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**Article**

# Induction of free-flowing gametes to establish a reintroduction broodstock of bloaters (*Coregonus hoyi*) for Lake Ontario

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With 5 figures

**Abstract:** Animals raised in captivity commonly exhibit reproductive dysfunction of varying degrees ranging from diminished to absent reproduction. This observation holds true for the bloater (*Coregonus hoyi*); a deepwater cisco extirpated from Lake Ontario. Efforts to rear bloaters in captivity for creating a broodstock for Lake Ontario reintroduction efforts have been met with complications, including a significant reduction of egg and sperm expression in females and males, respectively. Therefore, we examined whether the injection of an exogenous hormone (Leutenizing Hormone Releasing Hormone analog, LHRHa) at two times of the year (Early: December-January & Late: January-February), with two different concentrations (40 & 80 μg $kg^{-1}$; using N = 1,322 individuals) could induce gamete expression in males and females. We measured the proportion of males and females with free-flowing gametes and measured gonadosomatic index and egg diameter for all treatment groups. The exogenous hormone injections were more effective at inducing the gamete expression of females. LHRHa treatments had a significant effect as to whether females, but not males, were expressing free-flowing gametes. Furthermore, there were no significant differences between the bloaters' GSI for either sex amongst the treatment groups. Finally, egg diameters were significantly different among the free-flowing eggs of the females from the different treatments. Overall, the use of LHRHa is an effective tool in promoting reproduction for female bloaters and will be one tool among many to help produce a captive broodstock for reintroduction efforts for bloaters to Lake Ontario.

**Keywords:** *Coregonus hoyi*, reproductive dysfunction, egg quality, sperm, induction

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## Introduction

Many fish species around the world are bred in captivity for a variety of reasons. Some are bred for food, while others are bred for conservation purposes. A tremendous challenge often faced in captive breeding efforts is the establishment of a self-sustaining population of brood fish (Snyder et al., 1996). Often, this difficulty can be attributed to some form of reproductive dysfunction brought on by inadequate environmental conditions (e.g., rearing density), diet, behavioral incompatibility, or inbreeding depression (Danielle & Murray, 1986; Setchell et al., 1987; Milliam, et al., 1988; Yamamoto et al., 1989; Merola, 1994). However, the incorporation of reproductive biological strategies, such as the use of exogenous hormones, has improved the effectiveness of captive breeding efforts of many species including amphibians (Browne et al., 2006) and fishes (Crim et al., 1988; Mylonas, 1992; Wojtczak et al., 2005).

It is well documented that the reproduction of hatchery-reared fish can be controlled through the manipulation of various environmental aspects such as water temperature and photoperiod (King & Pankhurst, 2007; Malison et al., 1998). Species for which there is comprehensive information regarding necessary ecological and biological aspects of their reproduction have been shown to benefit from these environmental manipulations (King & Pankhurst, 2007; Lafferty et al., 1999). However, there are still some species among those in aquaculture today for which either the relevant information surrounding their reproduction is unknown or the appropriate environmental manipulations are neither practically nor economically feasible (e.g., migration, depth, or turbidity) (Mylonas et al., 2010). Alternatively, another common method of controlling fish reproduction that does not solely rely on environmental cues exists; the use of exogenous hormones. The use of exogenous hormones to manipulate and control fish reproduction has been utilized in aquaculture for many years and is founded on the understanding of reproductive endocrinology of fish (e.g., Noori et al., 2010; Crim et al., 1983).

The reproductive cycle of fish can be separated into two distinct phases. The first phase is referred to as spermatogenesis and vitellogenesis in males and females, respectively. These initial phases are primarily associated with the proliferation, growth, and differentiation of gametes while the second stage, spermiation and final oocyte maturation (FOM) constitute their maturation and preparation for release (Mylonas & Zohar, 2001). In general, to initiate the reproductive endocrine cascade there must first be adequate environmental stimuli (e.g., appropriate water temperature, depth, light conditions and photoperiod, and turbidity cues) present to elicit the release of gonadotropin releasing hormone (GnRH) from the hypothalamus. GnRH then stimulates the release of gonadotropic hormones such as follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary. Endocrine control of reproduction depends primarily on FSH during the first stage of reproduction while LH is the primary gonadotropic hormone of the second phase. By administering an exogenous hormone, the reproductive processes can be initiated without the presence of otherwise necessary environmental stimuli which, as mentioned previously, can be costly or impractical to create in a fish hatchery setting.

Often, the need to control the reproductive processes of cultured fish arises from the observation of some reproductive dysfunction of the broodstock. Mylonas & Zohar (2001) identified the unpredictable occurrence or absence of final oocyte maturation as the most commonly observed reproductive dysfunction in cultured fish. While females appear to be more

commonly affected by a dysfunction, males, too, can experience dysfunction in the form of reduced volume and increased viscosity of semen (Vermeirssen et al., 1998). Analogs of either gonadotropic hormone releasing hormone (GnRHa) or luteinizing hormone releasing hormone (LHRHa) are the most common exogenous hormones used in aquaculture when attempting to alleviate this reproductive dysfunction (Zohar & Mylonas, 2001).

