Received: 23 August 2023 | Revised: 4 April 2024 | Accepted: 6 May 2024
DOI: 10.1002/jez.2827

RESEARCH ARTICLE

JEZ-A ECOLOGICAL AND INTEGRATIVE PHYSIOLOGY WILEY

Intraspecific variation in thermal performance curves for early development in *Fundulus heteroclitus*

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Funding information
Canada Research Chairs; Natural Sciences and Engineering Research Council of Canada

Abstract

Thermal performance curves (TPCs) provide a framework for understanding the effects of temperature on ectotherm performance and fitness. TPCs are often used to test hypotheses regarding local adaptation to temperature or to develop predictions for how organisms will respond to climate warming. However, for aquatic organisms such as fishes, most TPCs have been estimated for adult life stages, and little is known about the shape of TPCs or the potential for thermal adaptation at sensitive embryonic life stages. To examine how latitudinal gradients shape TPCs at early life stages in fishes, we used two populations of *Fundulus heteroclitus* that have been shown to exhibit latitudinal variation along the thermal cline as adults. We exposed embryos from both northern and southern populations and their reciprocal crosses to eight different temperatures (15°C, 18°C, 21°C, 24°C, 27°C, 30°C, 33°C, and 36°C) until hatch and examined the effects of developmental temperature on embryonic and larval traits (shape of TPCs, heart rate, and body size). We found that the pure southern embryos had a right-shifted TPC (higher thermal optimum (T_{opt}) for developmental rate, survival, and embryonic growth rate) whereas pure northern embryos had a vertically shifted TPC (higher maximum performance (P_{max}) for developmental rate). Differences across larval traits and cross-type were also found, such that northern crosses hatched faster and hatched at a smaller size compared to the pure southern population. Overall, these observed differences in embryonic and larval traits are consistent with patterns of both local adaptation and countergradient variation.

KEYWORDS
countergradient variation, development, heart rate, local adaptation, temperature, Q_{10}

1 | INTRODUCTION

Understanding the effects of temperature change on organisms is likely to be critical for forecasting the impacts of climate change; however, most work examining the effects of temperature on aquatic organisms such as fish has been conducted on adults, and much less is known about the thermal sensitivities of embryos (Dahlke et al., 2020; Llopiz et al., 2014). This issue is of great importance because including information about the effects of temperature on early developmental stages can have substantial impacts on

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predictions of climate change vulnerability across populations (Kingsolver & Buckley, 2020; Levy et al., 2015; Radchuk et al., 2013). This is important because some of the currently available data for fishes suggest that early developmental stages are often more vulnerable to thermal extremes than later life stages (Dahlke et al., 2020; Flynn & Todgham, 2018; Rombough, 1997). However, the evidence for greater sensitivity to thermal extremes during early development is mixed (Collin et al., 2021; Pandori & Sorte, 2019; Przeslawski et al., 2015; Rebolledo et al., 2020; Tangwancharoen & Burton, 2014; Truebano et al., 2018). For example, in fishes, the conclusion from a meta-analysis that early developmental stages may be more sensitive to thermal extremes (Dahlke et al., 2020) has been challenged on several grounds including differences in the metrics chosen to reflect thermal tolerance across life stages, and the lack of comprehensive testing across multiple acclimation/developmental temperatures in embryos and larvae (Pottier et al., 2022a).

Thermal performance curves (TPCs) provide a conceptual framework in which to assess the effects of temperature on organismal performance and fitness across levels of biological organization (Schulte et al., 2011). These curves generally have a characteristic shape (Figure 1a), with performance increasing relatively gradually towards a maximum, followed by a steep decline with increasing temperatures (Schulte et al., 2011). TPCs can be described using a few simple metrics including the CT_{min} and CT_{max}, which are the low and high temperatures where performance is equal to zero, the T_{opt}, which is the temperature at which performance is maximized (P_{max}), and the thermal breadth T_{br}, which is the range over which performance reaches some specified percentage of P_{max} (often either 50% or 80% of P_{max}). Note that the terms CT_{min} and CT_{max} used in this context are not equivalent to the CT_{min} and CT_{max} measured as loss of equilibrium in a thermal ramping trial, and thus we and others prefer to use the terms upper and lower T_{crit} for the points at which the TPC reaches zero performance (Payne et al., 2016). However, in this paper, we retain the term CT_{max} for the upper T_{crit} to be consistent with the usage in the TPC program (Padfield et al., 2021) that we use to fit TPCs to our empirical data.

TPC shape and parameters can be altered by both plasticity and adaptation (Schulte et al., 2011; Seebacher & Little, 2021), and estimating the capacity for plasticity and adaptation in TPCs is likely to be vital for understanding population responses to climate change (Angilletta, 2009; Sinclair et al., 2016). However, relatively few studies in aquatic organisms, and particularly in early developmental stages, have assessed performance traits across a sufficient number of temperatures to provide good estimates of the shape of the TPC. As a result, studies of climate change vulnerability often rely on estimates of CT_{min} and CT_{max} at the whole organism level by assessing the temperature at which an organism loses equilibrium in a thermal ramping trial (e.g., Collin et al., 2021; Dahlke et al., 2020; Sunday et al., 2014, 2019). However, the relationship between CT_{min} and CT_{max} estimated in this way and the endpoints of a TPC for a given trait is not straightforward. In addition, changes in these parameters do not necessarily capture information about the nature of the shift across the entire TPC. Comprehensive characterization of TPC shapes has been suggested to be critical in determining which taxa will be winners and losers in the face of climate change (Sinclair et al., 2016; Tüzün & Stoks, 2018).

In this context, understanding how the shape of TPCs can be altered by plasticity or local adaptation is an important consideration for predicting a species likely resilience to climate change (Sinclair et al., 2016). There is a substantial body of theory that has attempted to describe potential changes in TPC shape in response to thermal adaptation or acclimation. One such change involves a horizontal shift of the curve (Figure 1b). For example, a right-shift of the TPC in a species living in warm environments could represent beneficial plasticity or local adaptation. If this horizontal shift occurs without a shift in peak height, this implies that thermal adaptation (or plasticity) is sufficient to completely compensate for the thermodynamic effects of temperature on performance. Alternatively, a variety of studies have detected an upwards vertical shift of the TPC (Figure 1c) with a higher P_{max} in warm-adapted taxa (Alruiz et al., 2023). This pattern is sometimes referred to as the "hotter is better" hypothesis, as it suggests that the performance of low-temperature adapted taxa may be constrained due to limits on the ability of acclimation or adaptation to fully compensate for thermodynamic restrictions imposed by low temperatures (Angilletta et al., 2010; Knies et al., 2009). On the other hand (Figure 1d), other studies have detected an upwards shift of the TPC in taxa from higher latitudes (i.e., colder environments) when compared at a common temperature with lower latitude populations, a pattern termed countergradient variation (Conover & Schultz, 1995). Countergradient variation is thought to represent an adaptation to seasonality, rather than temperature per se (Conover, 1990; Conover & Present, 1990; Yamahira & Conover, 2002). For example, it is hypothesized that an increased growth rate in organisms from higher latitudes compensates for the much shorter growing season, allowing individuals at high latitudes to reach a similar size as conspecifics at lower latitudes despite lower temperatures and shorter summer seasons. Finally, the breadth of the TPC could differ between populations (Figure 1e), which has been suggested as an adaptation to the extent of thermal variability in the environment. In addition, there has been much consideration given to whether there is a tradeoff between increased thermal breadth and increased maximum performance, which is a type of generalist-specialist tradeoff that has been termed the "jack-of-all-temperatures but master of none" hypothesis (Gilchrist, 1995; Huey & Hertz, 1984; Huey & Kingsolver, 1989).

