REARING AND INDUCING SPAWNING IN CAPTIVE PACIFIC SARDINE (*SARDINOPS SAGAX*)

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ABSTRACT

From 2014 to 2018, we conducted laboratory experiments to develop methods for inducing spawning in Pacific sardine (*Sardinops sagax*). Wild caught immature *S. sagax* were acclimatized for three months, reared until they were fully mature, and then induced to spawn. Fish reared at 14.5˚–18˚C and 4–12 h day-light, and fed a vitamin-enriched diet, were mature by 27 months. Fish treated with hormones prior to 24 months of rearing did not spawn, but those injected at 27 and 39 months spawned and naturally fertilized their eggs. The successful induction method consisted of human chorionic gonadotropin injections followed by a combination of carp pituitary extract and Domperidone injections 24 h later. Spawning occurred approximately 20 h after the second hormonal treatment. Eggs collected at 27 months and incubated at 11˚, 13˚, and 15˚C produced healthy larvae, but those spawned at 39 months did not survive to the larval stage. Ovaries were fully matured in February, but their oocytes regressed by May, suggesting that under controlled environment conditions *S. sagax* followed a seasonal maturity cycle similar to that of the naturally spawning population off California.

INTRODUCTION

The Pacific sardine, *Sardinops sagax* (Jenyns 1842), is one of the most important commercial and forage fishes in the California Current Ecosystem (CCE) (Hill et al. 2016; Nieto et al. 2014). The species is distributed from British Columbia, Canada, to the Gulf of California, Mexico, and is managed based on a three stock hypothesis, namely cold, temperate, and warm stocks (Félix-Uraga et al. 2005). The three stocks migrate almost synchronously, and thus their geographic locations along the Pacific coast differ seasonally (Demer and Zwolinski 2013). In most years the northern stock undergoes a migration in late spring to feed in waters off the Pacific Northwest (PNW) during summer and fall, and it returns south in late fall and winter to spawn in or near the Southern California Bight (SCB) during spring (Lo et al. 2011; Demer et al. 2012).

S. sagax is a fast-growing, short-lived and highly productive coastal pelagic species. Individuals may live up to 15 years (Dorval et al. 2015; Hill et al. 2016), but they accomplish most of their growth by their second year of life. During spring spawning season in the SCB, maximum lengths average 273 mm (SL) (Dorval et al. 2015). Annual recruitments fluctuate highly and are correlated with environmental conditions such as sea surface temperature (SST) and chlorophyll *a* (Reiss et al. 2008; Zwolinski and Demer 2013). Ontogenetic development, feeding, and reproduction requirements determine the magnitude of seasonal migration and the distribution of *S. sagax* along the Pacific coast (Hargreaves et al. 1994; Smith 1978; Zwolinski and Demer 2013). Young and small *S. sagax* (<200 mm) remain inshore off California, but larger, older fish (>200 mm, age 2+) begin the northern feeding migration (Lo et al. 2011). By late winter or early spring most fish have migrated back to the SCB, but occupy offshore spawning habitats (Zwolinski and Demer 2013). In the SCB 50% of females reach sexual maturity at 0.56 years old and 150.9 mm (SL), and all are mature by age 2 and 175 mm (SL) (Dorval et al. 2015). Northern stock fish spawn from January to July, but in the SCB peak spawning is in late March–April (Lo et al. 2010a; Dorval et al. 2014).

S. sagax eggs, larvae and post recruits have been studied in the CCE (Nieto et al. 2014; Smith and Moser 2003), but additional research remains to be conducted on the adaptive ecology of late larval and early juvenile stages. These life stages are critical in determining annual recruitment strengths into the fishery (Butler et al. 1993; Takahashi and Checkley 2008; Takahashi et al. 2009). Early juveniles recruit into nearshore waters where they are harvested by the California live-bait fishery (SAFE 2017), but remain inaccessible to most traditional surveys until the late juvenile and early adult stages (Ralston et al. 2015).

Although recent studies have shown that laboratory experiments can play a critical role in understanding adaptive divergence of fish in the wild as well as the evolution of life history traits that control their growth, recruitment, and productivity (Conover and Baumann 2009), very few such studies have been conducted on juvenile *S. sagax* (Pribyl et al. 2016). The absence of reliable methods to rear and induce spawning in cap-

tive *S. sagax* has made it difficult to develop controlled experiments on early life stages of this species. Because environmental cues play a critical role in regulating reproduction in teleost fish, under laboratory conditions these stimuli may be absent or weak, leading to physiological barriers that may prevent the initiation of reproductive processes (Stacey 1984).

Hormonal therapies have been used to alleviate these physiological blocks and accelerate vitellogenesis in fish species (Zohar and Mylonas 2001), although with various degrees of success in small pelagic fish. Consistent methods were developed to induce spawning in northern anchovy, *Engraulis mordax*, reared for several months in the laboratory (Leong 1971; 1989). Olmedo et al. (1990) applied similar methods to induce spawning in recently captured mature European pilchard, *Sardina pilchardus*, but these methods did not work after fish were acclimatized for one month in the laboratory. Shiraishi et al. (1996) used chronic hormone therapies to induce Japanese pilchard, *Sardinops melanostictus*, to spawn after one year in captivity by implanting mature females with luteinizing hormone releasing hormone (LHRH) pellets. However, no reliable methods have been reported to induce spawning in *S. sagax*.

In this paper, we report on the first steps in a series of exploratory experiments to establish a method for predictably inducing spawning in *S. sagax*, and as exploratory, the primary goal here was simply to determine whether spawning could be induced in a laboratory stock. A secondary goal was to determine the viability of experimentally spawned eggs and their potential usefulness in developing future studies on larval development and mortality, physiology, and adaptive behavior of *S. sagax* to environmental changes during the larval and juvenile stages. As the application of the daily egg production method (DEPM) to *S. sagax* has partly relied on egg-development rates at-temperature derived for *S. melanosticta* (Lo et al. 1996), a third goal was to start acquiring new data so that the calibration of DEPM models can be solely based on *S. sagax* age and stage parameters.

