INTRODUCTION

Individuals can respond to signals of predation by either innate or learned mechanisms (Ferrari et al., 2007). An innate anti-predator response is expressed in its entirety upon the first exposure to the signal of predation; subsequent exposure to the signal does not modify or otherwise enhance the response (Alcock, 1993). The signal of predation may originate from the predator itself, for example its appearance or odour, or it may originate from conspecifics, for example an alarm cue that is produced in response to a predator (Ferrari et al., 2010). For example, Seychelles warblers (Acrocephalus sechellensis) respond to the presence of an egg predator—the Seychelles fody (Foudia sechellarum)—by producing alarm calls and attacking the fody (Veen et al., 2000). This response is independent of the age of the warbler and its prior exposure to the fody, indicating that the anti-predator response is innate (Veen et al., 2000).

In contrast, a learned anti-predator response is not expressed in its entirety upon the first exposure to the signal of predation, but instead is modified or enhanced following exposure to the signal of predation (Alcock, 1993). A common example of a learned anti-predator response is the development of an association between an alarm cue that elicits an innate response and a predator cue that does not elicit an innate response (Alcock, 1993). For example, rainbow trout (Oncorhynchus mykiss) has no innate response to the odour of northern pike (Esox lucius) but reduce foraging and remain motionless in response to pike odour after the pike odour has been paired with the presentation of an alarm cue (Brown & Smith, 1998). Both innate and learned anti-predator responses have been shown to improve survival when individuals are exposed to predators and thus may represent adaptations (e.g., Mathis & Smith, 1993; Mirza & Chivers, 2000).

Chemical alarm cues are a reliable signal in aquatic environments where visual and auditory cues can be ineffective. Alarm cues have been observed in a wide range of aquatic taxa, including...
coral, invertebrates, amphibians and fishes (Ferrari, Wisenden, et al., 2010; Goddard, 2006; Hawkins et al., 2004; Huryn & Chivers, 1999). Alarm cues generally take the form of chemicals that are released when an individual is injured by a predator (reviewed by Chivers & Smith, 1998). The chemical composition of alarm cues are generally not well-characterized, but alarm cues derived from the skin of conspecifics are widely reported to decrease movement, increase shelter usage, increase shoal cohesion and reduce foraging, indicating a strong innate response to these cues (Chivers & Smith, 1998).

Captive breeding programmes are widely used to conserve and supplement wild populations (Fraser, 2008; Houde et al., 2015). However, captive breeding programmes remove the effect of predation during much of the life cycle, which can lead to reduced fitness when captive-bred individuals are released in natural environments (Fritts et al., 2007; Neff et al., 2011). Fitness differences may occur because captive-bred animals have a lower innate response to predator cues. For example, Houde et al., (2010) compared the innate response of Atlantic salmon (Salmon salar) to an avian predator cue across groups differing in the proportion of wild and hatchery ancestry and found that the response to predator cue decreased as hatchery ancestry increased. In a comparison of the innate response to an avian predator model in first- and second-generation captive-bred Atlantic salmon, de Mestral and Herbinger (2013) found that the second-generation salmon displayed more risk-taking behaviours than the first-generation salmon. It is less clear that if the capacity to develop learned anti-predator responses is affected by captive breeding. Captive-bred populations have previously been shown to maintain an innate response to alarm cue and the ability to form learned association with predator cues, even after several generations in captivity (Brown, Ferrari, Malka, et al., 2013; Ferrari et al., 2010; Mirza & Chivers, 2000). However, no study has explicitly examined the effect of captive breeding on the learned anti-predator response, although it has been hypothesized that that there may be evolved differences in anti-predator learning associated with captive breeding programmes (Griffin et al., 2000).

Our study used a classical conditioning paradigm (Rescorla, 1967) to evaluate the innate and learned anti-predator responses of Atlantic salmon from three populations that differ in the length of time that they have been maintained in captivity. Salmon were exposed to either an alarm cue, a predator cue, or both cues together. The first exposure was used to evaluate the innate response to these cues. Salmon were then exposed to either a paired or a unpaired training treatment. In the paired treatment, the alarm cue and predator cue were presented simultaneously, which simulates an environment in which the predator cue is a reliable signal of short-term predation risk. In the unpaired treatment, the alarm cue and predator cue were presented at different times, which simulates an environment in which the predator cue is not a reliable signal of short-term predation risk. Following training, salmon were exposed to the predator cue alone to evaluate their learned response.