The above hypotheses for adaptive and plastic variation in TPC are not an exhaustive list, and the various changes in TPC shape are not mutually exclusive, as horizontal, vertical, and breadth changes can occur in various combinations. The constraints and trade-offs reflected in these shape changes have been widely discussed (e.g., Angilletta et al., 2002; Buckley & Kingsolver, 2021; Gardiner et al., 2010; Huey & Kingsolver, 1989, 1993; Montagnes et al., 2022) but there remains limited consensus as to the dominant forces shaping the evolution of TPC shapes, with various studies supporting different interpretations (Angilletta et al., 2010; Conover et al., 2009;

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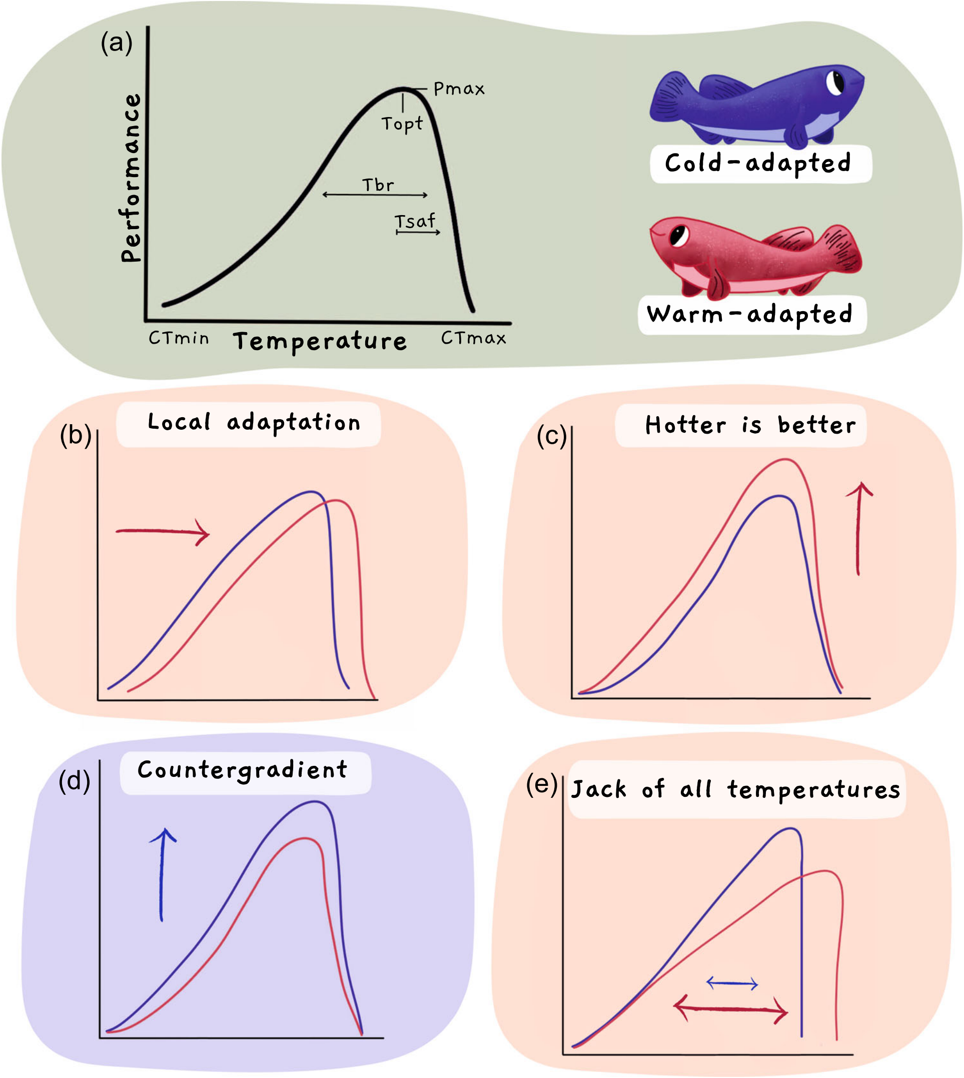

FIGURE 1 Shapes of thermal performance curves (TPC). (a) TPC parameters: CT_{min} and $CT_{max,}$ the low and high temperatures where performance is equal to zero (note that this is not necessarily equivalent to CT_{min} and CT_{max} measured as loss of equilibrium. Thus, many authors prefer to refer to these points as the upper and lower T_{crit}); T_{opt} the optimal temperature where performance is maximized (P_{max}); thermal breadth (T_{br}), the range where performance is greater than some specified percentage of P_{max}; thermal safety margin (T_{saf}), the temperature difference between T_{opt} and CT_{max}. The shape of the TPC between warm and cold acclimated populations may demonstrate (b) local adaptation, (c) "hotter is better" hypothesis, (d) countergradient variation, and (e) "jack-of-all trades but master of none" hypothesis.

Dwane et al., 2023; Frazier et al., 2006; Kontopoulos et al., 2020; Liu et al., 2022; McElroy, 2014; Morrison & Hero, 2003; Pettersen, 2020; Sanford & Kelly, 2011; Sinclair et al., 2012; Sørensen et al., 2018). Thus, the lack of comprehensive data for TPCs in organisms' early life stages, such as fishes, presents a major challenge for assessing these alternatives across life stages.

To address the limited data available for TPC shapes during early development in fishes, and to assess the nature of TPC evolution across latitudes, here we examine changes in the shape of the TPC for early developmental stages across populations of a small teleost fish, the Atlantic killifish, *Fundulus heteroclitus*. Atlantic killifish inhabit intertidal salt marshes along the Atlantic coast of North America. There are two recognized subspecies: *Fundulus heteroclitus macrolepidotus*, which lives in the northern part of the species range, and *Fundulus heteroclitus heteroclitus*, which lives in the south. There is a steep latitudinal thermal gradient along this coast such that the temperature experienced by southern populations is approximately 10°C warmer on average than their northern counterparts throughout the year (Schulte, 2007). Substantial work on the adults of this species demonstrates that the two populations differ genetically (e.g., Powers & Schulte, 1998), physiologically (e.g., Fangue et al., 2006; Fangue et al., 2009b; Healy & Schulte, 2012; McBryan et al., 2016) and behaviorally (e.g., Fangue et al., 2009a) with clear evidence of both local adaptation (Brennan et al., 2016; Fangue et al., 2006) and countergradient variation (Fangue et al., 2009a; Schultz et al., 1996) depending on the trait examined.