MATERIAL AND METHODS

Gonad classification

S. sagax gonads were initially categorized using gross anatomical observations, following a classification method established by Lo et al. (2010b). This method has been applied to classify gonads collected during spring surveys conducted by the SWFSC off California for selecting maturing and mature females for histological analyses of spawning rates. Gonads were evaluated visually, and then ovaries were preserved in 10% formalin solution. In addition to the visual evaluation, the formalin-preserved ovaries were processed and prepared as hematoxylin and eosin histological (H&E) slides. These slides were analyzed, and their oocytes, atresia, and postovulatory follicles categorized using descriptions in Macewicz et al. (1996), based on the Hunter and Macewicz (1985) classification methods. In addition, the maximum oocyte diameter (MOD) of the most developed oocyte type was recorded to determine how close mature fish were to spawning. Subsequently, the histological analyses were used to determine the phase in the reproductive cycle of each female using standardized terminology developed by Brown-Peterson et al. (2011). Females were either assigned to the immature phase or to one of four mature phases: developing (including the early developing subphase); spawning capable; regressing; and regenerating. Since *S. sagax* are batch spawners, and postovulatory follicle complexes can be identified (aged) as to time since spawning, the spawning capable phase includes the following subphases: actively spawning (that night); past-spawner (one night ago); and past-spawner (two nights ago) (Macewicz et al. 1996).

Broodstock

Young of the year (YOY) *S. sagax* were collected from stocking cages at the Mission Bay bait barge (Everingham Bros. Bait Co.) in San Diego in December 2014 and January 2015. Fish were transferred to the SWFSC aquarium, and were acclimated for 2–3 months in 2,800 L oval tanks. Before acclimation, randomly selected fish (n = 43) averaged 157.8 mm (SE = ±1.3) in fork length (FL; hereafter, fish lengths always are FL) and 35.8 g (SE = ±0.8) in weight. Ovaries were collected from 21 of the YOY females. Ageing from whole otoliths confirmed that all fish were less than 1 year old (age–0). After acclimation, all individual fish (N = 367) were measured for length and weight, and then stocked in a single 4,500 L circular broodstock tank (BST) on March 5 and 6, 2015. In all tanks, water depth was set to 0.9 m, and flow rate was maintained at 23 L/min, allowing the BST to turn over 7.4 times per day.

Broodstock fish were reared at approximately ambient temperature and fixed photoperiod until August 2016. Ambient temperatures approximated daily oceanic conditions off the Scripps Institution of Oceanography (SIO) pier in San Diego (Dorval et al. 2011), varying from 12° to 21°C. The photoperiod consisted of 12 h light (260–300 lx) and 12 h dark cycle. Light intensity was measured with a digital light meter (Extech Instrument, LT 300). Thereafter, with environmental controls, the temperature in the BST was maintained at about 14.7°C (± 0.11°C) and light level was reduced to 4–16 lx until May 2017 to simulate the cooler water and reduced light in the natural environ-

TABLE 1
Photoperiod and temperature conditions for all experimental trials prior and during hormonal injection. Duration is expressed in months (mo) and weeks (w).

Experiment	Tank	Period	Duration	Photoperiod	Temperature (SE)
				Hours of light (lx)	
I	BST	Mar 2015–Aug 2016	15 mo	12 (270-300)	17.7°C (±0.11)
I	A, B, C, D	Apr–May 2016	1 mo	12 (270-300)	16°C
I	A, B, C, D	May–Jun 2016	1 mo	8 (150)	14°C
I	A, B, C, D	Jun-Jul 2016	1 mo	10 (58)	12°C
I	A, B, C, D	Jul 2016	1 w	10 (58)	13°C
II	BST	Aug 2016–May 2017	10 mo	12 (4-16)	14.7°C (±0.02)
II	E	Jan 9–Jan 13, 2017	1 w	8 (58)	14.5°C
II	F	Jan 9–Jan 13, 2017	1 w	8 (58)	14.5°C
II	G	Jan 30–Feb 2, 2017	1 w	4 (58)	14.5°C
II	H	Feb 27–Mar 3, 2017	1 w	4 (58)	14.5°C
II	I	May 15–May 19, 2017	1 w	4 (58)	14.5°C
III	BST	Jun 12–Sep 7, 2017	3 mo	12 (270-300)	16.67°C (±0.08)
		Sep 8–Feb 12, 2018	5 mo	12 (4-16)	15.8°C (±0.10)
III	J	Feb 12–Feb 16, 2018	1 w	4 (58)	14.5°C
III	K	Feb 21–Feb 25, 2018	1 w	4 (58)	14.5°C

ment during winter. Note that mature *S. sagax* reside in waters as deep as 30–70 m (Dorval et al. 2011; Stieroff et al. 2019), and off southern California the euphotic depth, where light is 1% of surface value, tends to range slightly deeper than 30 m to a little over 50 m on average (Aknes and Ohman 2009). Therefore, *S. sagax* spend considerable amount of time in near to full darkness during daytime, before emerging at night in surface waters for feeding or spawning. In June 2017, temperature and illuminance in the BST were incrementally increased to reach 17.8°C and 300 lx, respectively. From September 2017 to February 2018, these parameters were progressively decreased to fixed values of 14.5°C and 58 lx, respectively (table 1). In the BST, environmental conditions were monitored daily during the whole study, and percent saturation of dissolved oxygen (DO) was measured as a proxy for water quality.

Throughout this study *S. sagax* were fed an experimental diet comprising 30% of high energy feeds (Oregon BioTrout) and 70% of protein and vitamin enriched gel cubes (Mazuri® Aquatic Gel Diet, MAG). The MAG primarily contained fish and krill protein and oil, essential vitamins including stabilized vitamin C (Stay-C®), vitamins E, K, D3, and B12, and required trace minerals. The MAG was prepared from a mixture of 40% of dried powder (Mazuri® Gel diet Formula Code-5B0C) to 60% water, heated near to boiling, by weight. Hence, the experimental feed approximately comprised 29.2% protein, 11.4% oil (fat), 1.2% crude fiber, 2.6% carbohydrate, 8.1% ash, 44.6% moisture, and at least 0.6% phosphorous, 0.2% selenium, 0.9% vitamins, 1.1% calcium, and 0.3% sodium. Fish were fed throughout the day and provided a daily ration equivalent to 1.5% of their body mass.

Prior to any biological measurement, tagging or hormonal injection, *S. sagax* were anesthetized by immersion in a solution bath of tricaine methanesulfonate (MS-222). Concentration of the solution bath (50 ppm) was similar across trials and, depending on their weight, fish were treated for two to five minutes. For euthanatizing fish prior to gonad and otolith extraction, the concentration of MS-222 solution was nearly tripled (140 ppm).

