design altered how Topeka shiners normally exhibit substrate preference; (iii) that our hatchery-raised fish exhibited substrate preferences different than those of wild fish; and/or (iv) silt-free fine substrates, such as those used in the present study, were not available at sites where Topeka shiners were studied previously. These are listed in order of what we consider to be most probable. However, we have no way to test these possibilities.

Our finding that territorial male Topeka shiners occurred most often over the Fine substrate seems robust. However, this finding does not demonstrate conclusively that Fines are preferred for actual spawning since (i) we did not see any spawning events during our relatively brief observation periods, nor did we allow time between runs for any spawned eggs to hatch, and (ii) upon inspection, many of the female ovaries appeared to be undergoing reabsorbtion. However, Kerns (2002) states that "while guarding its spawning territory, a male swam continuously in circular and figure-eight patterns". Consequently, our observations of male territoriality, and the males' performance of the Figure 8 and Charge behaviors, strongly suggest that spawning was the intended use of the established territories.

It is unclear whether or how differences in substrate color may have influenced our results. Although most of our substrates were collected from a common location and were composed of the same brown chert-limestone material, the Small Gravel contained fractionally more whitish-colored particles, and the Bare Floor of the tanks was a smooth sky-blue plastic. One thought is

that Topeka shiner males may select territories over substrates against which they are less visible to predators from above (Donnelly & Dill 1984). If so, a more natural-looking tank floor and darker-colored Small Gravel might have increased the use of these substrates. To the human eye, males were hardest to see over the Fines substrate, the one used the most (92 of 283 observations). However, because many territories were established over the remaining substrates, and fish activity levels over the Fines were relatively high, this "self-camouflage" idea seems untenable. Alternatively, Fine substrate might be preferred because small eggs resting upon many small multi-hued particles might be less visible to potential predators than if many eggs were adhered to a larger particle of a single color.

In several sunfish species, the males nest in aggregations that are considered "colonies" (Dupuis & Keenleyside 1988; Pflieger 1997), and Topeka shiners are nest associates of at least two of these, green sunfish (*Lepomis cyanellus*) and orangespotted sunfish (*Lepomis humilis*) (Pflieger 1997). Across a sunfish colony, it is apparent that some nest-to-nest variation in substrate size occurs (DBN, pers. obs.). Given our results, it is possible that in-colony nests with finer substrates may be more attractive to Topeka shiners, all else being equal. Similarly, where the nests of more than one species of sunfish are available, Topeka shiners may opt to disproportionately use those made where finer substrates predominate. Not to be discounted, however, is whether Topeka shiner males also establish territories based on attributes of the associated male sunfish.

Other factors that influenced substrate usage were sex ratio and fish length. Male occupation of Fines relative to No Territory was positively associated with increased sex ratio (i.e., greater numbers of female:males). In contrast, male occupation of Small Gravel was negatively associated with increased sex ratio. This difference indicates that the presence of more females per male prompts males to make increased use of Fines relative to that of Small Gravel, suggesting that males can detect differing levels of female availability and refine their substrate utilization accordingly. Why the occupation of Small Gravel and not Fines was influenced by male and female size remains unresolved, but it possible that larger fish may use larger substrate sizes that smaller fish do not.

Behavioral Responses

It was not surprising that the frequencies of occurrence of the Figure 8 and Charge behaviors were correlated since the two differed little, with Figure 8 not being directed at individual conspecifics whereas Charge was. These behaviors were exhibited more frequently as the probability of territories being established over Fine and Small Gravel substrates increased. This suggests that not only were Fines chosen more often, but they were also defended more actively.

Territorial males exhibited more frequent Charge behaviors when those females that were present were of greater total length. This suggests that males may behave aggressively towards any territorial intruders, scaling their efforts according to intruder size. Alternatively, larger, less gravid females (perhaps

intent on egg cannibalism) may have been less easy to drive away and thus elicited more Charges by the males. This difference suggests that, for males, it is only when a territory intruder is identified as being a receptive female that courtship supplants territorial aggression. Similar tendencies are exhibited by other species where males guard substrate territories (Noltie & Keenleyside 1987).