GnRHa and LHRHa are both classified as gonadotropic releasing hormones as their physiological function is to stimulate the release of gonadotropic hormones from the anterior pituitary. Their efficacy of relieving reproductive dysfunction in fish appears to vary among species and depends on many factors such as vector of administration, dosage, timing and duration of administration, and environmental influences (Crim & Glebe, 1984; Billard et al., 1984). Typically, the exogenous hormone is administered through one of two main methods: injection using a needle and syringe or the implantation of a slow-dissolving pellet. The appropriate dosage of a hormone can vary greatly among species but there is generally an accepted range of approximately 10–100 $\mu g\ kg^{-1}$. Pellets provide a slower and longer lasting release of hormone compared to injections, however, it is much more difficult to determine the exact dosage a fish may be receiving at a given point in time (Crim et al., 1988; Garcia, 1989). As different species may have different spawning seasons, a hormonal administration time point that is effective for one species may prove ineffective for another. Other studies suggest that environmental factors, such as water temperature, can play an important role in determining the efficacy of the administered hormone in the induction of reproductive processes in hatchery-reared fish (King & Pankhurst, 2007). Often, there is no single optimal potential strategy to overcome reproductive dysfunction in cultured fish, especially for those species whose ecological and biological aspects of reproduction are not well-known (Crim et al., 1983; Crim & Glebe, 1984; King & Pankhurst, 2007). Such is the case with the bloater (*Coregonus hoyi*), an important fish species native to four of the Laurentian Great Lakes.

The bloater was extirpated from Lake Ontario in the early 1950s (Clemens & Crawford, 2009). Multiple factors were implicated such as fishing pressure, predator-prey ratios (Christie, 1973), increased food competition with exotic species including alewives (*Alosa pseudolarengus*) and rainbow smelt (*Osmerus mordax*), as well as predation by sea lamprey (*Petromyzon marinus*), and reduced water quality (Smith, 1972; Christie, 1973). In the wild, bloaters spawn via external fertilization at depths ranging from approximately 40 to 100 m (Becker, 1983) with an average egg diameter of approximately 1.95 mm (Dryer & Beil, 1968). Additionally, adult bloaters have been found in water temperatures between 4 and 11 °C (TeWinkle & Fleischer, 1999). Given these natural environmental conditions, it is not surprising there are inherent difficulties (e.g., reproductive dysfunction) of raising these fish in captivity for conservation purposes.

The main goal of the Great Lakes Fish and Wildlife Restoration Act Regional Project entitled *Development of Propagation Strategies to Support Reintroduction of Deepwater Coregonids in Lake Ontario (2014)*, is "to establish a self-sustaining population of one or more species of deepwater ciscoes in Lake Ontario within 25 years". To achieve this goal, the project details two objectives: (1) to secure reliable sources of gametes from a self-sustaining broodstock by 2012 and (2) increase current culture capacity and stock 500,000 individuals by 2015. Since the launch of the restoration program in 2010, the principal source of gametes has been wild collections in the deep waters of Lake Michigan during the winter (late January to early March). Given the difficulty of collecting gametes at this time of year, and the

absence of well-developed husbandry practices, project participants decided to start developing a captive broodstock as a back-up strategy, beginning with the 2011 year class.

Conservation efforts via a partnership between the United States Fish and Wildlife Service, Ontario Ministry of Natural Resources and Forestry (OMNRF), the Great Lakes Fishery Commission, and the United States Geological Survey have been ongoing since 2010. As part of the initiative, the OMNRF White Lake Fish Culture Station (WLFCS) attempted to produce a self-sustaining broodstock of bloaters for their annual reintroduction into Lake Ontario, but experienced difficulties including reproductive issues. For example, hatchery-reared bloaters exhibited reproductive dysfunction in the form of absence of females expressing free-flowing eggs and males producing diminishing amounts of semen. Due to low levels of egg production in the 2011 year class during the 2014–2015 spawning season at WLFCS (compared to the consistent, albeit reduced semen expression in males), we elected to focus primarily on the quality of the eggs, rather than the sperm.

In this study, the efficacy of the use of an exogenous hormone (LHRHa) to overcome the observed reproductive dysfunctions exhibited by hatchery-reared bloaters was examined. During the development of the Ontario-based bloater broodstock it was commonly observed that there existed an asynchrony between sexes in terms of when they produced free-flowing gametes (usually 2–4 weeks in span between males and females, with males producing gametes prior to females) or a complete absence of free-flowing eggs in sexually mature females. We assume this asynchrony is due to a lack of normal ecological parameters in the fish culture facility, including density, sex ratio, lighting and temperature (among others), but at this time we have no quantitative data that can test these alternative hypotheses. Finally, because the production of eggs by the bloaters had been relatively low until recently, the egg diameter of both free-flowing eggs and eggs that have yet to be released were also examined and compared to the diameter of wild bloater eggs from Lake Michigan.