2 | METHODS

2.1 | Experimental fish

Three Atlantic salmon populations were examined in this study: the LaHave River population from Nova Scotia (44.3°N, 64.4°W), the Sebago Lake population from Maine (43.8°N, 70.5°W) and the Lac St. Jean population from Quebec (48.6°N, 72.0°W). These populations have all been targeted for reintroduction into Lake Ontario and differ in the length of time they have been maintained in captivity in the Ontario Ministry of Natural Resources and Forestry (OMNRF) hatchery system. LaHave has been propagated in the hatchery system since 1995, Sebago since 2006 and St. Jean since 2007 (OMNRF, unpubl. data).

The individuals used in this study were from mixed family production stock held at the OMNRF Normandale Fish Culture Station (42.7°N, 80.4°W). The individuals were raised at the Normandale facility until they became free swimming and at least 1 g in mass; then, they were brought to the University of Western Ontario. For each population, upon arrival to the university 50 salmon were placed into a population-specific 50 L flow-through housing tank that was maintained at 11°C throughout the experiment. Salmon were fed pelleted floating food (Corey Foods) ad libitum and maintained on a 12:12 hr light:dark cycle.

2.2 | Experimental trials

The protocol and procedures employed in this study were ethically reviewed and approved by Western University’s Animal Care Committee (protocol 2010–214) and were performed in accordance with National (Canadian Council on Animal Care) and provincial (Ontario Animals in Research Act) standards and guidelines. The experimental trials were conducted in 2014 and 2015 between August and December, when the salmon were 8–12 months of age. Predator recognition training and behavioural observation took place in 60 × 40 × 20 cm experimental tanks. Experimental tanks had a fresh water flow through of 6 L/hour to maintain water quality. Tanks included a gravel substrate and a 9 cm long refuge made of 3.8 cm diameter PVC pipe placed on the gravel opposite the inflow. A blind was placed around and above the experimental tanks to obscure the researcher, and the digital cameras used to record behaviour.

A summary of the training and observation timeline is provided in Table 1. At the start of each trial, two salmon from the same strain were randomly selected and moved from a housing tank into an experimental tank (day 0). Two salmon were included for each trial to facilitate normal social interactions during the behavioural observations. On days 1–3, salmon were fed 100 mg of floating food (Corey Foods) between 10:00 and 11:00 a.m. (i.e., following the daily feeding guidelines for juvenile Atlantic salmon in Farmer et al., 1983). On day 4, salmon were not fed to ensure that hunger levels were similar during the observation periods. The first observations were collected on day 5. Between 10:00 and 11:00 a.m., a digital video
camera was positioned above each experimental tank and individuals were recorded for 5 min with no cue present, during which time they were provided with 50 mg of floating food. Individuals were then exposed to alarm cue alone, predator cue alone or both cues together. The alarm cue was derived from the skin of juvenile salmon, and the predator cue was a belted kingfisher (Megaceryle alcyon) model. When exposed to the alarm cue, 10 ml of cue was injected over 1 s into the tank via a fixed piece of tubing located above the tank inflow. When exposed to the predator cue, the model was presented at the upper edge of the tank for 5 min. Individuals were recorded for 5 min starting at the beginning of the presentation of a cue, during which time they were provided with another 50 mg of floating food.

Predator recognition training began on day 5 and lasted a total of 3 days (i.e., on day 5, 6 and 7) and was conducted, while fish were in the experimental tanks. Individuals that were exposed to the alarm cue and predator cue together during the first behavioural observations were assigned to the paired treatment, whereas individuals that were exposed to either cue alone were assigned to the unpaired treatment. Individuals in the paired treatment were exposed to the predator cue and alarm cue simultaneously, twice a day, at intervals no less than 60 min apart. Individuals in the unpaired treatment were exposed to the predator cue and alarm cue at separate times, twice a day for each cue, at intervals no less than 60 min apart. All cues were presented between 10:00 a.m. and 5:00 p.m. On the day after the training period (day 8), all fish were given 24 hr during which no cues were presented and they were not fed.