In contrast, territorial males exhibited less frequent Charge behaviors when the females had greater GSI values. Here, with heightened GSIs, females may be more readily identified as potential mates, prompting males to scale back their territorial defense. Consistent with this interpretation, observations of spawning in the wild (Stark et al. 2002) indicate that Topeka shiner males will often shepherd select females into their territories; assumedly, the selected females are ones most apt to engage in spawning.

Although we recorded no feeding behavior data, most feeding would occur immediately after food was provided and while the flakes were still floating at the water surface. Bottom feeding occurred on occasion when fish bit at settled food particles as they occupied the Large Gravel and Small Cobble substrates. Consequently, occupation of the seldom-used Large Gravel and Small Cobble patches (only 5 observations out of 283) may have resulted from the feeding opportunities that the interstitial spaces within these patches provided.

Notable here is how our fish responded to inclement weather. On May 29 and May 31, 2006, afternoon thunderstorms caused rains that prevented making

some observations. Noticeable startle responses, in the form of high-velocity, short-distanced bursts of swimming, were observed following occasional episodes of thunder, which may have been felt more intensely in the experimental tanks than in a natural environment due to the sound being transferred through the walls of the tanks. As the storms approached, fish in all the tanks ceased any territorial or reproductive activities, formed a tight shoal, and began swimming around the tank. These findings suggest that Topeka shiner reproductive activity is negatively affected by stormy weather. This shift in behavior could have been prompted by the thick cloud cover, the fish having perceived the darkening of the sky as nightfall. Topeka shiners cease their territorial behavior during the night hours (CCW, pers. obs.). However, if this were the case, we would have expected to find either a difference in the frequency of Charge behavior between the sunset and other observational time periods, which our repeated-measures ANOVA did not find (see also Figure 4), or a significant relationship between cloud cover and Charge behavior from our stepwise regression analysis, which was also not apparent.

Fish Attributes

The fact that the GSI values for females in the present experiment were low, whereas the male GSI values were not (relative to these authors' previous unpublished data), suggests that the females were impacted more than were males by not having experienced an extended period of cold temperatures

preceding experimentation. Whether and how the apparently low female GSI values influenced male territorial behavior or substrate utilization is unknown.

Although we provided males with multiple females, it might have been enough to have a single reproductively mature female present to elicit male territorial behavior. To test this, we compiled the average and maximum female GSI values from each tank. The maximum female GSI values did vary across the tanks, but a correlation analysis showed that the maximum and mean GSI values were highly positively correlated ($r = 0.946$, $n = 20$, $p < 0.0001$). A consequence of this correlation was that we could not simultaneously include both variables in any analyses due to collinearity issues. However, substituting maximum female GSI into the territorial and substrate utilization analyses in place of average female GSI yielded the same results.

Methodologically, we measured GSI after the fish potentially had had an opportunity to spawn. We recognize that any such spawning could have lowered their GSI values. However, female Topeka shiners are what Heins and Rabito (1986) would consider as being multiple clutch spawners, in which breeding individuals carry ova in different stages of development within their ovaries. Therefore, even had females spawned during our experiment, we would have expected to find them with ovaries larger than in immature females, and so GSI would still be an acceptable measurement of reproductive development. For males, a study of the lemon tetra (*Hyphessobrycon pulchripinnis*) by Nakatsuru and Kramer (1982) found evidence that sperm depletion (and therefore possible testes mass reduction) can occur under intense spawning rates. However, due

to the lack of female gonadal development in our study, we doubt that our males released sperm sufficient to cause appreciable depletion. Consequently, for males, their GSI values should have been acceptable measures of their reproductive maturity.

Application

Our results are applicable to efforts to spawn Topeka shiners under laboratory or hatchery conditions: given acceptable substrates, eliciting Topeka shiner reproduction in the absence of sunfish may be logistically simpler, and might reduce space needs, feed costs, and the potential for interspecific disease or parasite transmission.