## Materials and methods

### Broodstock

Bloater brood stocks were held in 4 $m^3$ circular units that were 2 m in diameter and 1.3 m deep. Temperatures ranged between 12 °C during the summer months (mid-June to mid-September) and gradually decreased to 3 °C in the winter (mid-December to mid-March). Fish were fed a combination of Otohime EP1, EP2 and EWOS 2 mm salmonid diets. Fish were fed to satiation and amounts were reduced during spawning as dictated by feeding response. Feeding times and amounts were controlled and delivered using an Arvo-Tec feeding system. Lighting was controlled by a Sunmatch photoperiod controller and simulated sunrise, sunset, and daily light levels based on local latitude.

The sexually mature individuals from this study were derived from wild-caught bloater gametes collected from Lake Michigan in January–March 2012. In fall 2015, males and females from this broodstock population (N = 1,322 for the experiment) were randomly assigned to one of four experimental treatments; control, sham control, low dosage, and high dosage (see Figure 1). Following their respective treatments, each fish was checked in subsequent weeks to determine whether they were expressing free-flowing gametes. This

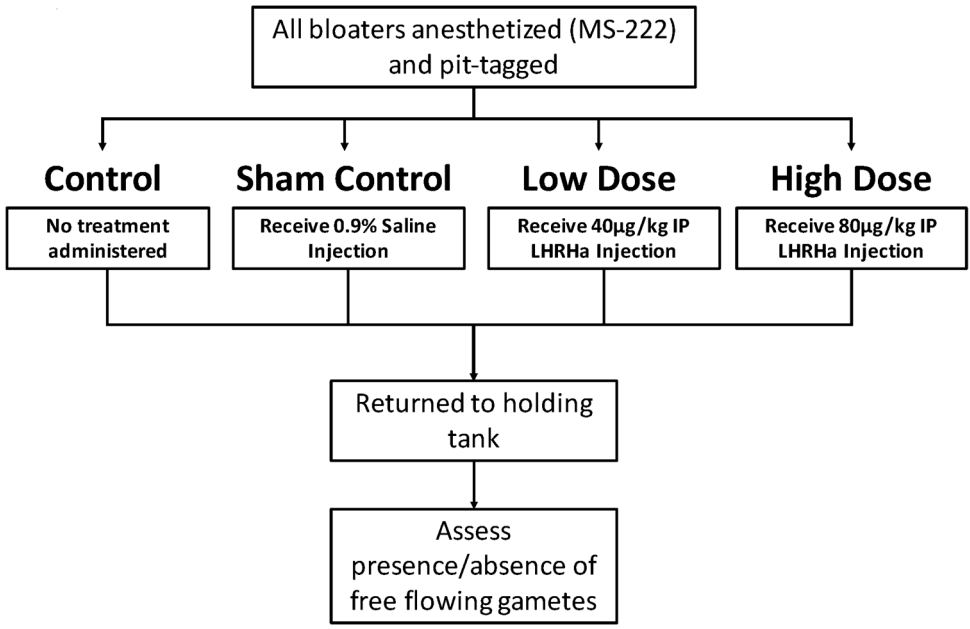


**Fig. 1.** Experimental design for the bloater (*Coregonus hoyi*) Luteinizing Hormone Releasing Hormone Analogue (LHRHa) treatment protocol. All fish were anesthetized and pit-tagged for identification purposes. There were four treatment groups: a control (no injection), a sham control (0.9% saline intraperitoneal (IP) injection), low dose (0.9% saline + 40 µg $kg^{-1}$ (LHRHa) IP injection) and high dose (0.9% saline + 80 µg $kg^{-1}$ LHRHa IP injection). Following treatment, fish were returned to holding tanks and the presence or absence of free-flowing gametes was assessed at regular intervals. The sample size for the "Early" time point was 241 males and 451 females for a total of 692 fish and for the "Late" time point it was 241 males and 399 females for a total of 640 fish.

experimental design was carried out twice, once between 14 December 2015 and 20 January 2016 (hereafter "Early"), and with another group of fish between 18 January 2016 and 24 February 2016 (hereafter "Late").

## LHRHa injection protocol

Treatment injections took place over two days (half the individuals on day one and half the individuals on the second day) for both time points (Early and Late). Each individual was anesthetized using tricaine methane-sulfonate (MS-222). Once each fish was anesthetized it was checked immediately for expression of free-flowing gametes (eggs or semen) by gentle abdominal massage. Any fish found to be expressing free-flowing gametes was placed in a recovery tank to be excluded from the study because the efficacy of exogenous hormone at inducing free-flowing gamete expression could not be examined in these individuals. Individuals first had their sex guessed (bloaters are sexually monomorphic) by an experienced technician in an attempt to randomly assign an approximately equal number of fish of each