In addition to being a model system for the study of thermal adaptation, *F. heteroclitus* was also an important model organism for

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developmental biology in the earliest years of the field, starting with work by Thomas Hunt Morgan in the 1890s (Atz, 1986; Morgan, 1893; Morgan, 1895). Subsequently, several studies have revealed latitudinal variation in embryonic characteristics including embryonic morphology and size (Able & Castagna, 1975; Brummett & Dumont, 1981; Brummett, 1966; DiMichele & Taylor, 1980; Marteinsdottir & Able, 1988; Morin & Able, 1983). Similarly, there is evidence for latitudinal variation in the thermal biology of *F. heteroclitus* embryos. For example, several studies have observed that northern embryos develop faster than those from the south when compared at a common temperature, which is consistent with a pattern of countergradient variation (DiMichele & Westerman, 1997; McKenzie et al., 2017), and there is some evidence that northern killifish have a lower maximum temperature for development than southern killifish (DiMichele & Westerman, 1997), suggestive of local adaptation. However, previous studies have not assessed embryonic development across a sufficient number of temperatures to model the shape of the TPC, which makes it difficult to make conclusions regarding local adaptation and prevents estimation of the thermal breadth for developmental processes.

To assess whether TPCs for northern and southern *Fundulus heteroclitus* demonstrate patterns consistent with local adaptation and/or countergradient variation, we characterized multiple traits (e.g., survival, heart rate, larval morphometrics) during embryonic and larval development in the northern and southern populations of *F. heteroclitus* and their respective reciprocal crosses. A total of eight developmental temperatures (15°C, 18°C, 21°C, 24°C, 27°C, 30°C, 33°C, and 36°C) were selected to capture the temperatures likely to be encountered during early development across the species range to ask:

1) Are patterns of TPC variation for early development across populations consistent with predictions for local adaptation or countergradient variation?
2) Is the breadth of the TPC for early development narrower than that observed in adults?
3) Does embryonic heart rate reflect observed differences in development rate across populations?
4) Are patterns of variation in the larval morphology across populations consistent with predictions for local adaptation or countergradient variation?

2 | METHODS

2.1 | Animal husbandry

Adult *Fundulus heteroclitus* were collected during the Fall of 2019 by Aquatic Research Organisms from Hampton, New Hampshire (42°55′ N, 70°51′W) and from Jekyll Island, Georgia (31°02′ N 81°25′W) using baited Gee's G-40 minnow traps. After transport to the University of British Columbia, Vancouver, Canada, fish were quarantined for 1 month in static tanks with daily water changes at 20°C, 12L:12D, and 25 ppt salinity, made with Instant Ocean Sea Salt (Instant Ocean, Spectrum Brands) and dechlorinated Vancouver tap water. The fish were then transferred to a recirculating system under the same conditions. Fish were fed once daily with PE Mysis Flakes Saltwater Fish Food (Piscine Energetics) until satiation. To achieve breeding conditions, fish from each population were transferred during the spring of 2021 to 60L tanks held at 18°C, 12L:12D, 25ppt that were well aerated with air stones. Males and females of each population were kept in separate tanks to reduce aggressive behaviors, which increased under spawning conditions. Each tank contained either five females (four tanks of females/population) or five males (two tanks/population) for a total of 12 tanks. To induce spawning, tank temperatures were increased by 1°C every other day until a final temperature of 25°C was reached, and photoperiod was simultaneously increased by 15 min/day to a photoperiod of 16L:8D. Breeding fish were fed a diet of blood worms and Mysis shrimp (Hikari) twice a day to satiation. Adult fish were held under these conditions (25°C and for 16L:8D) for 3 weeks before harvesting gametes. This period of time was sufficient to bring all individuals into reproductive conditions. Animal experimentation was performed in accordance with an approved University of British Columbia animal care protocol A20-0070.

2.2 | Crosses and experimental design

Fertilization was completed in vitro as previously described (McKenzie et al., 2017). In brief, both males and females were placed under light anesthetic (MS-222 1 g/L buffered with 2 g/L $NaHCO_3$). Light pressure to the abdomen was used to release the milt or eggs from adults. Twenty females and 10 males from each population were used for this experiment. Eggs from all spawning females were mixed from the same population and then randomly divided into Petri dishes containing a shallow layer of saltwater held at 25°C and 20ppt. Milt from all males was then mixed and divided across the petri dishes. Eggs and sperm were allowed to incubate for 1 h to achieve fertilization. Eggs were then examined under a microscope for the presence of a fertilization envelope (Armstrong & Child, 1965). A total of four different crosses of *F. heteroclitus* were made—pure northern, pure southern, and their reciprocal hybrid crosses (N ♀ x S ♂ and S♀ x N♂).

Eggs were then divided into pools of 60–130 from each cross and incubated in Petri dishes (2–6 Petri dishes per temperature and cross) containing 20ppt dechlorinated water with diluted (0.0003%) methylene blue (as a fungicide) at a range of temperatures from 15°C to 36°C in increments of 3°C and 12L:12D light cycle in temperature-controlled incubators (MIR-154; PHCbi). Water changes were performed daily with a 30-min air exposure period to induce hatching if the embryos were ready to hatch (stages 32–36; Armstrong & Child, 1965), as determined by visual inspection under a microscope (presence of buccal movement; Marteinsdottir & Able, 1992). Petri dishes were checked twice daily for nonviable embryos (identified by opaque white eggs or absence of heartbeat) and for new hatches. Once hatched, larvae were euthanized with MS-222 (3 g/L buffered with 6 g/L of $NaHCO_3$), fixed in 10% neutral buffered formalin for 24 h, and stored in 70% EtOH at 4°C until analysis.

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To assess whether the more rapid development and growth that has previously been observed in northern fish is associated with changes in metabolic parameters, a second set of clutches was generated to allow measurement of embryonic heart rate by breeding 15 females and 10 males from each population. These embryos were incubated at a range of temperatures from 15°C to 33°C in increments of 3°C. To account for differences in developmental timing across temperatures, heart rate measurements were performed at the same developmental stage across all temperatures: stages 30–31 as characterized by Armstrong and Child (1965), at which point the heart chambers are fully differentiated.

2.3 | Larval morphology

To assess the effects of temperature on larval morphology, fixed larvae (0dph) were digitally imaged using a DinoLite Digital Microscope (Dino-Lite Edge 5MP) at a magnification of ×40. Total length and yolk-sac volume (YSV) were measured for each larva using ImageJ, and all larvae were evaluated for deformities across all temperatures and crosses. Dry weight was measured by placing larvae in a drying oven at 60°C for 2 h. Dried larvae were then cooled for 20 min and weighed using a microbalance (Mettler Toledo XPR2). To estimate YSV, we used the formula $YSV = (\pi/6) YsL \cdot YsD^2$, in which YsL represents yolk-sac length and YsD represents yolk-sac diameter (Blaxter & Hempel, 1963). Embryonic growth rate was calculated by dividing length by the days to hatch for each larva.

