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BRIEF COMMUNICATION

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Advancing breeding in stickleback (*Gasterosteus aculeatus*) to produce two reproductive cycles per year

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Abstract

The effects of photoperiod and temperature manipulation on reproductive cycles in threespine stickleback *Gasterosteus aculeatus* were examined. The experimental "advanced group" conditions were adjusted to simulate two reproductive seasons within a calendar year by adjusting light and temperature cycles. *G. aculeatus* subject to advanced conditions had two reproductive cycles per year, grew at normal rates and suffered little additional mortality. The research of many stickleback scientists would benefit from faster generation times and our methods could potentially shorten the time required to produce fish for genetic, behavioural and morphological work.

KEYWORDS

breeding cycle, genomics, method, photoperiod, reproduction, threespine stickleback

Gasterosteus aculeatus are a model organism for the study of a wide array of biological and ecological research, including phenotypic variation, genomic variation, evolutionary change, speciation and learning (Hendry *et al*., 2013). Over the past 20 years *G. aculeatus* have become a powerful model for genetics and genomics research, which builds on a century's worth of data on the ecology, evolution and behaviour of natural populations, and on their rapid adaptation to diverse environments (Bell and Foster, 1994; Ostlund-Nilsson *et al*., 2006). The importance of *G. aculeatus* to genetics and genomics research is bolstered by the ease of making artificial crosses and raising the fish in a laboratory environment, but these pursuits are slowed down by the fairly long, typically 1 year, generation time of these fish, and some populations have even longer generation times (Baker *et al*., 2008; Gambling and Reimchen, 2012). Given that *G. aculeatus* typically have a single breeding season per year lasting about 2–3 months (Borg, 1982), we explored whether we could speed up the breeding cycle from once per year to twice per year, with the idea that this could be a boon, particularly to genetics and genomics work, if crosses resulting from these breeding cycles were able to reduce the length of time needed to produce new generations.

G. aculeatus are seasonal breeders and are thus sensitive to photoperiod and temperature when determining reproductive cycles (Giannecchini *et al*., 2012; O'Brien *et al*., 2012). Longer day length and warmer temperatures induce the onset of spermatogenesis and oogenesis in many fishes, while shorter day length and cooler temperatures cause fish to cycle out of reproductive mode. Photoperiod simulation has been a key component of commercial aquaculture for years, and there is extensive research on how photoperiod affects the growth, maturation and fecundity of commercial fishes (*e.g*., Appelbaum and Kalmer, 2000; Bromage *et al*., 2001; Howell *et al*., 2003). Applying these concepts to a research laboratory setting would provide useful information for scientists who seek to rear their own specimens for study and produce multiple generations of fish as efficiently as possible. In our study, the effects of photoperiod and temperature manipulation on reproductive cycles of captive laboratory-reared *G. aculeatus* were examined. It was also determined whether speeding up reproduction came at a cost to growth or survival for the fish because low mortality would be important to many research programs. Our work sets the stage for further research which can explore whether fast growth and maturation alter development or behaviour in breeding parents or their offspring.

Conditions in a controlled experimental room were adjusted to simulate two reproductive seasons within a calendar year, which typically contains a single reproductive season (Borg, 1982). Fish for this study were selected based on family size and families with a high number of individuals were selected to maintain as large a sample size as possible. We used both adult and subadult fish in our study to ascertain whether our methods would work independently of when

the method was initiated in the life cycle. Half of the families consisted of approximately 6-month-old subadult fish that reached sexual maturity during the experiment (termed growth group), while the other half consisted of 2-year-old fish that began the experiment as adults (termed adult group). In total, 10 unrelated families were included in this study, with five families comprising the growth group and the remaining five comprising the adult group.