sex to a treatment group (see details below). However, the sex ratio of the 2012 bloater cohort was approximately 2:1 in favor of females. Once it was determined a fish was not expressing free-flowing gametes, it was weighed (to the nearest gram) and total length (to the nearest mm) and girth just anterior to the dorsal fin (i.e., the widest part of the fish, to the nearest mm) were measured. Each fish received a pit-tag, which was injected lateral to the dorsal fin. Following these measurements, each fish (n = 692 [241 males, 451 females] in Early, n = 640 [241 males, 399 females] in Late) was randomly assigned to one of four treatments: control (C; n = 175 [52 males, 123 females] in Early, n = 160 [65 males, 95 females] in Late), sham control (S; n = 172 [65 males, 107 females] in Early, n = 160 [64 males, 96 females] in Late), low dose (L; n = 172 [58 males, 114 females] in Early, n = 160 [55 males, 105 females] in Late), and high dose (H; n = 173 [66 males, 107 females] in Early, n = 160 [57 males, 103 females] in Late). Each control fish was returned to a recovery tank after it was measured. Fish in the low dose group received an intraperitoneal priming injection (50%) of LHRHa (Syndel Laboratories Ltd., Vancouver, B.C.) in 0.9% saline, just below the pelvic fin, equivalent to 20 μg $kg^{-1}$ while high dose individuals received a similar injection but at a concentration of 40 μg $kg^{-1}$. Sham control individuals received an intraperitoneal injection of 0.9% saline of the same volume that a fish of identical mass would receive had it received an LHRHa injection (i.e., low or high dose treatments). Following treatment for both the Early and Late groups, half of the individuals (first day of injections) were put into one recovery tank and half (second day of injections) into another tank. Each tank contained individuals from all treatment groups to eliminate tank effects. This protocol was repeated for all fish from each of the treatments seven days following the initial handling and injections, this time to administer the resolving injection (50%) of LHRHa for the low and high fish, a second saline injection for sham fish and only anesthesia administration and handling for control fish. This brought the total amount of LHRHa injected to 40 μg $kg^{-1}$ for those individuals receiving a low dose and 80 μg $kg^{-1}$ for those receiving a high dose, which is consistent with other species in the literature (e.g., Billard et al., 1984; Crim & Glebe, 1984). To date, there is little data regarding appropriate injection concentrations of LHRHa for coregonids (but see Wojtczak et al., 2005) and as such we chose two concentrations within those used in the Salmonidae literature. Additionally, at this time, fish were first checked by abdominal massage to produce free-flowing gametes. All individuals were also checked again for free-flowing gametes at three, four and five weeks following their priming injections, which is consistent with other research regarding exogenous hormone injection in fish (e.g., Crim et al., 1988; Wojtczak et al., 2005) and is within the speculated spawning season of wild bloaters (Becker, 1983). Also, the average water temperatures during the Early and Late injections were 4 and 1 °C, respectively. For all treatment and checks following the initial treatment, fish were identified by pit-tag number and recorded for either expressing free-flowing gametes or not on the date of the check.

## Gonadosomatic index (GSI) measurement

Weighing of hatchery-reared bloater gonads for gonadosomatic index (GSI) measurements took place during the third week after the priming injection. Prior to handling, all individuals were euthanized using MS-222. All individuals were weighed to the nearest gram using an

electric scale and their girth was measured using a flexible tape measure, both, a second time in addition to the morphological measurements taken during the injection protocol. Each male (n = 38; 3 high, expressing and 8 control, 9 sham, 12 low and 6 high non-expressing) and female (n = 47; 7 control, 7 sham, 5 low and 6 high, expressing females and 7 control, 5 sham, 4 low and 6 high, non-expressing) were dissected using a sagittal incision along the ventral midline so that the gonads could be extracted. All gonads were placed into medium plastic weigh boats and weighed on an electronic scale (to the nearest milligram) for the calculation of GSI (mass of the gonads divided by whole body mass).

## Egg quality measurements

Eggs were collected from captive bloater females during the third week after the priming injection for both the Early and Late groups (i.e., same time as GSI measurement, see above). Additionally, unfertilized, wild bloater eggs from Lake Michigan were collected by staff from the United States Fish and Wildlife Service (USFWS) and delivered to WLFCS on 28 January 2016, approximately twenty-four hours later. These females, subsequently referred to as the "wild" treatment (n = 24), were anesthetized using MS-222, and processed in the field. Prior to handling, all experimental females at WLFCS (n = 26; 9 low and 7 high, expressing and 5 control and 5 sham non-expressing females for Early, and n = 47; 7 control, 7 sham, 5 low and 6 high, free-flowing females and 7 control, 5 sham, 4 low and 6 high, non-expressing females for Late), plus an additional group of non-experimental females from WLFCS (10 expressing and 10 non-expressing for each of Early and Late groups) were anesthetized using MS-222. These latter females, subsequently referred to as the "hatchery" treatment, were of the same 2012 cohort as the experimental fish but were not part of the LHRHa Injection Protocol, and thus, were anesthetized only once. The anaesthetized experimental and hatchery females had their morphological measurements taken as in the injection protocol (see above). If a female was expressing free-flowing gametes, they were collected by gentle abdominal massage into medium plastic weigh boats. Once the free-flowing eggs were extracted or the female was not yet expressing them, each female was dissected using a sagittal incision along the ventral midline.