2.4 | Heart rate

Heart rate measurements were performed by video recording live embryos in a petri dish using a DinoLite Digital microscope (Dino-Lite Edge 5MP). Temperature was maintained by placing the petri dish containing the embryo on a drilled aluminum block through which temperature-regulated water was circulated (Lauda Brinkmann RMT-6). A digital thermometer (Hanna Chektemp1) was placed in the Petri dish to ensure temperature remained stable during measurement (±0.5°C). Embryos were tested at their developmental temperature and were given 20 min to rest after transfer to the microscope before measurements began to account for handling stress. For all heart-rate measurements, 1-min videos were recorded in triplicate with 1-min intervals between each video. Heart rate was then determined by counting the number of heart-beats per minute (beats/min) in each video, and then a mean was calculated for the triplicate videos.

2.5 | Statistical analyses

Statistical analyses were performed using GraphPad Prism (version 9.3.0) and RStudio (version 4.2.1). We estimated TPC shape for survival (%), developmental rate (1/days), and embryonic growth rate (mm/days) using the program rTPC (Padfield et al., 2021). We ran a total of 10 best-fit models (Supporting Information S1: Figures 1–3), based on an initial run of the 24 total models, for each curve, and the best-fit models were determined using Akaike information criterion (AIC) for each curve. We then chose a single model with the lowest mean AIC and a best-fit TPC curve between all crosses for comparison among crosses. Different models were then selected for each phenotype: survival ("Ratkowsky_1983"), developmental rate ("ONeill_1972"), and embryonic growth rate ("Weibull_1995"). From each model (see Supplementary Materials), key parameters were predicted for each curve (e.g., T_{opt}, CT_{max}, Q_{10}, safety margin (T_{safety}), and T_{br}. Note that Q_{10} is modeled with an exponential fit and considers only temperatures below the estimated T_{opt}. Thermal breadth is calculated at 80% of P_{max}. For embryonic growth rate and survival, some parameters could not be estimated due to the shape of the curve. To obtain confidence intervals for the estimates, we used non-parametric bootstrapping case resampling implemented in rTPC to estimate model uncertainty (Padfield et al., 2021).

Significant correlations between heart rate and developmental temperature were identified using a pairwise Spearmen's correlation test ($\alpha = 0.05$). For data on the effect of developmental temperature on embryo and larval phenotypes, normality and homogeneity of variance were checked Shapiro–Wilk and Bartlett's tests respectively, before the application of parametric statistical analyses. For YSV assumptions were not met, and thus, we log-transformed the data, which allowed assumptions of parametric testing to be met. We then used a two-way ANOVA with cross and temperature as factors to detect effects. If significant effects were detected, we used a Tukey's post hoc test for multiple comparisons. All data are presented as mean ± S.E.M., and tests were all evaluated at an alpha level of 0.05.

3 | RESULTS

3.1 | Survival to hatch

TPCs for survival estimated using rTPC are displayed in Figure 2, and TPC parameters and 95% confidence intervals between the four crosses are shown in Table 1 and Supporting Information S1: Figure 4. Regardless of cross-type, *F. heteroclitus* embryos did not survive to hatch when incubated at 36°C. In general, both cross-types with southern mothers had a higher T_{opt} than the pure northern cross (indicated by nonoverlapping confidence intervals). The pure northern cross had the lowest T_{opt} (25.2°C), whereas the pure southern cross had a T_{opt} of 30.3°C and the reciprocal southern cross (S♀ x N♂) had a T_{opt} of 30.9°C. In contrast, the reciprocal northern cross (N♀ x S♂) displayed an intermediate T_{opt} of 28.9°C with confidence intervals that overlapped the estimates for all other crosses. This pattern between cross-types demonstrates a right-shift of the TPC in populations with a southern mother. Embryos from the pure northern crosses also displayed a marked decrease in survival from 30°C to 33°C (Figure 2a). However, at low temperatures, embryos of the pure northern cross performed well, whereas survival was low for embryos of the pure southern cross below 24°C (Figure 2d). Furthermore, the

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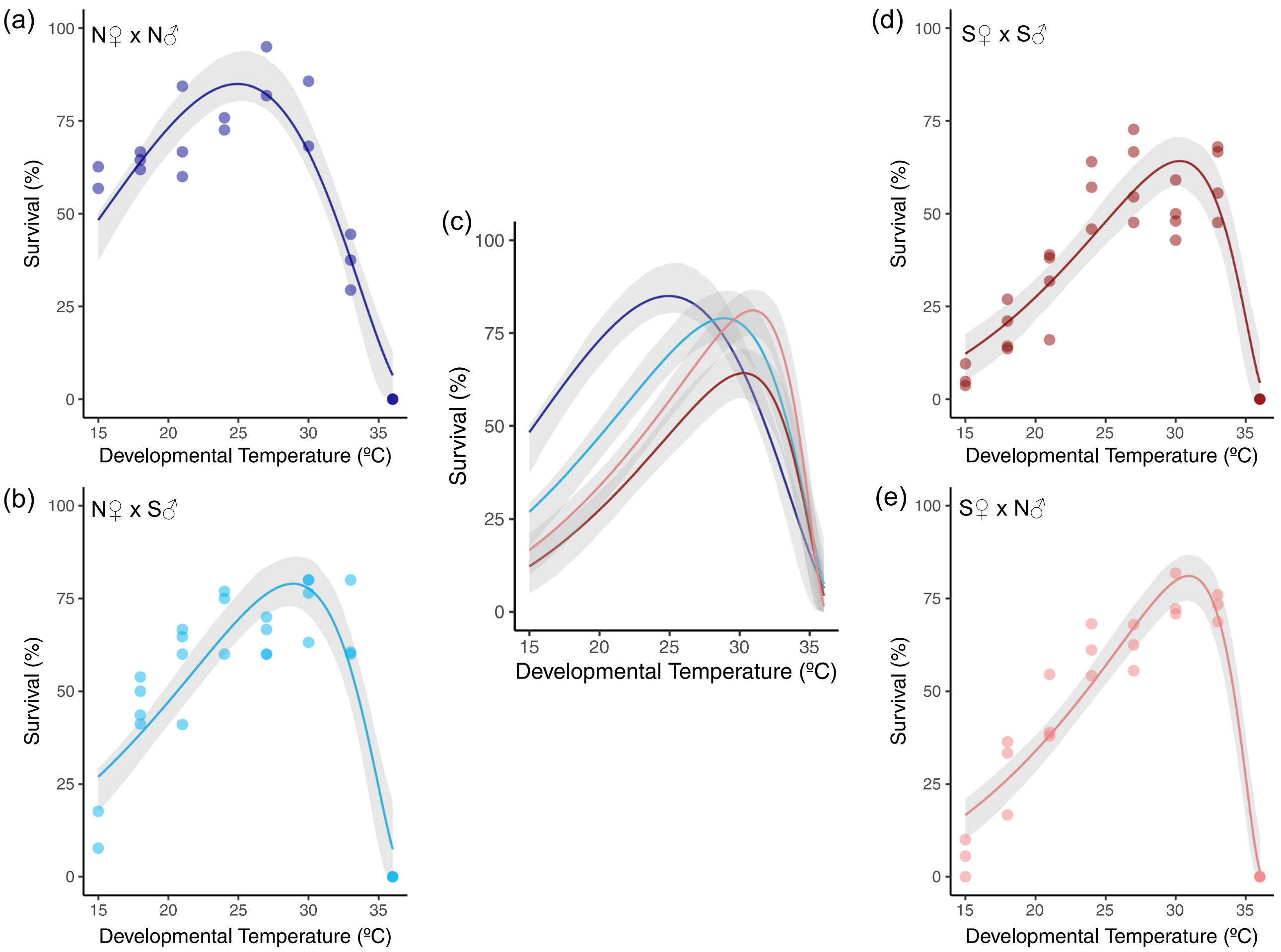