The five growth and five adult laboratory-raised families were split in half, resulting in a total of 20 tanks being observed for this study: 10 advanced group tanks and 10 control tanks each holding half of the 10 different families. On splitting the selected families, half of these fish were kept in their original 110 l tanks (the controls), while the other half were transferred into new precycled 110 l tanks in the room with altered photoperiod and temperature (the advanced group). Families were split as evenly as possible. In families with an uneven number of fish, the odd fish were randomly assigned to either the control or advanced group tank so control and advanced groups from a particular family might differ by one fish. Families were initially between 6 and 55 fish before being split, and once families were split they ranged from 3 to 38 fish per tank, our lowest and highest density

TABLE 1 Paired analysis of weight gain, length gain, mortality and average proportion of reproductive *Gasterosteus aculeatus* between control and advanced groups

	Mean ± S.D.			
	Control	**Advanced**	**t**	***P* value**
Weight gain (g)	0.73 ± 0.451	0.73 ± 0.493	−0.042	0.968
Length gain (mm)	16.8 ± 13.496	16.68 ± 14.801	0.119	0.908
First period mortality	**0.06 ± 0.128**	**0.27 ± 0.214**	**−2.773**	**0.022***
Second period mortality	0.08 ± 0.118	0.08 ± 0.164	−0.024	0.981
Total mortality	0.14 ± 0.138	0.33 ± 0.22	−1.933	0.085
First period reproductive fish	0.06 ± 0.045	0.08 ± 0.108	−0.951	0.367
Second period reproductive fish	0.04 ± 0.03	0.13 ± 0.137	−2.0	0.077
Total reproductive fish	0.09 ± 0.147	0.12 ± 0.111	−0.409	0.692

Note: The significant difference is shown in bold.

TABLE 2 Estimates for the beginning of the study and the first and second 6 month periods for number of fish per tank, average weight, average tank density (number of fish in tank/l) and mortality rate (number of fish deaths per tank per week divided by total number of fish in tank) for *Gasterosteus aculeatus*

	Average number of fish per tank	**Average weight (g)**	**Average tank density (fish per tank/l)**	**Mortality rate (deaths per tank/fish per tank)**	**Correlation (*r*) density and mortality**	**Correlation (*r*) density and weight gain**
Beginning of study						
Advanced growth group	16.0	0.09	0.14			
Advanced adult group	5.0	1.62	0.04			
Control growth group	20.2	0.09	0.18			
Control adult group	4.8	1.62	0.04			
First period (first 6 months)						
Advanced growth group	13	0.67[a]	0.12	0.18		
Advanced adult group	3.2	1.77[a]	0.03	0.35		
Control growth group	19.6	0.65[a]	0.18	0.04		
Control adult group	4.4	1.79[a]	0.04	0.08		
Second period (last 6 months)					**Over full experiment**	
Advanced growth group	10.8	1.25	0.10	0.17	−0.53	−0.38
Advanced adult group	3.2	1.93	0.03	0.0	0.08	−0.50
Control growth group	18.0	1.21	0.16	0.06	0.32	−0.51
Control adult group	3.4	1.95	0.04	0.11	−0.44	−0.09

Note: The Pearson correlation coefficients for the full study period between average tank density and both mortality and weight gain are also shown. None of these correlations were significant.

[a]Weights were recorded at the beginning and end of the study. We estimated fish weight in the middle of the study by taking the median value between the start and end weights.

tanks, respectively. We realize that density affects growth, so we ensured that density did not differ significantly for fish exposed to control *versus* advanced temperature and photoperiod. However, density did differ between age class. There were generally more fish per tank for the growth group and thus a higher density than for the adult group (Table 2).

Before the families were split, standard length (tip of snout to caudal peduncle) was measured on each fish with callipers accurate to 0.02 mm, while placing fish on a wet sponge. In addition, fish were weighed on a digital scale accurate to 0.01 g, and average standard length and weight were calculated for each family. These measurements were repeated at the end of the study and were conducted in the same manner.