Egg quality was measured as diameter, dry mass, and total lipid content. One subset of eggs from each female was carefully placed on filter paper cut to fit inside a small petri dish. These eggs were carefully separated using steel forceps and were then photographed using a Celestron Handheld Digital Microscope Pro with each photograph including approximately 20 eggs (n = 352 subsets, mean = 22 eggs per subset, range = 10–37). Following this imaging, the Celestron Portable Capture Pro software was used to measure the diameters of each individual egg (mm). A second subset of eggs (~ 5 g) from each female was used for determinations of dry mass and total lipid content. Dry mass per egg was used as an alternate measure of egg size, to determine if observed patterns in egg diameter may be related to hydration status of the eggs. These eggs were frozen, then freeze-dried for one week. For each female, three replicates of 30 eggs each were weighed to determine mean dry mass per egg. The freeze-dried eggs were then ground to a powder with a mortar and pestle, and total lipid content (% of dry mass) was estimated gravimetrically following a chloroform:methanol

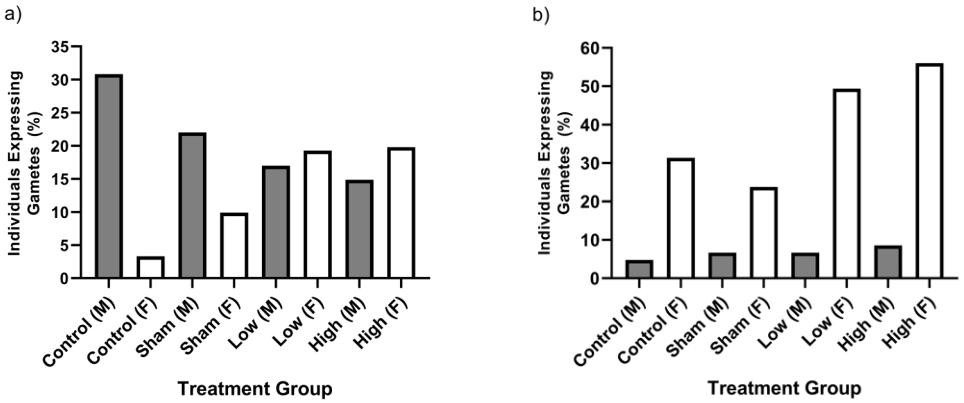


**Fig. 2.** Percentage of bloaters (*Coregonus hoyi*) that expressed free-flowing gametes during the Early (a) and Late (b) time points (see Methods for details) at White Lake Fish Culture Station in Sharbot Lake, Ontario. Males are indicated by dark gray bars and females by open bars for each of the treatment groups (control, sham, low and high). Note that in (a) the y axis ranges from 0–35% while in (b) the y-axis ranges from 0–60%.

extraction procedure outlined previously (Moles et al., 2008). Ova lipid extractions were performed in duplicate, and a third extraction was performed if the CV exceeded 10%.

## Results

### LHRHa injections

Cumulatively, LHRHa treatments of the Early bloaters had a significant effect as to whether females (chi-squared test: $X^2 = 19.355$, $p < 0.0005$, Figure 2a), but not males (chi-squared test: $X^2 = 4.76$, $p = 0.19$, Figure 2a), were expressing free-flowing gametes. Similarly, treatment of the Late bloaters had a significant effect as to whether females (chi-squared test: $X^2 = 23.07$, $p < 0.0001$, Figure 2b), but not males (chi-squared test: $X^2 = 0.94$, $p = 0.82$ Figure 2b), were expressing free-flowing gametes.

### Gonadosomatic index (GSI)

The GSI of non-expressing male bloaters (i.e., not producing free-flowing milt) was not significantly different among the treatment groups (One-way ANOVA: $F_{3,31} = 0.96$, $p = 0.42$). Similarly, the GSI of both expressing (One-way ANOVA: $F_{3,21} = 2.20$, $p = 0.12$) and non-expressing (One-way ANOVA: $F_{3,18} = 0.61$, $p = 0.62$) female bloaters was not significantly different among the treatment groups (Figure 3).

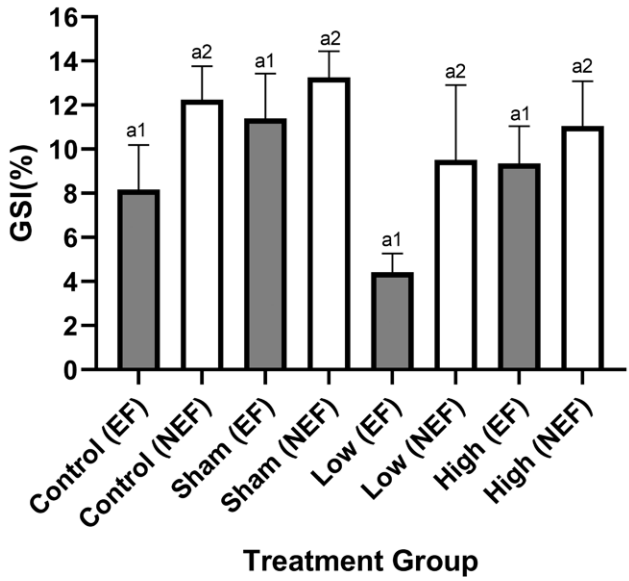


**Fig. 3.** Mean (+ standard error) gonadosomatic index (GSI) of female bloaters (*Coregonus hoyi*) at White Lake Fish Culture Station in Sharbot Lake, Ontario. GSI is the ratio of gonad mass to total body mass for an individual. Individuals that expressed free-flowing gametes between 25 January and 24 February (Late sample) are indicated by dark gray bars and individuals that did not (gonads were surgically excised) are indicated by open bars. Treatment means without a letter in common (with the same numbered subscript) for the same expression status were significantly different ($p < 0.05$) and subscripts correspond to individual one-way ANOVAs.