Experimental trials

As fish grew in the BST, occasionally a few were removed for visual examination of gonad maturation. All gonads examined were identified as clearly immature, intermediate, active, or hydrated based on the visibility of yolk-oocyte developmental stages or visible milt (Lo et al., 2010b). Experimental trials were initiated in March 2016 when gonad stage was intermediate or active. The BST fish were sequentially removed from the tank for three experiments to evaluate the effects of photoperiod, light intensity, temperature, hormone combination, and concentration on the gonad maturation. The three experiments were divided into several trials, where fish were reared under varying levels of environmental factors. Because gender cannot be reliably determined based on external examination of *S. sagax*, the number of each sex in the experimental trials was unknown. Prior to each experiment, small samples of fish (2–10) were sacrificed to visually evaluate gonads. In addition, samples of 5–20 were sacrificed 2, 3, or 5 days after hormone injection, except in the last trial where all fish (46) were sacrificed. These samples were dissected to visually evaluate gonads and ovary tissues were collected for histological analyses. Experimental trials were completed in

TABLE 2
Combination of hormone and concentration of solution injected per mean fish body length and weight during each trial.

Experiment	Trial	Homone	Concentration[a]	Injection date	Dose per fish	Volume injected per fish (ml)	Location	Fish (N)	Sex ratio[b]	Mean weight (g, ±S.E.)
I	I.1	HCG	600 IU/9 ml	7/12/16	30 IU	0.45	Muscular/ Dorsal fin	20	1	104.78 (5.58)
	I.1	CPE	72.4 mg/13.5 ml	7/13/16	1.21 mg	0.20				
	I.2	HCG	600 IU/9 ml	7/12/16	30 IU	0.45		20	2.2	109.42 (8.69)
	I.2	SPE	72.12 mg/13.5 ml	7/13/16	1.21 mg	0.20				
	I.2	D122	3.65 mg/13.5 ml	7/13/16	0.06 mg	0.20				
	I.3	HCG	1,800 IU/22.5 ml	7/19/16	30 IU	0.50		28		108 (4.80)
	I.3	CPE	72.31 mg/13.5 ml	7/20/16	2.58 mg	0.50				
	I.4	HCG	1,800 IU/22.5 ml	7/19/16	30 IU	0.50		27		108 (4.80)
	I.4	SPE	72.07 mg/13.5 ml	7/20/16	2.58 mg	0.50				
	I.4	D122	5.08 mg/18 ml	7/20/16	0.14 mg	0.50				
II	II.1	HCG	18,000 IU/12 ml	1/9/17	300 IU	0.20	Peritonial/ Pelvic fin	17	1	138.74 (6.88)
	II.1	CPE	125 mg/12 ml	1/10/17	5 mg	0.50				
	II.2	HCG	18,000 IU/12 ml	1/9/17	300 IU	0.20		16	1	132.58 (6.82)
	II.2	SPE	125 mg/12 ml	1/10/17	5 mg	0.50				
	II.2	D122	1.6 mg/12 ml	1/10/17	0.064 mg	0.30				
	II.3	HCG	10,000 IU/8 ml	1/30/17	250 IU	0.20		32	0.67	149.68(16.59)
	II.3	SPE	200 mg/ 12 ml	1/31/17	6 mg	0.40				
	II.4	HCG	10,000 IU/8 ml	2/27/17	250 IU	0.20		40		174.98 (9.08)
	II.4	CPE + D122	((400 mg CPE) + 11 mg D122)/24ml	2/28/17	8.33 mg CPE + 0.23 mg D122	0.50			0.9	
	II.5	HCG	10,000 IU/8 ml	5/15/17	250 IU	0.20		38		160.95 (9.70)
	II.5	CPE + D122	((400 mg CPE) + 11 mg D122)/24ml	5/15/17	8.33 mg CPE + 0.23 mg D122	0.50			0.6	
III	III.1	HCG	10,000 IU/8 ml	2/12/18	313 IU	0.25	Peritonial/ Pelvic fin	30	1.14	193.82 (6.44)
	III.1	CPE + D122	((360 mg CPE) + 9.44 mg D122)/24ml	2/13/18	10.5 mg CPE + 0.28 mg D122	0.70				
	III.2	HCG	15,454 IU/12.5 ml	2/21/18	309 IU	0.25		46	0.77	167.64 (5.12)
	III.2	CPE + D122	((490 mg CPE) + 13.04 mg D122)/38.8ml	2/22/18	8.84 mg CPE + 0.24 mg D122	0.70				

[a]Concentrations are in IU/ml for HCG and mg/ml for all other hormones. IU is the international unit used to indicate the amount of biological activity of a substance, e.g. the hormone HCG.
[b]Sex ratio = Female/male

2840 L oval tanks located in an enclosed section of the aquarium, where light intensity and photoperiod were controlled. In all trials, fish were not fed the day prior to the start of hormonal injections.

Experiment I: This experiment was designed to simulate environmental conditions that *S. sagax* may experience during the last three months prior to their spring spawning in the SCB. These conditions, decreasing temperature, and photoperiod followed first by increasing photoperiod and then increasing temperature, simulate conditions that would be experienced during migration, beginning in the fall and early winter to spring, from feeding areas off the PNW to spawning in the SCB (Lo et al. 2010a; Dorval et al. 2014). In April 2016, 120 fish were subsampled from the BST and assigned randomly to four trial tanks (A, B, C, D) at a density of 30 fish per tank. For three months the fish in all four tanks were reared under the same changing photoperiod and temperature conditions (table 1). At the end of the three-month period, the fish in each tank were treated with a different hormone combination, potentially to induce spawning, as described for trials I.1 to I.4 in Table 2.

Experiment II: The aim of this experiment was to maintain the BST within a range of temperatures that varied little from the mean temperature (14.5˚C) measured from plankton (pairovet net) tows that captured *S. sagax* eggs during summer surveys in 1986 and 1987 and spring surveys in 1994 and 2004–13 in the SCB (Dorval et al. 2014). Thus, temperature in the BST was decreased from an average ambient temperature of 17.7˚C to a mean of 14.7˚C from August 2016 to May 2017. Photoperiod in the BST was set at a 12 h cycle and light intensity during the day was lowered to 4–16 lx to approximate environmental condition during winter and up to the spawning season. Trials began when a sampled female was classified as having an active ovary with yolked oocytes visible. Randomly selected fish samples (20–40) from the BST were apportioned among five tanks, and trials (II.1–II.5) were conducted using five combinations of hormones (table 2). Trials II.1 to II.4 were conducted at the beginning of the normal spawning season (January–February), whereas II.5 was conducted near the end of the season (May).