The length of photoperiod was adjusted to simulate British Columbia, Canada in our regular laboratory rooms due to the fish originating from lakes in coastal British Columbia, and the temperature was set at approximately 15.5°C year-round, as most researchers have a set temperature in their fish rooms that does not vary across the year (Figure 1). These were the light and temperature settings used as the control for this study. In the experimental room, this cycle was sped up to allow us to simulate 1 year in the span of 6 months. A 6-month cycle was chosen because of the need of many researchers for fish in reproductive mode at specific times of year. The temperature was adjusted in single degree increments from a "winter" low temperature of approximately 13°C to a "summer" high temperature of approximately 18°C over each 6-month period. The length

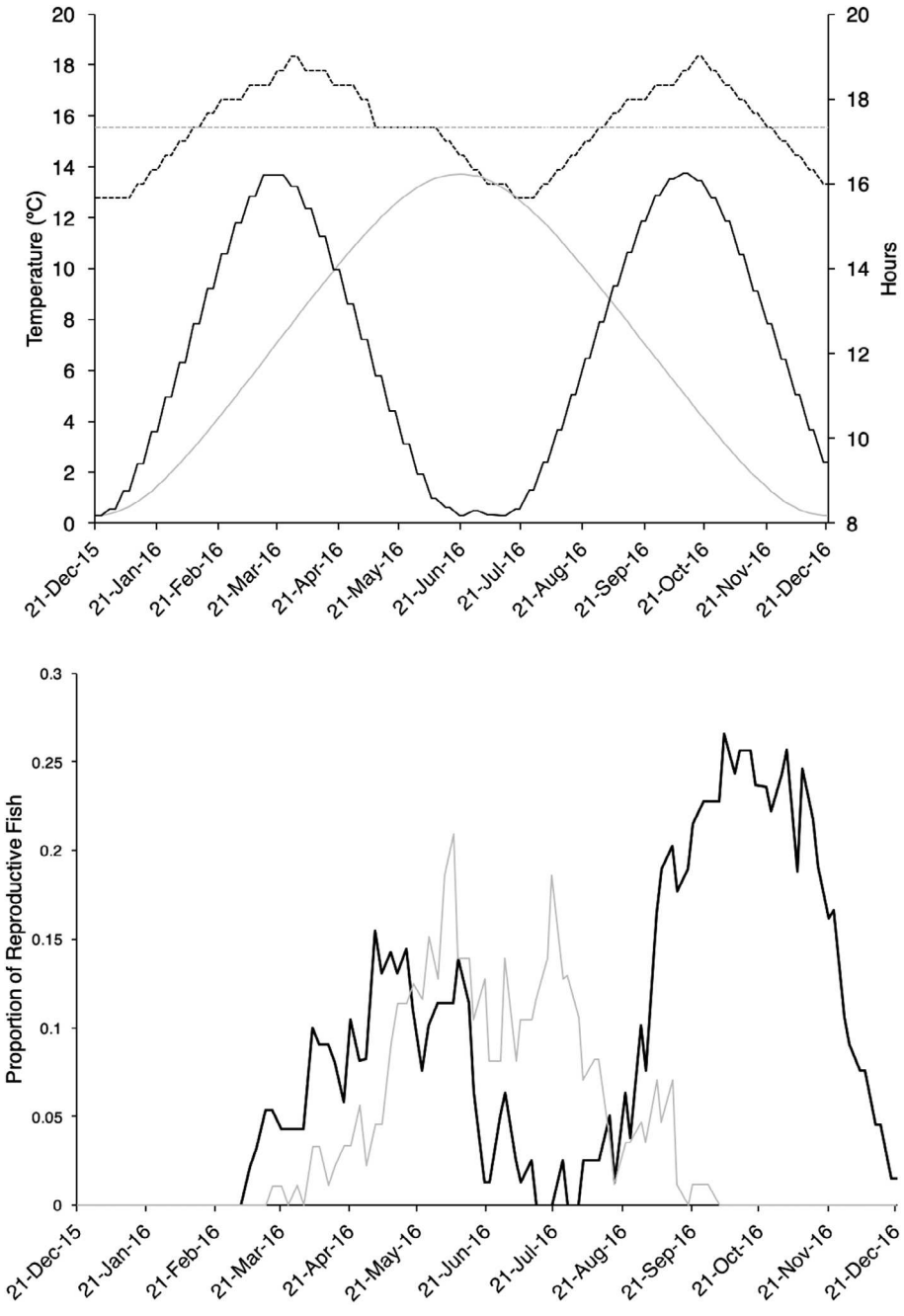

FIGURE 1 (a) Temperature and daylength settings over the duration of the experiment for advanced and control conditions. (------), advanced temperature; (------), advanced daylength; (——), control temperature; (——), control daylength. (b) Proportion of reproductive advanced group and control group sticklebacks (*Gasterosteus aculeatus*) throughout the period of the study. (——), advanced group fish; (——), control group fish