## Egg quality

Early bloaters of the control, sham, and hatchery groups that were non-expressing, showed no significant differences in mean egg diameter (One-way ANOVA: $F_{2,17} = 0.45$, $p = 0.65$). However, mean egg diameter was significantly different among the expressing females of the low, high, and wild groups (One-way ANOVA: $F_{2,37} = 16.90$, $p < 0.0001$), with wild eggs being significantly larger than both low and high dose eggs. Similarly, non-expressing Late bloaters of the control, sham, low, high, and hatchery groups showed no significant differences in mean egg diameter (One-way ANOVA: $F_{4,27} = 0.77$, $p = 0.56$, Figure 4). But, for expressing Late bloaters, mean egg diameter was significantly different among the control, sham, low, high, hatchery, and wild groups (One-way ANOVA: $F_{5,53} = 6.74$, $p < 0.0001$, Figure 4) with wild, low, and control eggs being significantly larger than hatchery high and sham eggs.

Our analyses of egg dry mass and lipid content included models with maternal TL as a covariate to account for possible ontogenetic effects. Wild fish were generally smaller than the captive fish examined in this study (mean TL of 210 mm and 256 mm, respectively). For the wild Lake Michigan bloater (all females expressing), egg dry mass was positively related to female TL (regression analysis, $F_{1,22} = 5.24$, $p = 0.032$, $R^2 = 0.19$), and egg lipid content was negatively related to female TL ($F_{1,22} = 5.91$, $p = 0.024$, $R^2 = 0.21$). In contrast, for the

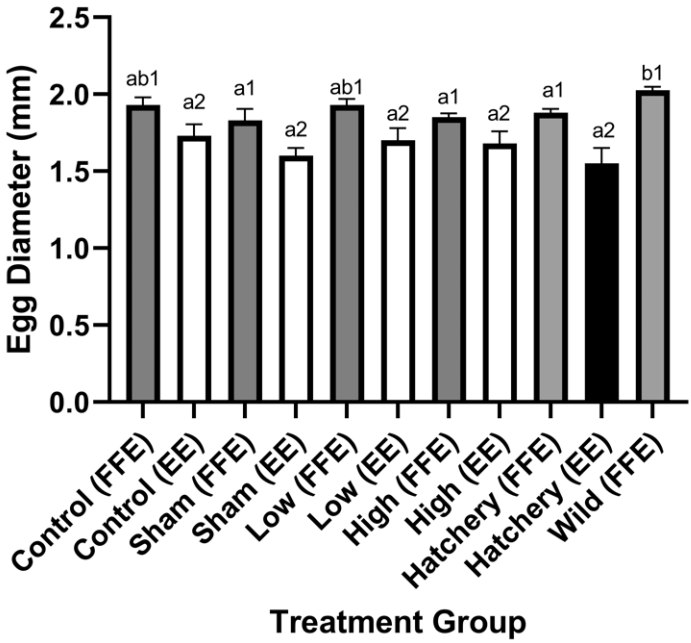


**Fig. 4.** Mean (+ standard error) egg diameter (mm) of bloater (*Coregonus hoyi*) from both captive broodstock at White Lake Fish Culture Station (WLFCS) in Sharbot Lake, Ontario and wild females from Lake Michigan. Egg diameters measured from free-flowing eggs are indicated by dark gray bars while egg diameters measured from surgically excised eggs are indicated by open bars for each treatment group (control, sham, low, high, hatchery and wild, defined in text) for the Late sample. In the case of an absent bar, no data could be collected for that group. Treatment means without a letter in common (with the same numbered subscript) for the same egg status were significantly different ($p < 0.05$) and subscripts correspond to individual one-way ANOVAs.

captive bloater in the WLFCS, there was a significant negative relationship between egg dry mass and maternal TL for expressing females (partial-$F_{1,41} = 5.87$, $p = 0.020$), but not for non-expressing females, and no significant relationship between egg lipid content and maternal TL in either expressing or non-expressing females. In subsequent analyses, egg traits were analyzed by ANCOVA, and *post hoc* tests were carried out on least-square means adjusted to a common maternal size of 245 mm TL.

Analyses of egg dry mass and lipid content indicated that there was very little variation attributable to treatment timing (i.e., Early versus Late injection timepoints), somewhat more variation attributable to the state of female ovulation at the time of sampling (i.e., expressing vs. non-expressing), and the most variation attributable to female treatment (i.e., injection treatment, hatchery, wild). Similar to the pattern observed for egg diameter (Figure 4), both egg dry mass and lipid content tended to be slightly higher in expressing than non-expressing females, and thus, tests among female treatments were conducted separately for these two groups. For non-expressing females, there were no significant differences in either egg dry mass (Tukey-Kramer adjusted multiple comparisons, $p > 0.39$) or egg lipid content ($p > 0.11$) between any treatment pairs. In contrast, for expressing females, significant