Experiment III: The objective of this experiment was to determine whether fish that were previously treated or not with hormones could again mature their gonads in the laboratory the following year, and whether trial II.4 could be replicated. In June 2017, all surviving fish from Experiment II were mixed with the remaining fish in the BST. Temperature in the BST was then progressively increased from 14.5˚C to 18˚C by the end of August 2017, before being decreased to 14.5˚C by mid-December and held at that level through February 2018. From June to August 2017, light intensity ranged from 270 to 300 lx to simulate summer environmental conditions, and from September 2017 to February 2018 it was lowered to <5 lx, approximating winter conditions (table 1). Two experimental trials were conducted (III.1 and III.2) using the same combinations of hormones and the same number of injections as in trial II.4 (table 2).

Hormone Induction

Hormones used in trials were selected based on previously successful spawning experiments of small pelagic fish (Leong 1971; Leong 1989; Olmedo et al. 1990), and other cultured species (Zohar et al. 2001). Four types of hormones were used: (1) human chorionic gonadotropin (HCG, Sigma-Aldrich®), prepared as a lyophilized powder from human pregnancy urine; (2) carp pituitary extract (CPE, Argent Aquaculture®), purified from the pituitary gland of *Cyprinus* spp.; (3) salmon pituitary extract (SPE, Argent Aquaculture®), purified from the pituitary gland of salmonid species; and (4) Domperidone (D122-100MG, Sigma-Aldrich®) a dopamine antagonist prepared as dried powder.

HCG solutions were prepared less than 1 h before injection, whereas solutions of CPE, SPE, and D122 were prepared 2–3 h prior to injection. Hormonal powders were weighed using a dual range analytical balance (Mettler Toledo XS205) with a repeatability of 0.1 mg. All hormonal powders were dissolved in sterilized 0.9% saline water (Teknova®). For all trials, the induction method consisted of: HCG injections during the first day followed by CPE, SPE, and Domperidone 24 h later. The combinations and concentrations of these hormones for each trial are provided in Table 2. Solutions of HCG were injected using #23 gauge needles, whereas for all other hormones #22 gauge needles were used. After each day of injection, the photoperiod was set according to the objective of the experiment (table 2). During the first experiment, hormones were injected intramuscularly below the dorsal fin, whereas during the second and third experiments, hormones were injected in the peritoneal cavity about half way between the pelvic fins and the anal pore.

Egg Incubation

After spawning, eggs were collected and incubated to verify their viability and development up to hatching and early larval stage through yolk absorption. The incubation system consisted of three Cole-Parmer® (Polystat R6L) incubators, each of which controlled temperature in a separate bath. Each bath consisted of a Styrofoam box containing fresh water and a copper coil connected via plastic tubing to the incubator. A 6-L beaker containing approximately 5 L of seawater was placed within

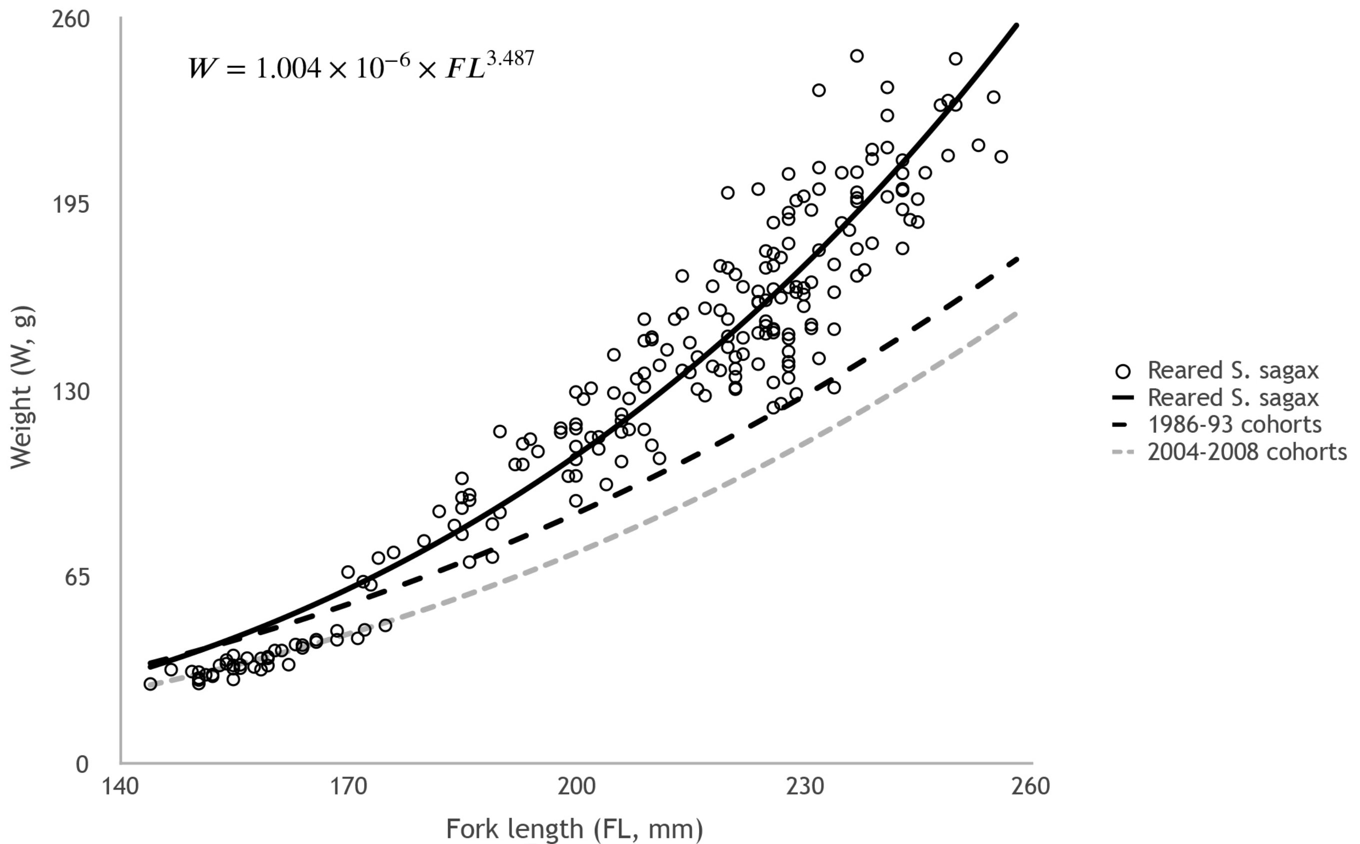

Figure 1. Comparison of predicted weight-at-length of *S. sagax* reared over 39 months during the spawning experiment and wild caught fish (1986–93 and 2004–08 cohorts) collected off California during spring surveys. The equation shown on the plot is for laboratory-reared *S. sagax*. Predicted curves for the two aggregated cohorts were derived from weight-length relationships in Dorval et al. (2015).