FIGURE 2 Thermal performance curves (TPCs) for survival (%) of embryos in four cross-types of *F. heteroclitus*: (a) northern female x northern male, (b) northern female x southern male, (d) southern female x southern male, and (e) southern female x northern male across eight developmental temperatures (15°C, 18°C, 21°C, 24°C, 27°C, 30°C, 33°C, and 36°C). (c) Displays overlayed TPC for all four crosses. All curves are fitted with the "Ratkowsky 1983" model. Bootstrapped data shows the 95% confidence intervals. Individual data points represent replicate Petri dishes ($N = 2–4$).

cross N♀ x S♂ had improved survival at high temperature and poor survival at the lowest temperature, similar to the pure southern cross (Figure 2b). However, at moderately low temperatures (18°C and 21°C), this cross displayed an intermediate pattern with survival declining by ~25% compared to ~50% declines in survival in the pure southern cross. There were also slight differences in curve shape depending on the population, with pure northern crosses having a higher (i.e., higher survival) and broader shape than the pure southern cross, which had a lower and narrower shape, resulting in a larger thermal breadth for the pure northern cross.

3.2 | Developmental rate

Developmental rate (calculated as the reciprocal of time to hatch; Supporting Information S1: Table 1) was significantly affected by temperature ($p < 0.0001$), cross ($p < 0.0001$), and their interaction ($p < 0.0001$). We directly compared the maximum developmental rate observed regardless of temperature among the crosses. The pure northern cross had the highest maximum developmental rate (0.130 ± 0.001 1/days), which was observed at 30°C, whereas the pure southern cross had the lowest maximum developmental rate (0.096 ± 0.003 1/days), which was observed at 33°C. The reciprocal crosses tended to be most similar to the development rate of their maternal parent, with the N♀ x S♂ reciprocal cross having a maximum developmental rate of 0.112 ± 0.002 1/days at 30°C, and the S♀ x N♂ reciprocal cross having a maximum developmental rate of 0.096 ± 0.003 1/days at 33°C.

3.3 | TPCs for developmental rate

TPCs for developmental rate estimated using rTPC are displayed in Figure 3, and TPC parameters and 95% confidence intervals between the four crosses are shown in Table 1 and Supporting Information S1: Figure 5. In general, both crosses with southern

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TABLE 1 Calculated mean values and 95% confidence intervals (C.I.) for TPC parameters of survival (%), developmental rate (1/days), and embryonic growth rate (mm/days) between four cross-types of *F. heteroclitus*[a].

	Northern♀ x Northern♂	Northern♀ x Southern♂	Southern♀ x Northern♂	Southern♀ x Southern♂
	Survival (%)			
Thermal optimum (°C)	25.2	28.9	30.3	30.9
95% C.I.	24.80–26.75	26.54–30.30	28.15–32.51	29.29–31.74
CT_{max} (°C)	36.1	36.3	36.1	35.8
95% C.I.	35.26–26.51	35.80–38.28	35.82–44.27	35.62–36.59
Safety margin (°C)	10.9	7.32	5.80	4.91
95% C.I.	8.86–11.03	5.60–11.61	2.96–9.22	4.08–7.23
Thermal breadth (°C)	10.7	8.80	7.28	6.83
95% C.I.	9.55–10.85	7.70–9.86	6.08–8.64	6.00–7.77
	Developmental rate (1/days)			
Thermal optimum (°C)	29.15	30.41	31.56	31.65
95% C.I.	28.91–29.32	30.17–30.70	31.12–31.93	31.43–32.02
CT_{max} (°C)	35.96	35.85	35.99	35.99
95% C.I.	35.74–36.38	35.78–35.89	35.97–36.00	35.97–36.00
Q_{10}	3.55	2.48	1.96	2.00
95% C.I.	3.41–3.72	2.28–2.66	1.80–2.13	1.81–2.19
Safety margin (°C)	6.82	5.42	4.44	4.34
95% C.I.	6.50–7.41	5.20–5.67	4.07–4.85	3.98–4.49
Thermal breadth (°C)	6.92	6.95	8.11	7.93
95% C.I.	6.76–7.07	6.63–7.30	7.88–8.44	7.42–8.25
	Embryonic growth rate (mm/days)			
Thermal optimum (°C)	28.8	29.0	31.2	32.57
95% C.I.	28.16–29.16	28.34–29.40	29.35–33.00	29.55–33.00

[a]Note that Q_{10} is calculated for temperatures below T_{opt} and T_{br} is estimated at 80% of P_{max}.

mothers had a higher T_{opt}, and a smaller safety margin (calculated as $CT_{max} - T_{opt}$) than did the crosses with northern mothers, with nonoverlapping confidence intervals, suggesting a right shift of the TPC in the southern population. Curve shape also differed depending on the population of origin of the mother, with crosses from northern mothers having a taller and narrower shape and crosses from southern mothers having a lower and broader shape, resulting in a larger thermal breadth. The overlapping confidence intervals and very similar means suggest that there were no differences in CT_{max} between the four crosses, although making accurate estimates of CT_{max} is difficult as we have no data above 33°C because embryos did not survive to hatch at 36°C, and thus our estimates of CT_{max} reflect extrapolation of the curve beyond the available data. Overall, the shapes of the TPCs differed between crosses as a result of both vertical and horizontal shifts, and we also observed changes in thermal breadth.

3.4 | Embryonic heart rate

Heart rate was positively correlated with developmental temperature (Figure 4a; $R^2 = 0.99$; $p < 0.0001$ for all crosses). We predicted that embryos from the northern cross would have a more rapid heart rate than those from the southern cross to support their more rapid developmental rate. However, only the reciprocal northern cross (N♀ x S♂) had a significantly different slope relative to all other crosses, which was most likely driven by this cross-type maintaining a higher heart rate at the higher developmental temperatures. Q_{10} values for heart rate were ~4 for both maternal southern crosses from 15°C to 21°C then decreased to ~2.5 from 21°C to 24°C (Figure 4b). By contrast, the Q_{10} values for both maternal northern crosses decreased from 4 to 3 between 15°C and 21°C. From 24°C to 30°C, Q_{10} values for heart rate were stable at ~2.0 for all crosses other than the pure southern cross, which has a Q_{10} value of ~1.6. At 33°C, all crosses had a Q_{10} value of less than 2.0.