In addition, our use of behavioral responses to demonstrate spawning substrate preference in Topeka shiners has identified some interesting inconsistencies. First, the association between spawning Topeka shiners and nesting sunfish has long been recognized and is considered by many to be "typical" of the species. However, the willingness of our males to establish breeding territories in the absence of a nesting sunfish suggests that this association may be more facultative than obligate. Second, our "sunfish-free" Topeka shiner males preferred to establish territories above finer substrates than would seem characteristic of most sunfish nests. This preference may reflect the species' "fundamental" preference, one that would be demonstrated when spawning Topeka shiners find themselves without nesting sunfish. Given that nest parasitism is possibly a derived (potentially recent) trait, the coarser

substrates of sunfish nests may actually be “suboptimal” for the Topeka shiner, with the protection of the guarding sunfish counterbalancing the consequences.

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Figure 1: Experimental tanks used for observing Topeka shiner substrate occupation and behavior. To prevent startling the fish, small horizontal slits were cut in the perimeter blind for viewing. The mesh tank covers prevented fish escape and predation by birds, but did not impede viewing.

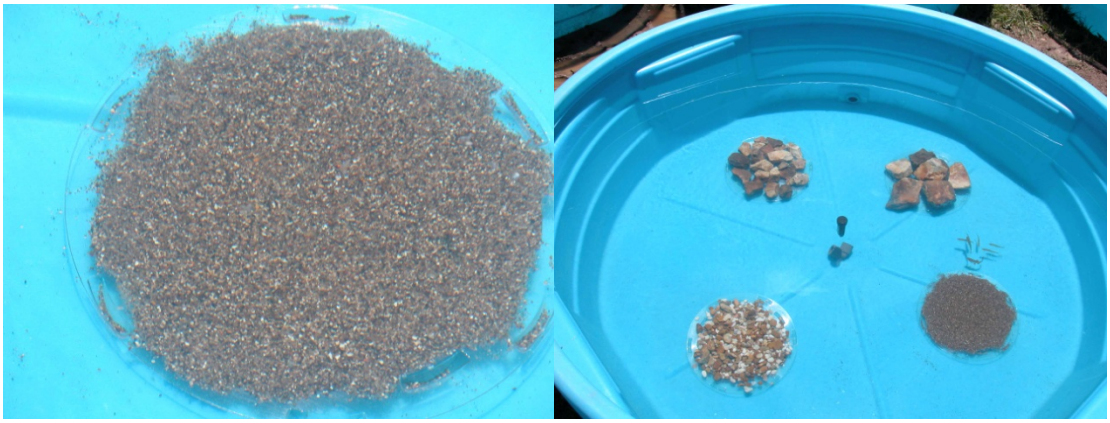

Figure 2: Left: Close-up photograph of Fine substrate patch (diameter 30 cm). Right: Photograph illustrating the substrate patches randomly assigned to positions in an experimental tank (floor diameter 150 cm), with a temperature logger anchored mid-depth at the center. Substrates: upper left = Large Gravel; upper right = Small Cobble; lower left = Small Gravel; lower right = Fine.