each coil and maintained at a constant preset temperature (±0.1˚C). Prior to egg collection, the incubators were set at 11˚, 13˚, and 15˚C, respectively, to represent the range of oceanic temperatures likely experienced by *S. sagax* eggs in the SCB during spring.

Eggs were collected in an egg trap consisting of a five-gallon bucket with drainage holes covered by 150 µm mesh. The trap was connected to the drainage system of the spawning tank. Recently spawned eggs were pipetted from the egg trap and transferred to the 6-L beakers, at a density of approximately 400 eggs per beaker, but remaining eggs in the trap were not counted. Because relatively few eggs were spawned in experiment III, only the 15˚C bath was used for that experiment. At the start of the incubation five eggs from each beaker were sampled and preserved in 3% formalin, and the time of preservation was recorded as sample 1. Eggs were initially sampled every hour for the first 3 h of the incubation. Thereafter, egg samples were collected every 2 h in each beaker until the completion of hatching. After hatching, yolk-sac stage larvae were collected daily until their eyes were fully pigmented and the yolk reserve was depleted.

RESULTS

Rearing and growth

In the laboratory-controlled environment, juvenile *S. sagax* grew and matured following a different growth trajectory than in wild-caught fish (fig. 1). Body weight was nonlinearly related to length with an allometric coefficient of 3.487 and an intercept of 1.004×10^{-6}) ($SE = 0.52 \times 10^{-6}$). The fish nearly quintupled in average weight from the time of capture (35.8 g ± 0.8) to the end of the experiment (178.0 g ± 4.2). Mature *S. sagax* averaged 216 mm (*SE* = ±2.3) and 234 mm (*SE* = ±1.3) at 27–30 and 39 months, respectively (fig. 2). The fish experienced low mortalities (<35% in 30 months) during the course of the experiment. The ratio of females to males ranged from 0.6 to 2.2 in the trials. Percent saturation of DO averaged 100% (*SE* = ±0.27), indicating high level of water quality was maintained throughout the experiment.

Gonad development

S. sagax collected at the San Diego bait barge in December 2014 and January 2015 were largely domi-

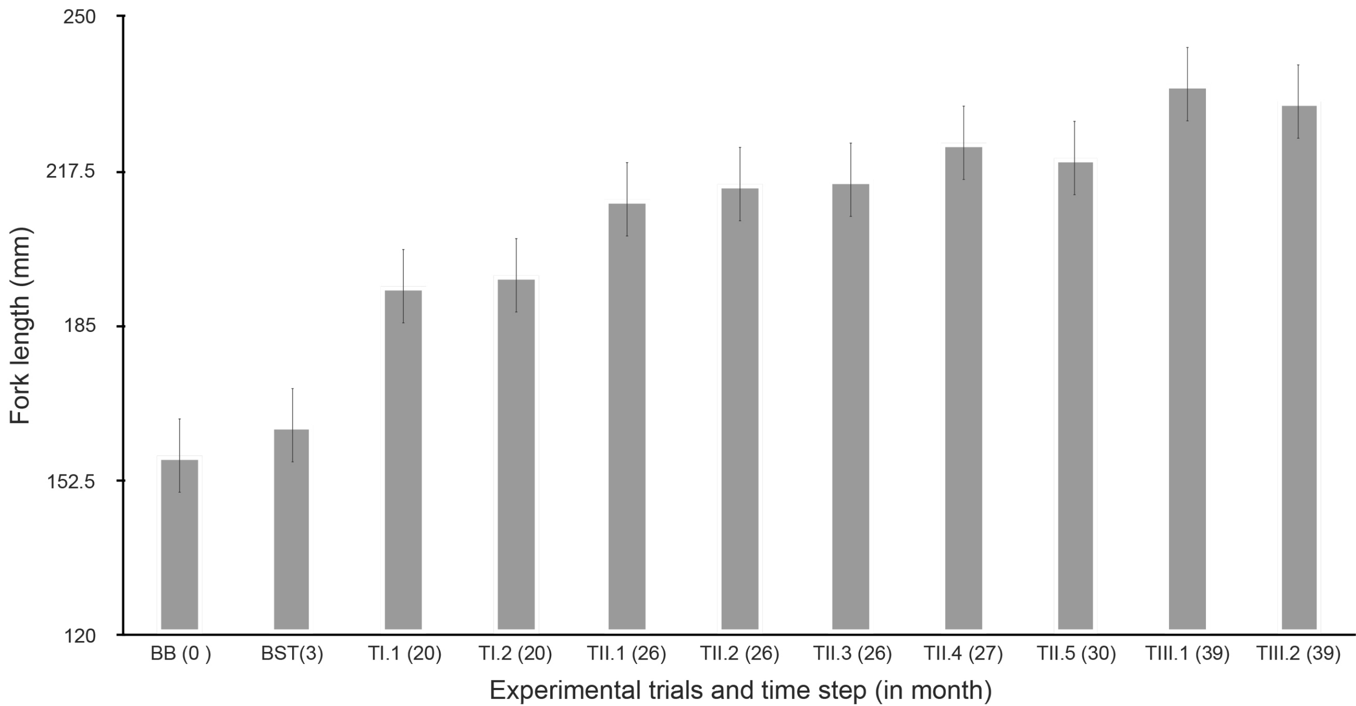

Figure 2. Mean fork length of *Sardinops sagax* sampled from the San Diego bait barge (BB) and from the broodstock tank (BST) during each experimental trial. Values in parenthesis are number of months since initial fish collection. Error bars are ± 1SD.

nated by immature individuals or individuals entering sexual maturity for the first time. Histological analysis of ovarian tissue showed that 38% of these females were immature (table 3). Mature females (62%) were in the developing phase, with most in the early developing subphase (n = 11), and their ovaries contained non-yolking oocytes less than 0.3 mm in diameter. In gross anatomical evaluation of testes, most males (71%) were identified as clearly immature.