On day 9, there was a second observation day, in which behaviour was recorded for 5 min in the absence of either cue. All salmon were then exposed to the predator cue alone, and behaviour was recorded for 5 min. Salmon were then euthanized with an overdose of MS-222, and their body mass and fork length were measured. In total, we examined 40 salmon from the LaHave population (22 unpaired, 18 paired), 34 salmon from the Sebago strain (20 unpaired, 14 paired) and 24 salmon from the St. Jean strain (14 unpaired, 10 paired). An additional 10 trials were excluded from the analyses due to technical problems during filming that led to incomplete behavioural data for the individuals involved.

Based on the considerations outlined in the STRANGE framework (Social background, Trappability and self-selection, Rearing history, Acclimation and habituation, Natural changes in responsiveness, Genetic make-up, and Experience; Webster & Rutz, 2020), unintentional bias among treatment groups is unlikely to have been introduced based on the selection of experimental animals. All individuals were sourced from the same hatchery and then reared under the same social conditions, there was limited opportunity for self-selection during sampling, and all individuals were naïve to the predator stimulus used during training.

### 2.3 Behavioural measures

Each 5-min video recording was scored for anti-predator response behaviours by an observer who was blind to the experimental treatment. Behaviour was scored separately for each fish in a pair. Three behaviours that are commonly associated with the anti-predator response in Atlantic salmon were examined as follows: time spent motionless, number of feeding acts and number of aggressive acts (Brown & Smith, 1998; Leduc et al., 2007; de Mestral & Herbinger, 2013). Time spent motionless was calculated as the total amount of time that a salmon spent stationary on the gravel bottom of the tank or inside the PVC refuge. Number of feeding acts was calculated as the total number of floating food pellets consumed. The number of aggressive acts was calculated as the total number of rapid movements or biting motions directed towards the other salmon.
2.4 | Preparation of cues

As in previous studies on salmonids, alarm cue was derived from the skin of juvenile Atlantic salmon. Following Ferrari, Brown, et al., (2010), salmon were euthanized using an overdose of MS-222; the skin was removed, homogenized and filtered through cheesecloth into water collected from the housing tanks to a concentration of 10 ml water per 1 cm² of skin. Alarm cue was prepared in 400 ml batches by pooling alarm cue produced by individuals from all strains and then frozen in 50 ml aliquots at −20°C until needed.

The predator cue was a belted kingfisher taxidermy model that was 20 cm tall. When presented to salmon in an experimental tank, the model was placed on a raised stand such that the top 12 cm of the model (most of the body and head) was visible over the edge of the tank (Figure 1). The belted kingfisher is an important predator of juvenile Atlantic salmon, and along with the common merganser (Mergus merganser), is estimated to cause between 21% and 45% of all mortality in 0- to 2-year-old Atlantic salmon (Cairns, 2001).

2.5 | Statistical analysis

Body mass and fork length were compared between populations (LaHave, Sebago and St. Jean) and treatments (paired, unpaired) using linear models that included both population and treatment as fixed factors.

The three behaviours (time spent motionless, number of feeding acts and number of aggressive acts) were combined into a single variable to capture overall activity. To do so, we first calculated a Z-score value for each behaviour across all 5-min observation periods. These Z-scores were combined into an index of overall activity using Z-score (log[number of feeding acts + 1]) + Z-score(log[number of aggressive acts + 1])−Z-score(time spent motionless). After calculating the Z-scores, the minimum Z-score (i.e., corresponding to motionlessness for the entire trial with no feeding or aggression) was subtracted so the activity values were always positive. In general, high activity values were associated with high feeding and aggression and low time spent motionless. Across the four observation blocks, the Z-scores showed consistent positive correlations between feeding and aggression (Pearson $r^2 = .04$ to $.13$, $p < .051$ to <.001, $n = 98$ individuals per block), consistent negative correlations between aggression and time spent motionless (Pearson $r^2 = .04$ to $.10$, $p = .046$ to .001, $n = 98$ individuals per block) and consistent negative correlations between feeding and time spent motionless (Pearson $r^2 = .27$ to .50, $p < .001$, $n = 98$ individuals per block).