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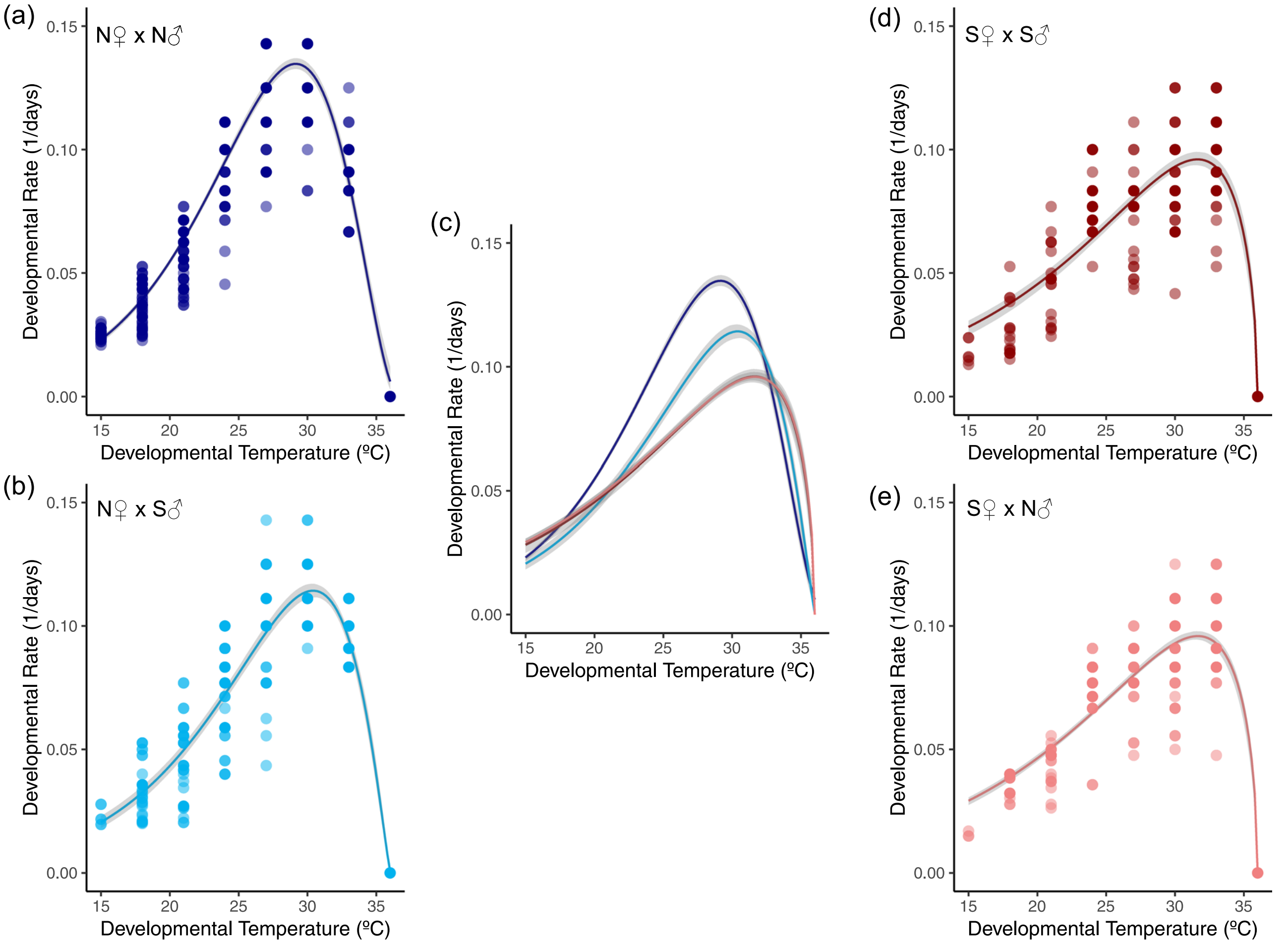

FIGURE 3 Thermal performance curves (TPC) for developmental rate (1/days) in four cross-types of *F. heteroclitus*: (a) northern female x northern male, (b) northern female x southern male, (d) southern female x southern male, (e) southern female x northern male across eight developmental temperatures (15°C, 18°C, 21°C, 24°C, 27°C, 30°C, 33°C, and 36°C). (c) Displays overlayed TPCs for all four crosses. All curves are fitted with O'Neill 1972 model. Bootstrapped data shows the 95% confidence intervals. Each point represents the days to hatch of a given individual. Note that *F. heteroclitus* tends to hatch synchronously so the points for many individuals are overlayed ($N = 7–100$, depending on cross and temperature).

3.5 | Larval morphometrics

Larval length at hatch (Figure 5a; Supporting Information S1: Figure 7A) was significantly affected by temperature ($p < 0.0001$), cross ($p < 0.0001$), and their interaction ($p < 0.0001$). Across all temperatures, the pure northern cross was significantly smaller in length at hatch compared to the pure southern cross. On average, the length of pure northern crosses ranged between 5 and 6.5 mm at hatch across temperatures, whereas the pure southern cross hatched at 6–8 mm in length. The reciprocal cross with a northern mother had an intermediate length at hatch across most temperatures (6–7 mm), whereas the reciprocal cross with a southern mother was more similar in length to the pure southern cross across most temperatures. Higher temperatures resulted in smaller length at hatch for all crosses. The effect of temperature on length at hatch was particularly striking for the pure southern crosses at lower temperatures (18°C and 21°C). The *p* values for larval weight at hatch for all pairwise comparisons of the two-way ANOVA are shown in Supporting Information S1: Tables 2 and 3.

Larval weight at hatch (Figure 5b; Supporting Information S1: Figure 7B) was significantly affected by temperature ($p = 0.014$), cross ($p < 0.0001$), and their interaction ($p < 0.0001$). In general, temperature had a smaller effect on weight at hatch within a cross compared to its effects on length. In both maternal northern crosses, larvae hatched with a significantly lower weight at 18°C relative to 33°C ($p < 0.05$). In the maternal southern crosses, the only significant effect of temperature on larvae weight was found between larvae hatched at 27°C and 33°C ($p < 0.05$). As well, patterns for larval weight among crosses were similar to those for length, with pure northern larvae having significantly lower weight (~0.40 mg) at hatch compared to pure southern larvae (~0.60 mg) across most developmental temperatures. However, near their thermal limits of development (33°C), there were no significant differences in weight between the northern and southern crosses ($p > 0.05$). For the reciprocal

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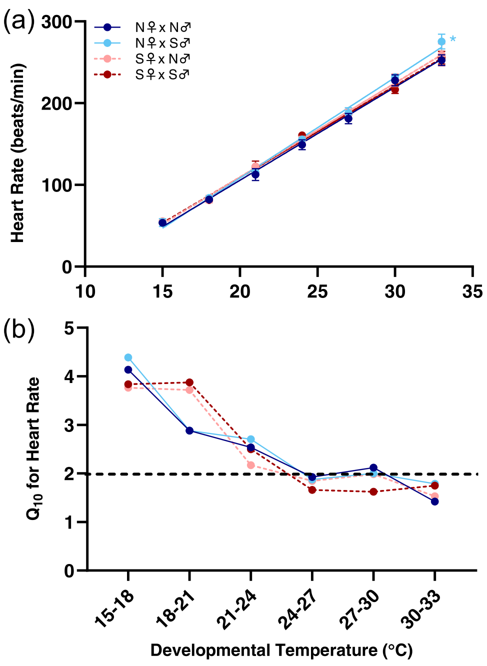

FIGURE 4 Relationship between (a) heart rate (beats/min) and developmental temperature and (b) Q_{10} values for heart rate and developmental temperature between four cross-types of *F. heteroclitus*. (a) Data are presented as means ± SD. Significant relationships were identified using a pairwise Spearmen's correlation test ($\alpha = 0.05$). *Denotes that the slope is significantly different from all other slopes ($p < 0.001$). (b) Points represent mean values calculated from panel (a) for each developmental temperature. The dotted line represents a Q_{10} value of 2.0 for reference.

crosses, their weights at hatch were comparable to their maternal parent cross for most temperatures (Supporting Information S1: Figure 2B). The *p* values for larval weight at hatch for all pairwise comparisons of the two-way ANOVA are shown in Supporting Information S1: Tables 2 and 4.