between these incremental changes varied based on the projected average temperature in British Columbia for the simulated date, but was changed once per week or once every 2 weeks. Light and temperature were not manipulated separately, and we did not attempt to mimic the exact temperatures in nature, as the goal was to determine the efficacy of accelerated generation time rather than identify the specific effects of these two factors.

Both the advanced and the control groups were fed *ad libitum* a 50/50 mixture of thawed pre-packaged frozen Chironomidae larvae and brine shrimp (*Artemia salina*) once each morning using a nylon baster tube, adjusting the amount of food to account for fish density. Routine tank maintenance was conducted on the same schedule for both control tanks and advanced group tanks.

Data on each advanced group tank and its corresponding control tank were gathered twice a week to determine the total number of fish in each tank and mortality, as well as the number of reproductively active fish, beginning on 21 December 2015 and ending on 21 December 2016. Reproductive activity was determined based on the outward appearance of the fish. Females were evaluated by distention of their abdomen to determine whether or not they were producing eggs. Males were evaluated by the development of nuptial coloration, a secondary sexual characteristic which corresponds to reproductive activity. This includes the presence and intensity of blue or green body colour, intensity of eye colour, and intensity and area of red throat colour (Lewandowski and Boughman, 2008). Ethical methods were followed and the work was conducted under approved animal care protocols at Michigan State University.

Analyses of data regarding weight gain, length gain, and first and second period mortality rates between control and advanced conditions were performed in SPSS. Statistical tests used paired *t*-tests, with data paired by family. There was no significant difference in weight gain, length gain, second period mortality, or average proportion of reproductive fish ($P > 0.05$; Table 1). There was, however, a significant difference ($P < 0.05$; Table 1) in first period mortality between the control and advanced groups, with higher mortality occurring in the advanced group, therefore the advanced treatment appears to have had fairly little negative effect. Family-level analysis on reproductive and mortality rates was not the subject of focus for this study, but family-level genetic differences within control and advanced groups may have impacted how individuals included in this study responded to treatment. The mortality rate for this analysis was created by counting the number of fish deaths in each tank per week by treatment group (control *versus* advanced), divided by the total number of fish in that tank. Mortality was recorded each day throughout the length of the study.

We compared control and advanced groups for the proportion of fish that became reproductively mature during the 12-month period of the experiment. Overall, there was no statistically significant difference between our control and advanced groups in terms of visibly reproductive traits. Analysis revealed a trend towards a higher mean proportion of reproductive advanced group fish in the second 6 month period (Table 1).

Importantly, reproduction tracked both light and temperature changes. Tests of linear regression revealed a strong relationship between the proportion of reproductive fish and control day length ($r = 0.83$, $r^2 = 0.69$, $P < 0.001$). Fish subjected to the advanced treatment conditions showed a significant relationship between the proportion of reproductive individuals and advanced day length conditions ($r = 0.54$, $r^2 = 0.30$, $P < 0.001$) and a slightly higher relationship with advanced temperature conditions ($r = 0.62$, $r^2 = 0.38$, $P < 0.001$). A relationship between reproductive activity and control temperature conditions could not be statistically evaluated because control temperature did not vary over the course of the study.

No significant difference was found for weight gain or length gain between advanced fish and control fish over the course of the study ($P > 0.05$; Table 1).