After 20 months of rearing, less than 18% of females were categorized as immature, suggesting that the broodstock was largely comprised of sexually mature fish. Although the mature females were in the developing phase, less than 50% were in the early developing subphase, and 20% had begun vitellogenesis (table 3). By 25 months (January 2017), all *S. sagax* were mature with all females producing yolking oocytes and 20% were spawning capable. In late February, 2017, 50% of observed ovaries contained hydrated oocytes, and milt was present in all testes. Females sampled in February 2017 had substantially larger gonads than those collected in January (table 3). In May 2017, most females had reduced gonads, which weighed less than 1.6 g. Most male testes were still secreting milt, but 10% had regressed to the intermediate category (table 4).

In February 2018, all observed males but one were secreting milt and all females had mature ovaries. Nine out of 15 females from trial III.1 were spawning capable, including five that had postovulatory follicles, indicating past spawning (table 3). Five out of 18 females were spawning capable in trial III.2, with two females exhibiting postovulatory follicles. Most of the developing and all of the spawning capable females had moderate to major atresia of vitellogenic oocytes.

Induced spawning

Neither females nor males injected in experiment I responded to hormonal induction. The fish were relatively small, with mean weights ranging from 105 to 113 g. The means of realized concentrations of HCG ranged from 27 to 29 IU per 100 g of body weight, and those for CPE and SPE ranged from 1.07 to 2.11 g per 100 g of body weight. In trial I.2 fish were injected with a separate dose of Domperidone at a concentration of 0.05 mg per 100 g of fish, but this hormone apparently had no effects.

Concentrations of hormone solutions were increased in experiment II (table 2). During trials II.1–II.3, mean realized concentrations of HCG ranged from 116 IU to 216 IU per 100 g of body weight, whereas 3.6 g to 4 g of CPE or SPE were injected per 100 g of body weight. Domperidone was separately injected in fish from trial II.2 at a concentration of 0.05 mg per 100 g of body weight. Although female *S. sagax* did not respond to hormonal treatment during these trials, 88% of males in trial II.1 and 50% males in trial II.2 secreted milt.

TABLE 3
***Sardinops sagax* reproductive cycle phases and range of ovary wet weight (g) and maximum oocyte diameters (MOD in mm) observed from ovaries of fish collected at the San Diego bait barge (BB) and during each experimental trial.**

							Mature phases			
			Ovary	Ovary wet weight[a]	Immature phase		Developing		Spawning capable	
Experiment	Trial	Sample date	*N*		*n*	MOD	*n*	MOD	*n*	MOD
BB		12/04/2014 or 01/15/2015	21		8	0.13-0.15	13	0.15–0.27		
I	I.1	7/18/16	12		2	0.16-0.16	10	0.19–0.37		
	I.2	7/18/16	11		2	0.13-0.15	9	0.21–0.40		
II	II.1	1/11/2017 or 1/13/2017	5	2.60–3.60			5	0.35–0.44		
	II.2	1/11/2017 or 1/13/2017	9	1.50–5.30			7	0.29–0.56	2	0.61–0.63
	II.3	2/3/17	2	1.60–3.00			2	0.30–0.48	1[b]	0.67
	II.4	3/3/17	9	2.30–7.90			3	0.35–0.58	6[c]	0.48–0.63
	II.5	5/19/17	3	1.00–1.50			3	0.21–0.47		
III	III.1	2/15/18	15	3.22–13.85			6	0.30–0.48	9[c]	0.49–0.65
	III.2	2/23/2017 or 2/26/18	18	1.82–7.99			13	0.18–0.51	5[c]	0.50–0.57

[a]Spawning female ovary weight ranged from 6.4 to 7.9 g and their body weight ranged from 143-170 g.
[b]Ovaries were dissected before hormonal injection.
[c]Number of females that spawned after hormonal injection: 5 in II.4 and III.1, and 2 in III.2

TABLE 4
Gross anatomical evaluation of *Sardinops sagax* testes after hormonal injections during experiment II and III.

		Percentage of testes in each category		
Experiment	Trial	Clearly immature	Intermediate	Active
II	II.1	0	20	80
	II.2	0	43	57
	II.4	0	0	100
	II.5	0	10	90
III	III.1	0	0	100
	III.2	0	0	100

In trial II.4, within 24 h after the second injection, *S. sagax* spawned and newly spawned eggs were observed in the traps. On the first day of the trial, fish were injected with 143 IU of HCG per 100 g of body weight on average. On the second day, fish were treated with a mixed solution of CPE and D122 at a dosage of 4.76 g and 0.13 mg per 100 g of fish, respectively. Spawning and fertilization occurred naturally. Nineteen *S. sagax* were sacrificed from this trial, two days after spawning, comprising nine females and ten males. Based on visual and histological analyses, five females did spawn two nights prior to sampling, whereas six males were actively secreting milt.

Hormonal induction had no effects on females injected during trial II.5. Although fish were not sampled between trials II.4 and II.5, ovaries of mature females were in the developing phase which indicated that within two months ovaries could pass through the regressing phase and be back in the developing phase of the reproductive cycle. In contrast, 90% of males were still oozing milt.

Mature *S. sagax* treated with hormones in trials III.1 and III.2 successfully spawned. Similarly to trial II.4, spawning occurred 20 h after the second injection. On average, fish were injected with 161–184 IU of HCG, and 5.35 mg of CPE plus 0.14 mg of Domperidone per 100 g of body weight, respectively, during the first and second day of these trials. Histological analyses showed that five females in trial III.1 and two in trial III.2 did spawn prior to sampling (table 3).

Egg rearing

Live fertilized eggs siphoned in trial II.4 from the egg-trap were at the 2- and 4-cell stage of embryogenesis, approximately 4–8 h post-fertilization, at the time of collection and transfer to the incubation baths. The rate of subsequent embryonic development and time until hatching was a function of incubation temperature, with eggs reared at 15˚C hatching approximately 63 h to 77 h after fertilization, and those at 11˚C developing much more slowly, hatching approximately 111 h to 127 h after fertilization (table 5). Egg samples collected every 2 h up to hatching showed no obvious visual morphological differences from those collected in the wild.