Indexes that combine Z-scores for multiple related measures are a common approach in behavioural studies to increase sensitivity and reliability (Guiloux et al., 2011; Labots et al., 2017)

A general linear mixed model (GLMM), which assumes a Gaussian error distribution, was used to analyse activity. The number of feeding acts and number of aggressive acts were log-transformed before calculating activity to meet the model assumptions. Individual ID was included as a random effect to account for the repeated behavioural measurements of each fish (day 5 with no cue, day 5 with predator, alarm or both cues, day 9 with no cue, day 9 with predator cue). Body mass was included as a covariate. The fixed factors included in the model were population (LaHave, Sebago and St. Jean), training treatment (paired, unpaired), predator cue (present, absent), alarm cue (present, absent) and observation day (day 5 [before training], day 9 [after training]). Pairwise and higher interactions were included in the model with the exception of interactions involving alarm cue, for which only population × alarm cue was included. Body mass and fork length were highly correlated ($r^2 = .89$, $p < .001$), and similar results were observed when fork length was instead included in the model (results not shown). In this model, an innate response to either alarm cue or predator cue would be indicated by a significant main effect of the corresponding term. A learned response to the predator cue would be indicated by a significant interaction that includes training treatment, observation day and predator cue (e.g., if the paired and unpaired treatments differed after training in the presence of the predator cue). All statistical analyses were performed using JMP (v4.0.2, SAS Institute Inc.).

3 | RESULTS

3.1 | Physical comparisons of test fish

Body mass was significantly higher in the Sebago population (mean ± SE: $5.3 ± 0.5$ g) than in either the LaHave ($2.8 ± 0.2$ g) or the St. Jean populations ($2.6 ± 0.2$ g; $F_{2,91} = 17.5$, $p < .001$). Fork length was also significantly higher in the Sebago population ($7.9 ± 0.2$ cm) than in either the LaHave ($6.7 ± 0.2$ cm) or the St. Jean populations ($6.5 ± 0.2$ cm; $F_{2,91} = 15.4$, $p < .001$). As expected, there was no significant difference in body mass ($F_{1,91} = 0.11$, $p = .74$) or fork length ($F_{1,91} = 0.49$, $p = .48$) between the salmon assigned to the paired versus the unpaired treatment.

FIGURE 1  Belted kingfisher (Megaceryle alcyon) taxidermy model showing the portion visible above the top of the experimental tank during the presentation of the predator cue. [Colour figure can be viewed at wileyonlinelibrary.com]
3.2 | Behaviour

Differences in activity were observed among individual fish, with individual ID showing a highly significant effect in the general linear mixed model (Table 2). The model showed a significant positive relationship between body mass and activity, which is consistent with larger fish having an advantage during aggressive interactions and feeding competition. Activity was significantly lower when alarm cue was present than when it was absent, which is consistent with a strong innate response to alarm cue (Table 2, Figure 2). There was no significant interaction between population and the presence of alarm cue, suggesting that the innate response to alarm cue did not differ among populations. In contrast, there was no significant main effect of the presence of predator cue, nor was there an interaction between predator cue and population, suggesting that there was no innate response to the predator cue across the three populations (Table 2, Figure 2).

Activity increased between day 5 and day 9 of the experiment and showed the largest increase in the St. Jean population, as indicated by significant effects of observation day and the interaction between observation day and population (Table 2, Figures 2 and 3). These increases in activity were independent of whether an individual was part of the paired or the unpaired training treatments between day 5 and day 9. After training, fish in the paired treatment had 25% lower activity when the predator cue was present than when it was absent, whereas fish in the unpaired treatment had 7% lower activity when the predator cue was present than when it was absent (Figure 3). However, neither the interaction between training treatment, observation day and predator cue nor the interaction between training treatment, population, observation day and predator cue was significant, suggesting that training was not associated with a statistically significant learned response to the predator cue. Similar trends can be seen with the individual behaviours (Tables 3 and 4).

4 | DISCUSSION

Exposure to alarm cue commonly elicits an innate behavioural response in salmonids (Brown, 2003). Examining three populations of Atlantic salmon, we found that exposure to an alarm cue derived from skin homogenates elicited a decrease in activity levels. This result is consistent with previous studies on salmonids that have shown an innate response to skin-derived alarm cue is conserved, even across multiple generations of captive breeding (reviewed by Ferrari, Wisenden, et al., 2010). Indeed, one study of rainbow trout found an innate response to alarm cue even after 100 years (~15 generations) in a predator-free environment (Scheurer et al., 2007). Innate responses to alarm cues are thus well-conserved in salmonids and provide a mechanism through which individuals may form learned associations with novel cues.