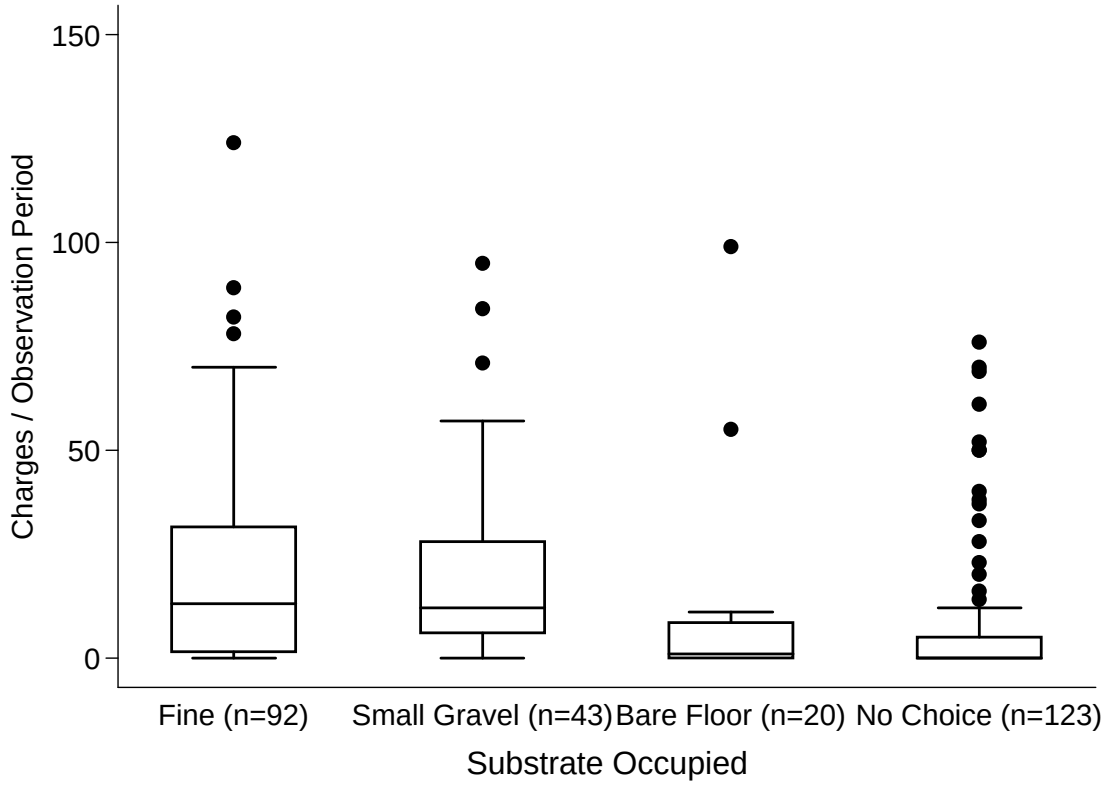

Figure 3: Box plot illustrating the number of Charge behaviors exhibited per 5-min observation period (total n = 283) above each substrate patch type, pooled across both runs of the entire experiment. Each substrate patch type is identified on the X axis, followed by the number of times it was occupied (in parentheses). Boxes span the 75th and 25th percentiles; center line depicts the median; whiskers represent the adjacent values; dots depict outside values (Tukey 1977).