Duration of the yolk-sac stage of larval development was, like the rate of embryonic development, a function of water temperature, with larvae at 15˚C completing yolk absorption within seven days after hatching, those at 13˚C completing yolk absorption within nine days after hatching, and those at 11˚C not completing yolk absorption by ten days after hatching, when the experiment was terminated.

TABLE 5
Estimated hours following fertilization to hatching and yolk absorption at 11˚, 13˚, and 15˚C during the egg rearing experiment.

	Hatching		Yolk sac absorption
Temperature (˚C)	Start time (h*)	End time (h*)	End time (h*)
11	111	127	> 167**
13	83	97	239
15	63	77	167

*Estimated number of hours were approximate due to the intervals of egg collection.
**Few embryos survived to hatching and yolk-sac larvae were not collected after 167 hours although a very few, still in yolk-sac stage, found in the final collection at termination of the experiment may have survived until then.

In trial III.1 far fewer eggs were available for collection compared with trial II.4, and in trial III.2 even fewer were available. Although eggs initially appeared to be viable in both trials, in trial III.1 most eggs apparently were not fertilized and embryos did not develop. The few eggs that did begin embryogenesis mostly developed abnormally and did not survive past the first few cleavages, dying after 16 h; none survived through the morula stage of development. In trial III.2, although there were fewer eggs, a larger proportion were fertile and began normal embryogenesis; however, few survived past the morula stage and none survived through the blastula stage of development. Most of these eggs died after 23 h of incubation, although one stage-3 egg, showing the segmentation cavity between the blastodisc and the yolk mass, was collected alive in the final sampling.

DISCUSSION

For the first time YOY *S. sagax*, reared over two years, were induced to spawn under experimentally controlled temperature, diet, photoperiod, and hormonal treatments. Female *S. sagax*, fully spawning capable after 27 months of rearing, were induced to spawn using a combination of HCG, CPE, and Domperidone. Eggs were fertilized naturally, and were reared through hatching and the yolk-sac larval stage. This study demonstrated that *S. sagax* can be induced to spawn in captivity, and can produce healthy eggs and larvae, although repeated hormonal treatments on the same broodstock may affect egg quality. These experiments establish a basis for designing a subsequent experiment series to refine the technique so that spawning can be efficiently and reliably induced, and for developing laboratory studies to enhance the understanding of *S. sagax* early life history strategies and adaption to changes in environmental conditions.

Fish rearing and growth

Compared to past experiments, *S. sagax* reared in this study were healthier, lived longer, and reached similar length-size to wild-caught spawning fish in the SCB (Dorval et al. 2015; Dorval et al. 2011; Javor and Dorval 2016). Although the experiment was not set to specifically contrast the effects of biological and environmental parameters, several factors contributed in improving the quality of rearing conditions. In this study, we transitioned from a high-energy feed (BioTrout pellets) to a more balanced diet combining these pellets with the MAG, which provided additional proteins and oils from fish and krill and essential vitamins and minerals. The experimental feed contained nearly 24% less fat than BioTrout pellets. It also contained stabilizing vitamin C (Stay-C), and at least 2% of essential minerals, including selenium. Stay-C is more digestible than other ascorbic acid polymorphs, and has been shown to enhance growth rates, survival rates, and immune responses of juvenile fish (Liang et al. 2017; Khan et al. 2017; Zhou et al. 2012). Selenium can improve lysozyme activity, hence boosting the immunity of fish (Burk et al. 2003). One potential direct impact of these nutrients on *S. sagax* health was the near disappearance of the puffy snout syndrome (eye and mouth occlusion which limits feeding), which was frequently observed in *S. sagax* and other pelagic fish reared during past experiments that lacked these supplements. No puffy snouts were observed during the first three years of experiments, and only three fish exhibited this syndrome during the last two experimental trials.

Beyond the reduction in fat content and availability of essential vitamins, the experimental feed also provided proteins and oils from a more diverse source of marine animals, including different fish and krill species, than is available in the high-energy feed. During the 30 months of the experiment, *S. sagax* more than quadrupled their growth. The weight and length relationship had an allometric coefficient (3.5 ± 0.1, fig. 1) higher than observed in wild-caught spawning fish (2.8 ± 0.01) suggesting that fish reared in this study accumulated more mass per unit length than those from the natural population (Dorval et al. 2015). Hence, using a diet formulation that contains nearly all essential nutrients facilitated the maintenance of a healthy broodstock during the experiment. Further, compared to Dorval et al. (2011) daily ration was reduced from 5% body weight to only 1.5%, leading to significantly reduced accumulation of wastes and improved water quality in rearing tanks.

To optimize growth rate while minimizing the risk of diseases, YOY *S. sagax* were reared at an average temperature of 17.7˚C (±0.11). However, for stimulating gonad development and maturation, older *S. sagax* were raised in water averaging 14.7˚C. These experimental temperatures simulated the average SSTs likely to be experienced by the northern stock, which mostly resides in water below 17˚C. Dorval et al. (2011) found that at 17˚C *S. sagax* grew faster than at 13˚C, and achieved

better survival rates than at 21°C. However, the stock appears to have adapted to spawn primarily between 13°and 15°C. From 1994 to 2013 *S. sagax* eggs were collected in the SCB in surface waters ranging from 13.4°to 18.9°C on average, but mean temperature exceeded 15°C in only two (1986 and 1987) of 12 years of surveys (Dorval et al. 2014).

Controlling photoperiod levels was also critical to *S. sagax* growth, particularly before they fully reached sexual maturity. YOY were reared at 12-h-day light intensity ranging from 270 lx to 300 lx during the first year. To simulate winter environmental conditions light intensity was set to 4–16 lx; whereas prior to hormonal induction illuminance was set to be equal or less than 4 lx in the successful trials. Previous studies have demonstrated that photoperiod can determine the optimal temperature of growth for juvenile *Gadus morhua* and *Hippoglossus hippoglossus* (Imsland et al. 2007; Jonassen et al. 2000). However, the effect of light intensity is less predictable as it may greatly vary with species and environmental conditions (Boeuf and Le Bail 1999). To our knowledge no studies have determined the optimal combination of photoperiod and temperature for juvenile *S. sagax* growth, warranting the development of new growth experiments in this area.