The advanced growth group had a consistent mortality rate (0.16 in the first 6 months, followed by 0.17 in the last 6 months), while the advanced adult group had the highest overall mortality rate in the first 6 months of the study (0.35) followed by no deaths at all in the last 6 months of the study. As previously stated, a significant difference in mortality between advanced and control fish was found in the first 6 months of the experiment, as a significantly higher mortality rate was found for advanced fish than for control fish ($P < 0.05$; Table 1).

Reproduction in *G. aculeatus* was successfully sped up by a simple manipulation of light and temperature, generating two reproductive seasons in a 12 month period (Figure 1). This occurred for both adult and growth groups of fish, suggesting that researchers can both speed reproductive cycles in their current breeding stock and speed up growth and maturation for juvenile fish. Faster reproductive cycling came at minimal cost in terms of fish growth, suggesting that the fish develop normally given these advanced conditions. Pearson correlations between overall tank density and weight gain as well as tank density and mortality were performed for each treatment and life stage category (Table 2). There was no correlation between tank density and weight gain, suggesting that fish growth was not affected by tank density. There was also no significant correlation between tank density and mortality for any age or treatment group. Due to the discrepancy in density between growth and adult tanks (Table 1), there is a confound between group type and density. However, this does not appear to have influenced our conclusions, in part because we used paired *t*-tests to compare split families experiencing control or advanced treatments.

Although mortality was significant in the first portion of the study for the group subject to the advanced conditions, the mortality rate fell to the same level as for the group subject to control conditions by the last half of the study, indicating that there may be some loss of fish early on, but this should stabilize over time. An adjustment period allowing for the fish to more slowly adapt to the increasing cycling rate may be one way to mitigate this issue.

The proportion of reproductive fish was high in the second breeding season for the advanced fish. Thus, scientists should be able to implement the protocol outlined in the methods description of this study to produce multiple generations yearly to support breeding experiments and genetics or genomics work. It may be possible to

speed up reproduction even more, given the close tracking of reproduction to the light cycle. The only caution would be to confirm that development and growth are normal, fecundity is not overly depressed and mortality rates are not accelerated.

Male reproductive activity is known to be affected by both the presence of females, as well as available nest-building materials (Wooton, 1976). Nest-building materials were not provided to the males in this study. Egg size or overall fecundity of females were not measured, and these factors can sometimes be affected by photoperiod manipulations (Campos-Mendoza *et al*., 2004). Even though actual fecundity cannot be evaluated, given that advanced group fish were not smaller on average than control fish and that clutch size typically scales with body size, fecundity is not likely to differ substantially. In most cases, small reductions in fecundity should not limit research when the main goal is to produce F1 or hybrid offspring for genetics or behavioural work. Generating F2 populations for mapping work depends more on family size, and researchers may want to evaluate fecundity and egg size for certain types of projects. In a similar vein, the capacity for accelerated reproduction is largely restricted by female productivity. Males maintain spermiogenesis and nuptial colour throughout the breeding season in natural populations, whereas females produce clutches of eggs episodically (Wootton, 1985; Sokolowski and Kulczykowska, 2006). Other studies suggest that energy reserves constrain female fecundity (Ali and Wootton, 1999; Wootton and Fletcher, 2009), especially late in the breeding season (Brown-Peterson and Heins, 2009), so scientists can consider providing supplemental food to increase female fecundity and overall egg production.

While this study used only a *G. aculeatus* freshwater ecotype originating from British Columbia, Canada as the population of interest, it appears likely the accelerated treatment would produce similar results on ecotypes throughout the species' range due to photoperiodic control of reproduction that has been observed in the species (Borg *et al*., 2004).

Our experiment manipulated conditions only for breeding parents and did not evaluate the phenotypes of potential offspring, but we have no reason to anticipate that the offspring will not be normal. For research projects that study the development of phenotypes, we recommend using the offspring from the breeding adults and allowing them to mature under normal conditions.

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AUTHOR CONTRIBUTIONS

J.L.S. helped design the experiment, generated the data, performed analyses and wrote the first draft of the paper. J.W.B. helped design the experiment, contributed to analysis and writing, and provided funds.

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