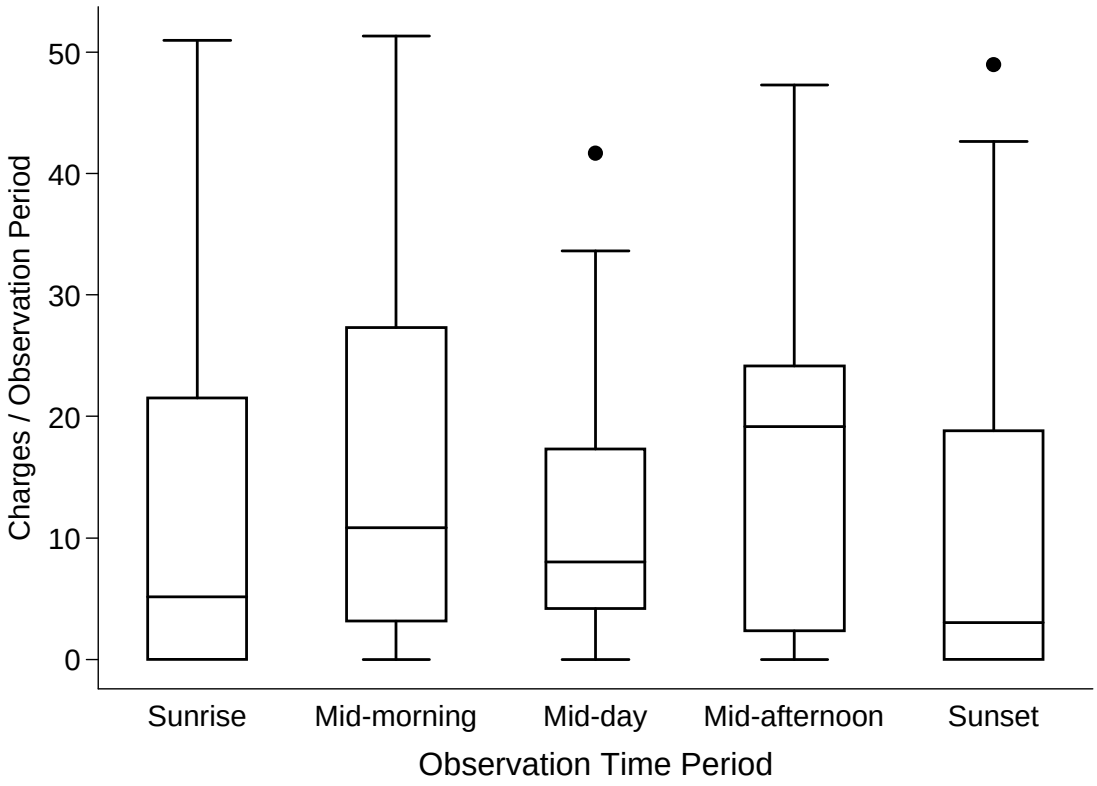

Figure 4: Box plot illustrating the number of Charge behaviors exhibited per five-minute observation period (n = 20) at intervals across the day. Boxes span the 75th and 25th percentiles; center line depicts the median; whiskers represent the adjacent values; dots depict outside values (Tukey 1977).

Table 1: Proportional usage of substrate patches, associated tests for normality, and the corresponding test for statistical significance of use. See text for analysis details. Among the observations when males established territories, occupation of the Fine substrate patch occurred significantly more often, and Large Gravel and Small Cobble significantly less often than random chance (0.1117).

				μ = 0.1117	
Substrate Occupied	Mean of Proportions (n=20)	Shapiro-Wilk Normality Test (W, p)	Normal (Y/N)	Student's t-test (t, p)	Sign Test (M, p)
Fine	0.320	0.919, 0.096	Y	3.331, 0.004	-
Small Gravel	0.151	0.694, < 0.001	N	-	-2, 0.503
Large Gravel	0.007	0.236, < 0.001	N	-	-9, < 0.001
Small Cobble	0.011	0.446, < 0.001	N	-	-10, < 0.001
Bare Floor	0.070	0.857, 0.007	N	-	-4, 0.115
No Territory	0.442	-	-	-	-

Table 2: Multinomial logistic regression result for the relationships between substrate occupation and associated environmental and fish measurement variables. The "N" outcome (No territory) was used as the reference group. TL = total length. GSI = gonadosomatic index. Regression coefficients are listed with their respective p-values in parentheses. Significant comparisons are bolded (model: n = 278, clustered by 20 tanks; Wald Chi-square = 381.27, $p < 0.001$, Count pseudo-$R^2 = 0.572$).

	Substrate Patch		
Variable	Fines	Small Gravel	Bare Floor
Male TL	0.003 (0.981)	**0.613 (0.003)**	0.074 (0.440)
Female TL	0.275 (0.157)	**0.833 (< 0.001)**	-0.174 (0.215)
Male GSI	**1.660 (0.028)**	**-3.500 (0.051)**	0.673 (0.384)
Female GSI	-0.227 (0.533)	-0.343 (0.203)	0.104 (0.546)
Sex Ratio	**0.894 (0.004)**	**-1.377 (0.004)**	0.313 (0.150)

The Influence of Temperature, Photoperiod, and Substrate Size on the Reproductive Development and Behavior of the Topeka Shiner (*Notropis topeka*): Applications and Avenues for Future Research

The goal of this research was to determine which temperature, photoperiod, and substrate conditions would best facilitate Topeka shiner (*Notropis topeka*) spawning under culture conditions and in the absence of sunfish.

My results show that male Topeka shiners prefer to establish spawning territories over substrates of relatively small particle sizes. Thus, for culture purposes, one approach to encourage spawning might be to place a removable patch of course sandy substrate within the spawning tank. This purpose of the sandy patch is to mimic a sunfish nest and to concentrate where eggs might be deposited in the tank. Removing the patch would allow it to be inspected for eggs.

Fertilized eggs would need to be removed from the spawning tank as soon as possible, given that minnows often feed on their own eggs if left unprotected (Gale & Gale 1977; Kaya 1991; Vives 1993). Topeka shiners also raid the nests of other species and feed on their eggs (Stark et al. 1999; Dahle 2001), and would most likely consume the eggs of conspecifics if given the opportunity. Because the eggs of congeneric species require 4-6 d incubation before hatching (Kaya 1991; Rakes et al. 1999), any spawned Topeka shiner eggs should be held at least this long to determine if they hatch or not.