Gonad development and maturation

S. sagax reared during this experiment appeared to follow similar seasonal patterns in maturation and regression of their gonads as in wild-caught fish. The patterns of gonad development and regression followed closely those of the northern stock of *S. sagax* that develop in late winter and early spring in the SCB, spawn from March to late April, and begin gonadal regression by early May when little spawning occurs off California (Dorval et al. 2014; Macewicz et al. 1996). Further, these results demonstrated that the development of gonads in *S. sagax* is strongly tied to their natural biological clock and environmental conditions, and that hormonal induction played little role until the near completion of this process when they were in the spawning capable phase. Similar to other pelagic fish such as *E. mordax*, *Sardina pilchardus*, and *S. melanostictus*, it appeared that hormones only act as a stimulator of final oocyte maturation (FOM) and ovulation in *S. sagax*. Finally, these results showed that photoperiod does play a role in *S. sagax* maturation, although its optimal level and interactions with temperature and other environmental parameters remain to be quantified.

Induced-spawning

In this study, various exploratory experimental trials (I.1–I.4, II.1–II.3) were conducted, prior to determining the timing of hormonal induction and the combination of hormones that could be replicated to induce spawning in *S. sagax*. Three spawning trials, II.4, III.1, and III.2, successfully induced *S. sagax* to spawn using a combination of HCG, CPE, and Domperidone injections (table 2). Doses of HCG (143–184 IU) and CPE (4.8–5.5 g) per 100 g of body weight used in the three trials were within the range of concentrations used for *E. mordax* (Leong 1971, 1989). HCG was also within the range administered to most captive fish, which may vary from 10 to 400 IU per 100 g of body weight (Zohar and Mylonas 2001). Single treatments with HCG can stimulate FOM and ovulation in many cultured fish, for example cobia (*Rachycentron canadum*) at very low dose (i.e., 27.5 IU per 100 g) (Caylor et al., 1994), and mangrove red snapper (*Lutjanus argentimaculatus*) at very high dose (i.e., 1,500 IU per 100 g) (Emata et al. 1994). The potency of HCG in fish species is likely due to its long retention time in blood vessels (Ohta and Tanaka 1997; Zohar and Mylonas 2001). However, for small pelagic fish such as *E. mordax* and *S. sagax*, its effectiveness seems to depend on the addition of other hormones. HCG did not work alone in *E. mordax* (Leong 1971), and its combination with CPE and pregnant mare serum was necessary to induce spawning in *Sardina pilchardus* (Olmedo et al. 1990). Similarly, in the successful trials of this study, fish were injected with a mixed solution of CPE and Domperidone, 24 h after the HCG injection. The CPE used in this study provided additional gonadotropins purified from carp pituitary (Yaron 1995). The Domperidone, a dopamine antagonist, injected at 0.13–0.14 mg per 100 g of body weight, helped remove the inhibition on gonadotropins and thus further enhanced the effects of HCG and CPE on FOM and ovulation (Zohar and Mylonas 2001).

It is difficult to identify the exact causes for lack of FOM and ovulation in trial II.3, which combined HCG, SPE, and Domperidone injections in fish with closely similar gonad maturation as in trial II.4. However, poor handling in trial II.3 might have increased stress levels, and consequently reduced the effectiveness of the hormones. There could be also species specificity for these hormones. If so, it is possible that SPE gonadotropins may not be useful for *S. sagax*. The timing of injections might be a determining factor as well. For example, it has been shown that the effects of dopamine antagonists, such as Domperidone, may change over the course of the reproductive cycle, and the magnitude of changes may be species-specific (Peter et al. 1986; Trudeau and Peter 1995). Finally, it is important to note that the determination of optimal hormone combinations, dosages, and species-specificity was beyond the scope of this study. However, experiments are being planned to determine their optimal levels for inducing spawning in *S. sagax*.

Egg incubation

Reared eggs and yolk-sac stage larvae from trial II.4 were qualitatively similar to wild-caught eggs and larvae, and appear to have developed normally. Rates of embryogenesis that we observed were consistent with findings from previous studies. For example, Lasker (1964) found that at 11˚C hatching may occur 140 h after spawning, whereas at 21˚C it took only 34 h for eggs to hatch post-spawning. Yolk-sac stage larvae at 11˚C were largely inactive and did not complete yolk absorption before the experiment ended, while those at 15˚C became increasingly active after the first day post-hatching, which undoubtedly contributed to their higher rate of yolk utilization and completion of the yolk-sac stage within seven days after hatching.

We made no attempt to quantify mortality rates during embryonic and larval development following trial II.4, but visual inspection during sample collections suggested that mortality rates of eggs and larvae reared at 11˚C were somewhat higher than those of eggs reared at 15˚C and 13˚C, however, they were not noticeably different from rates observed during rearing of wild-caught eggs and larvae in other experiments (unpublished data). Given that *S. sagax* batch fecundity may range from 17,662 to 60,916 oocytes for females averaging 67 to 192 g (Dorval et al. 2016), the relative paucity of eggs resulting from trials III.1 and III.2 was not likely due to the sex ratio or the number of females that spawned in either trial. Although there were more males than females in trial III.2 (table 2), histological analysis of observed samples showed 2 females (out of 5 spawning capable) spawned during this trial. Further, the number of spawned females in trials II.4 and III.1 was similar, i.e. $n = 5$ (table 3). The preponderance of unfertilized eggs resulting from trial III.1 is also difficult to explain because all males collected from that trial were actively secreting milt, and environmental conditions were similar to trial III.2. Although initially viable eggs resulted from both trials, the abnormal development and very early complete mortality of embryos in trial III.1, and apparently normal development but complete mortality of embryos by only a few hours later in embryogenesis during trial III.2, suggests the possibility that repeated hormone-induced spawning of *S. sagax* will not yield viable progeny. Additional induced spawning and egg incubation trials will be required to explore this possibility. In the future, we also plan to attempt rearing larvae resulting from induced spawning experiments through a complete life cycle to maturity. Furthermore, the temperature-dependent embryogenesis experiment conducted here is part of a separate, larger study of the relationship of temperature with the rate of embryonic development in *S. sagax* over a much broader temperature range (9˚–21˚C) to which the northern stock is likely adapted (Pribyl et al. 2016). This study will be treated in a separate paper, which will provide new data to calibrate and improve the parametrization of the daily egg production method for *S. sagax*.

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