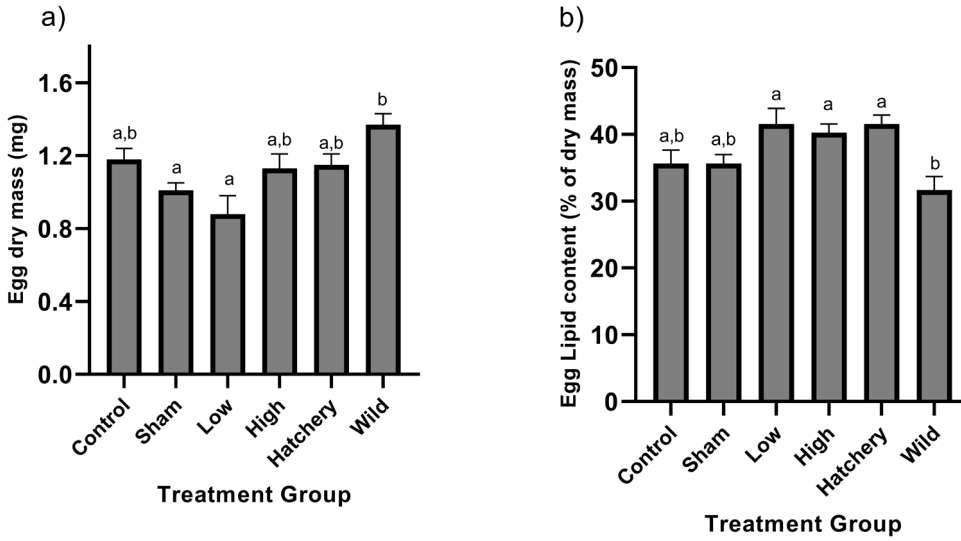


**Fig. 5.** Mean (+ standard error) egg size (mg dry; left) and egg lipid content (% of dry mass; right) of bloater (*Coregonus hoyi*) from both captive broodstock at White Lake Fish Culture Station (WLFCS) in Sharbot Lake, Ontario and wild females from Lake Michigan. All measurements were on eggs of expressing females (i.e., eggs free-flowing), and means were adjusted to a standard female size of 245 mm TL. Treatment groups are defined in text. Treatment means without a letter in common were significantly different (Tukey-Kramer adjustment for multiple comparisons, $p < 0.05$).

differences were observed among treatments, primarily due to the inclusion of wild females in the comparisons (Figure 5). Captive bloater tended to produce eggs that were both smaller and of higher lipid content than their wild counterparts, but there were no significant differences in either trait among the five captive treatments (Figure 5).

## Discussion

We have shown that intraperitoneal injections of leuteinizing hormone releasing hormone analog (LHRHa) are effective in inducing the expression of free-flowing gametes in hatchery-reared bloaters, but only for females. Expression of free-flowing eggs from hatchery-reared bloaters at the levels observed in this study, have not been previously reported. Injection of LHRHa did not appear to have a significant effect on GSI for either sex, and did not appear to significantly alter egg size or lipid content. However, we did observe differences in egg quality among the treatment groups of this study, and the contrast was greatest between wild fish and all other treatments in captivity.

## Male response to LHRHa injection

Males of the Early group of bloaters did not appear to benefit from the LHRHa injections. Results indicated that the prevalence of free-flowing gametes in males was higher in control (31%), and sham control (22%) treatments than in low (17%) and high (15%) LHRHa dosage treatments in the five weeks following injection. Similarly, for males of the Late group the prevalence of free-flowing gametes following treatment was not markedly higher for low (7%) or high (9%) dosage treatments compared to control (5%) and sham control (6%) treatments. These results are contradictory to what is typically observed following exogenous hormone injection of other hatchery-reared fish such as European whitefish (*Coregonus lavaretus*) (Wojtczak et al., 2005). Wojtczak et al. (2005) injected 33 whitefish with GnRHa (25–32 μg $kg^{-1}$) at the beginning of the spawning season (November) and all fish produced free-flowing semen – mostly within 16 days after injection.

The difference between the responses of male European whitefish (Wojtczak et al., 2005) and male bloater to hormone injection may be related to the timing of injection relative to the natural spawning cycle. The spawning cycle of European whitefish is well known, and injections were delivered just prior to the expected spawn time. However, the exact spawn timing of bloater is not well documented, either in the field or captivity, and our hormone injections may have been too late in the spawning cycle to be effective. It is possible that the males (Early) experienced reduced levels of plasma gonadotropins due to increased plasma androgen levels via a negative feedback loop within the HPG axis (Soma et al., 1996). That is, while the males were producing free-flowing semen (i.e., during their natural spawning season), their plasma androgen levels may have already been elevated, and thus, by injecting LHRHa, a gonadotropin which eventually triggers the release of androgens from the testes, the abnormally high androgen levels reduced natural gonadotropin release from the pituitary, lowering plasma androgen levels and, consequently, stopping free-flowing semen expression in some males (Nagahama, 1994). Finally, most of the males (Late) may also have spent their semen reserves earlier in the season, preventing the LHRHa injections from inducing further semen expression.