YSV at hatch (Figure 5c; Supporting Information S1: Figure 7C) was significantly affected by temperature ($p < 0.0001$), cross ($p < 0.0001$), and their interaction ($p = 0.002$). In the pure northern cross, YSV increased with temperature, whereas in the pure southern cross, YSV increased up to 24°C but then remained stable as temperature increased. Both reciprocal crosses exhibited similar patterns in response to temperature as the pure southern cross increased up to 24°C and then remained stable. As well, at 33°C, the pure northern cross had a larger YSV than the pure southern cross ($p < 0.05$). In general, the reciprocal cross with a northern mother hatched with a smaller yolk sac compared to all the other cross-types. The p-values for larval YSV at hatch for all pairwise comparisons of the two-way ANOVA are shown in Supporting Information S1: Tables 2 and 5.

3.6 | Embryonic growth rate

TPCs for embryonic growth rate (larval length divided by days to hatch) estimated using rTPC are displayed in Figure 6, and 95% confidence intervals for T_{opt} between the four cross-types are shown in Table 1 and Supporting Information S1: Figure 6, as this was the only curve parameter that could be accurately estimated due to the shape of the TPC. Overall, crosses with a southern mother had higher T_{opt}, with pure southern having a T_{opt} of 31.2°C and the reciprocal southern cross (S♀ x N♂) having a T_{opt} of 32.7°C. On the contrary, both the reciprocal northern cross (N♀ x S♂) and the pure northern cross displayed lower and similar T_{opt} of 29.0°C and 28.8°C, respectively. Embryos from crosses with a northern mother also displayed a marked decrease in growth rate from 30°C to 33°C (Figure 6a,b). In contrast, embryos from crosses with a southern mother maintain growth rate at higher developmental temperatures (Figures 6d,e), highlighting a potential right-shift of the TPC in populations with a southern mother. There were also slight differences in curve shape depending on the population, with crosses with a northern mother having a taller TPC (i.e., higher growth rate) than crosses with a southern mother (i.e., lower growth rate) across most developmental temperatures.

4 | DISCUSSION

The thermal tolerance of earlylife stages in fishes is likely to be an important factor in predicting a species vulnerability to climate change, but there are limited data examining the thermal tolerance of development in fishes, with most data available for species that are used in aquaculture or for which there is hatchery breeding (e.g., Beacham & Murray, 1989; Burt et al., 2011; Flynn & Todgham, 2018; Llopiz et al., 2014; Murray et al., 1990; Nissling, 2004; Ojanguren and Braña, 2003; Pauly & Pullin, 1988; Rombough, 1997). In this study, we examined the thermal tolerance during embryonic development in two populations of *F. heteroclitus* and their reciprocal crosses. Our TPC modeling revealed a vertical shift (with the northern cross having a higher P_{max} for developmental rate), and a horizontal shift (higher T_{opt} in the southern crosses for survival, developmental rate, and embryonic growth rate), as well as a change in thermal breadth (with the southern crosses having a wider T_{br} for developmental rate but not for survival). These data suggest that both local adaptation and countergradient variation are factors influencing the thermal tolerance and development of *F. heteroclitus* embryos.

4.1 | Are patterns of variation in the TPC for early development across populations consistent with predictions for local adaptation or countergradient variation?

There have been relatively few studies examining latitudinal or other geographic variation in the shape of TPCs for early developmental stages, and most of the available work has been performed in insects (Alruiz et al., 2023; Khelifa et al., 2019). By contrast, there are limited data for geographical variation in TPC shapes for the early developmental stages of fishes, although there is some evidence

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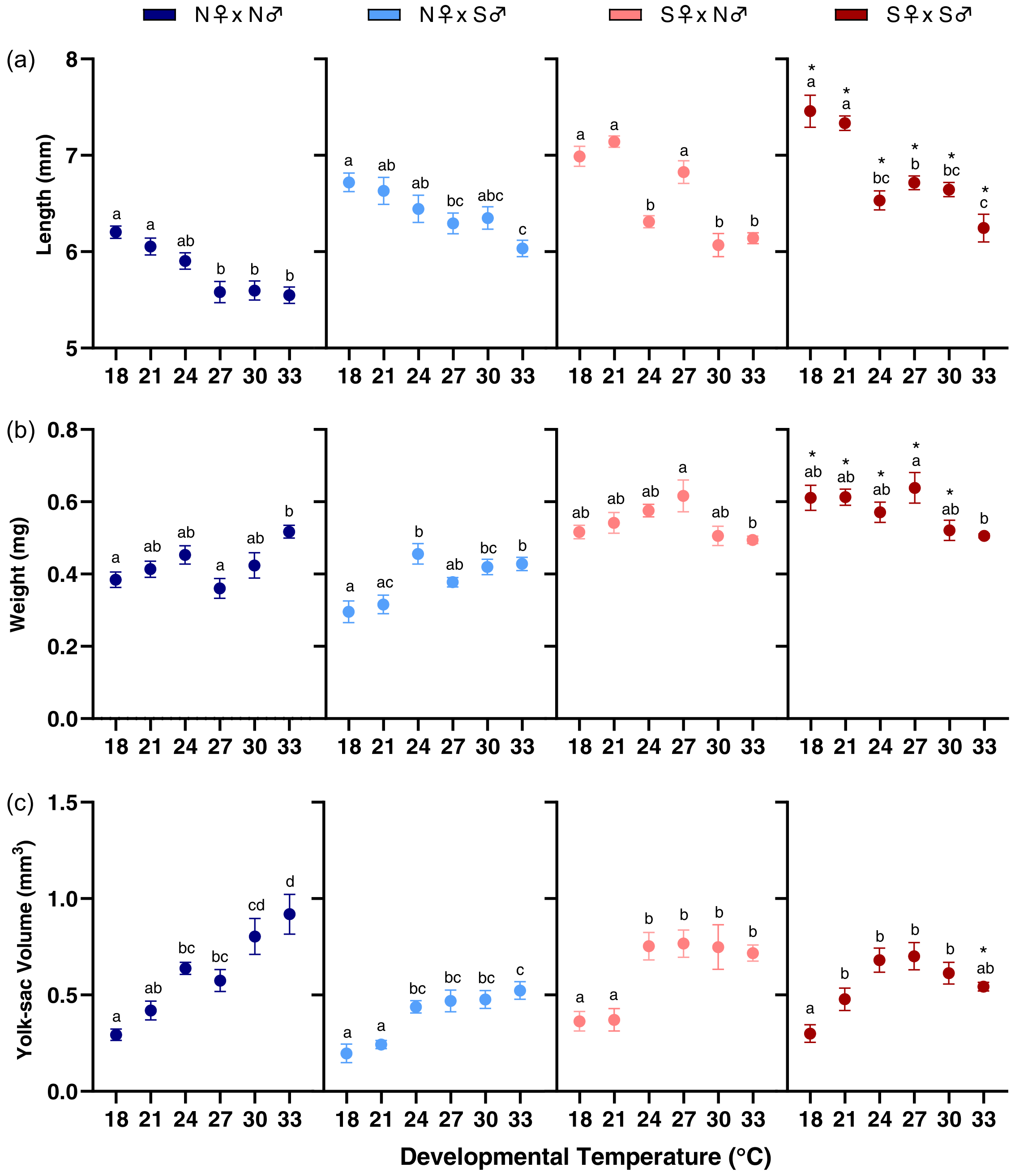