Innate responses to predator cues are typically less consistent than innate responses to alarm cues (Brown & Smith, 1998; Ferrari,
Brown, et al., 2010; Scheurer et al., 2007). For example, an innate response to odour cues of a predatory fish has been observed in some studies of fishes (Hawkins et al., 2004; Jackson & Brown, 2011), but not in others (Brown & Smith, 1998; Ferrari, Brown, et al., 2010). We found no innate response to an avian predator cue in three populations of Atlantic salmon. Interestingly, avian predator cues were associated with an innate anti-predator response in previous studies of Atlantic salmon (Houde et al., 2010; de Mestral & Herbinger, 2013). However, those studies of avian predators had the predator model strike the surface of the water, integrating a disturbance component to the presentation of the avian predator cue that was not present in our study. Taken together with our data, we suggest the disturbance and not necessarily the visual cue of the bird may have elicited the behavioural response in the Atlantic salmon. In the absence of consistent innate responses to predator cues, predator cues may instead contribute to predator recognition through learned associations.

The consistent innate response to alarm cue but not predator cue allowed us to assess learning across three populations of captive-bred Atlantic salmon. After training in which the predator cue and alarm cue were paired, we did not find significant evidence of a learned anti-predator response. It is somewhat surprising that a learned anti-predator response was not observed, as learned anti-predator responses have been shown in many other studies of captive-bred salmonids (Berejikian et al., 2003; Brown et al., 2013; Hawkins et al., 2008). However, these previous studies used an odour cue of a predator, whereas our study used an overhead visual cue of a predator, and it is possible that visual cues are less likely than odour cues to elicit the development of a learned anti-predator response. Alternatively, the absence of a learned anti-predator response in our study could result from the repeated presentations of the alarm and predator cues during training if individuals habituated to the presence of these cues. However, previous studies have generally shown that repeated presentations of paired alarm and predator cues lead to reinforcement of the learned anti-predator responses rather than habituation (Crane et al., 2016; Vilhunen, 2006) and that learned irrelevance occurs most frequently when the predator cue is also presented in the absence of the alarm cue (Ferrari & Chivers, 2006; Hazlett, 2003). Our ability to detect a learned response may also have been limited by statistical power. The activity trends were in the direction expected if a learned response had occurred, but a power analysis indicated that approximately

![Figure 2](image1.png)

**Figure 2** Activity in three populations of Atlantic salmon (*Salmo salar*) on day five of the experiment, which tested the innate response upon first exposure to predator and alarm cues. The activity was calculated as the combined Z-score of three behavioural measures (time spent motionless, number of feeding acts and number of aggressive acts). Means ± SE are plotted. Different letters indicate significant differences among groups.

![Figure 3](image2.png)

**Figure 3** Activity in three populations of Atlantic salmon (*Salmo salar*) on day nine of the experiment, which tested the learned response to the predator cue following predator recognition training. The activity was calculated as the combined Z-score of three behavioural measures (time spent motionless, number of feeding acts and number of aggressive acts). Predator recognition training consisted of a five-day protocol in which alarm cue and predator cue were presented together to enable learning (paired) or presented separately (unpaired). Means ± SE are plotted.
When captive-bred animals are released into novel environments, as is the case in reintroduction programmes, the choice of source population may be an important determinant of a programme’s success (reviewed by Houde et al., 2015). In this study, we tested the innate and learned anti-predator response in three captive-bred populations of Atlantic salmon. All three populations demonstrated an innate response to an alarm cue, suggesting that these populations have the capacity to develop learned anti-predator responses. No population showed an innate response to the visual predator cue, nor was a significant learned anti-predator response observed following predator recognition training with the visual predator cue. As potential differences in anti-predator behaviour populations among populations could influence the success of reintroduction programmes, more work is warranted to better understand the role of captive breeding and population differences in anti-predator responses.
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CONFLICT OF INTEREST
The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT
The datasets generated and analysed during the current study are available as a Supplementary Data file.

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REFERENCES


**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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