## Female response to LHRHa injection

Contrary to the male bloaters, females experienced significantly greater prevalence of free-flowing gamete expression following the LHRHa injections. Females from the Early group that received low (19%) and high (20%) doses of LHRHa experienced greater levels of free-flowing gamete expression as compared to sham control (10%) and control (3%) groups over the five weeks post-treatment. Similarly, in the Late females, low (49%) and high (46%) dose females experienced significantly higher frequency of gamete expression as compared to sham control (24%) and control (30%) females. The higher level of gamete expression in all treatments in January–February compared to Early may indicate that the natural spawning season of the bloater is closer to the former time period. This increased level of free-flowing gamete expression following exogenous hormone injection is what is typically observed in hatchery-reared fish such as brown trout (*Salmo trutta*) (Noori et al., 2010) and Atlantic salmon (*Salmo salar*) (Crim & Glebe, 1984). Noori et al. (2010) achieved

cumulative percent ovulations ranging from approximately 50% in control to 100% (in under 30 days post injection) in individuals who received a 100 μg $kg^{-1}$ injection. Similarly, Crim & Glebe (1984) found that 94% of female Atlantic salmon had expressed free-flowing gametes within 9 days of LHRHa pellet implantation (27 μg $kg^{-1}$) when it was conducted 4 weeks prior to the natural spawning season.

## Gonadosomatic index

There were no significant differences observed in GSI of bloater among treatments in this experiment for either sex. This observation is consistent with earlier studies that have examined exogenous hormone application and GSI. For example, Trudeau et al. (1991) observed that after implanting testosterone or estradiol (both 100 μg $g^{-1}$) pellets into female goldfish (*Carassius auratus*), there was no significant difference in GSI at any point during its spawning season. Additionally, there appears to be a poor correlation between GSI and the different stages of reproductive development (i.e., expressing free-flowing gametes or not), depending on which species of fish was examined (DeVlaming et al., 1982). Not accurately knowing the spawning season of captively-reared bloaters prior to this experiment made GSI measurement for male bloaters difficult. In this study, it appeared that most male bloaters had stopped producing free-flowing semen by the time the GSI measurements took place (8 February 2016). However, in the future, it would be beneficial to measure the reproductive hormone levels (e.g., estradiol, testosterone, vitellogenin) in conjunction with the GSI and gamete expression (i.e., free-flowing or not) status of the bloaters to better characterize and determine the physiological effects of LHRHa on the bloater reproductive cycle.

## Egg quality

For the captive bloater females, we found that egg size, and possibly lipid content, tended to be higher for expressing females (i.e., eggs free-flowing) than for non-expressing females, regardless of treatment. This trend was evident for egg size both when measured as diameter and dry mass, suggesting that the size effect is not related to hydration at the time of ovulation. The implication is that either captive female bloaters continue to provision their eggs right up to the time of ovulation, or those producing larger eggs are also those most likely to ovulate, either naturally or through hormonal induction. We also found that egg quality did not vary greatly among the various hormonal treatments applied to captively-reared bloater, consistent with similar research on other salmonids (Arabaci et al., 2004). This is encouraging, as it suggests that hormonal treatment can be used to promote ovulation without compromising egg quality.

We found that the greatest contrast in bloater egg quality was between wild females and captively-reared females of all treatments. Relationships between egg quality and maternal size differed between captive and wild bloater, and captively-reared females appeared to produce smaller and more lipid-rich eggs than their wild counterparts at a standard body size. Captive-rearing has been shown to influence egg quality in a variety of fish species (Mylonas & Zohar, 2001; Heath et al., 2003; Mylonas et al., 2010; Johnston, 2018). The divergence in

egg traits between female bloater in captivity and those in their wild source population was evident over a single generation, suggesting a phenotypically plastic response, possibly due to changes in environmental conditions (temperature, photoperiod) and/or nutritional status. The observed differences may also be attributable to age and size structure differences between the two groups, as egg quality appears to be related to combined effects of age, size, and growth in some salmonids (Johnston, 2018). In this study, all captive female bloater were the same age (3.5 years) whereas the wild female bloater, though of a smaller mean size, may have represented a wider diversity of age classes, growth histories, and spawning histories.

## Conclusions

Captive-rearing and broodstock development of bloater is still in the early research stages. Reliable gamete production and spawning is a key element in this process. Intraperitoneal LHRHa injections can effectively induce free-flowing gamete expression in hatchery-reared female bloater with minimal (< 1%) mortality, and minimal effects on GSI and egg quality. We could not demonstrate a similar effect on gamete expression in male bloater. Further research into the timing of spawning for bloater in captivity and whether males and females should be housed separately prior to spawning may allow us to optimize the timing of hormone injection to increase the frequency of ovulation further in females and increase the success of semen release in males. Future studies could benefit from injecting males even earlier than our "Early" treatment (in early November) and females in mid-January (i.e., at the beginning of their respective observed spawning seasons) (Crim & Glebe, 1984; Billard et al., 1984). Another prominent factor which affects the reproduction of fish in captivity is their diet (reviewed in Izquierdo et al., 2001). For example, Duray et al. (1994) observed that when dietary lipid level was increased for rabbitfish (*Siganus guttatus*) there were significant increases in both fecundity and hatching percentage. All of these avenues of research should prove useful in the successful development of a bloater broodstock.

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