Knowing what environmental factors control the timing of Topeka shiner spawning can be applied to a culture setting as well, the goal being to maximize production efficiency. A possible approach to spawning Topeka shiners in

captivity, using my results, would be to first simulate a short winter by lowering water temperatures as low as are feasible to maintain (10 °C was sufficient in my experiments) for at least one and perhaps as long as two months. If possible, culturists should separate males from females, but still allow water to circulate between the sexes; this should minimize the occurrence of unwanted spawning events. Allowing the water to circulate between the sexes would permit any potential reproductive chemical cues to be transferred from females to males and *vice versa*. Next, one would want to raise water temperatures incrementally to near the lower end of the range of spawning conditions (approximately 20-22 °C) and maintain them there under a relatively long photoperiod (15 h of light /day) for at least a month. This should allow females to maximally develop their ovaries (i.e., increase their GSIs by maturing more ova). Then, when most females appear very gravid, temperatures should be increased slowly (over a span of a week or two) up to, but not to exceed, 28 °C. Based on my findings that higher temperatures increase the presence of empty follicles, this increase in temperature should trigger more intense spawning activity than having no increase in temperature at all.

Overall, these results are apt to be most useful to a culturist wanting to propagate the species in captivity, where maintaining sunfish would require additional time, space, resources, and impose additional logistical constraints. Although there are sure to be other environmental factors that influence Topeka shiner reproduction (for example, the presence of sunfish nests), managers could use my temperature results to predict the time of year when spawning activity is

apt to be greatest in individual streams. This window of time should occur when average daily temperatures in the pool habitats reach 25 to 28 °C.

Future Research

Laboratory: As extensions of the current work, future research efforts could explore how Topeka shiner reproduction is affected by conditions that even more closely resemble those in nature. First, water temperatures in the headwater streams Topeka shiners inhabit exhibit wide daily fluctuations (Matthews 1988). Mimicking these daily fluctuations, as Albers (2001) did with Neosho madtoms (*Noturus placidus*), might yield greater numbers of successful spawning attempts.

Second, different sex ratios than I used might also increase the likelihood of eliciting spawning. Relatively high female to male sex ratios were used in this study in hopes of providing each mature male with at least one mature female, while minimizing male-to-male aggressiveness or territoriality. However, there are conflicting reports regarding what sex ratios occur in the wild: Dahle (2001) found that females were more common than males among age 1 and 2 individuals, whereas Kerns & Bonneau (2002) reported that males were more common among age 2 fish. Using more "natural" sex ratios might yield higher rates of successful spawning (Kvarnemo & Ahnesjo 1996).

Field: To the author's knowledge, no previous study has been aimed at determining whether substrate size preferences exist for reproduction in broadcast spawning minnows. Additional field research could explore whether

Topeka shiners, in a natural setting, display affinities for spawning over relatively small particle substrates, akin to those they preferred experimentally. This preference could manifest itself through associations with sunfish nests comprised of particles relatively smaller than surrounding sunfish nests. Also, more vigilance could be applied in the field for detecting Topeka shiner reproduction away from sunfish nests. If this behavior occurs, the substrate (both beneath the territory and in the surrounding area) should be measured and described.

Even though this species is known to be a nest associate (Pflieger 1997) of both orangespotted (*Lepomis humilis*) and green sunfish (*Lepomis cyanellus*), this research (in addition to Katula 1998, J. Candrl, pers. comm.) further supports the notion that Topeka shiners will spawn in the absence of sunfish. Therefore, the specific benefits that Topeka shiners acquire from this nest association behavior should be further explored. Again, whether Topeka shiners also spawn in the absence of sunfish nests in nature also merits investigation.

Overview

The Topeka shiner is federally-listed as endangered, due to the precipitous reduction (approximately 80%) in its distribution (Tabor 1998). Efforts to conserve the species can benefit from increased knowledge of its basic life history traits, and the environmental conditions that positively (and negatively) affect reproduction being among these. My research took advantage of laboratory and outdoor experimental environments to test how temperature,

photoperiod, and substrate size influence Topeka shiner reproduction, while controlling other factors. Such studies cannot be performed in nature because of the inability to control extraneous environmental influences. However, given the artificial conditions of my study, validating my results in the field may be necessary to convince managers of their applicability and before management decisions are made based on this research.

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