FIGURE 5 Effects of developmental temperature and cross on larval morphology (a) length (mm), (b) weight (mg), and (c) yolk-sac volume (mm^3) between four cross-types of *F. heteroclitus* at hatch. All data presented as means ± s.e.m. ($N = 8$). Different letters denote significant differences between temperatures within a cross ($p < 0.05$). * Denotes significant differences between southern and northern cross at the same developmental temperature ($p < 0.05$).

for geographic variation in embryonic TPCs for salmonids (Beacham & Murray, 1989); however, this variation is not associated with latitude. In this study, we found clear differences in the shape of the TPC for survival, developmental rate, and embryonic growth rate between the northern and southern population of Atlantic killifish. Consistent with the prediction of local adaptation to temperature on the shape of TPC, the pure southern cross had a slight horizontal shift for all TPCs compared to the pure northern cross such that the T_{opt} for the southern cross was ~2.5°C higher (i.e., developmental rate, embryonic growth rate) or ~5.7°C higher (i.e., survival) than that of the northern cross, with nonoverlapping confidence intervals on the estimates. Note that this difference in T_{opt} is similar in extent to the observed differences in CT_{max} at the adult stage between these populations (Fangue et al., 2006). In contrast, our data did not fit the prediction for local adaptation in maximum developmental rate or embryonic growth rate (P_{max}). According to the "hotter is better hypothesis," locally adapted warm-water populations would be predicted to have a higher maximal performance than populations

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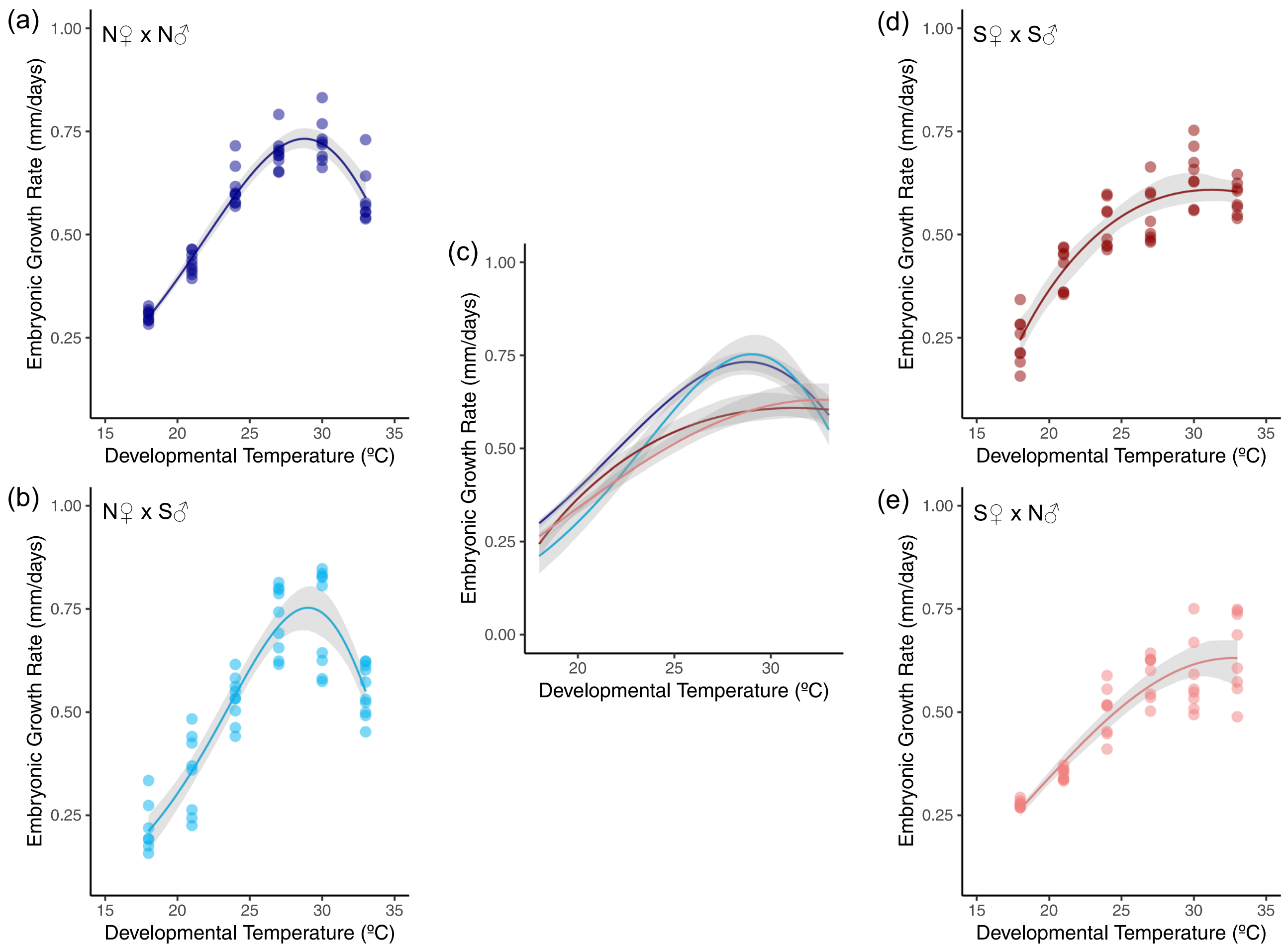

FIGURE 6 Thermal performance curves (TPCs) for embryonic growth rate (mm/days) in four cross-types of *F. heteroclitus*: (a) northern female x northern male, (b) northern female x southern male, (d) southern female x southern male, and (e) southern female x northern male across six developmental temperatures (18°C, 21°C, 24°C, 27°C, 30°C, and 33°C). (c) Displays overlayed TPCs for all four crosses. All curves are fitted with the "Weibull 1985" model. Bootstrapped data shows the 95% confidence intervals. Individual data points represent each individual hatched larva (N = 8–10).

from colder temperatures (Huey & Kingsolver, 1989). By contrast, we found that the pure northern cross had a higher P_{max} compared to the pure southern cross. Furthermore, the pure northern cross had a faster developmental rate and higher embryonic growth rate across all developmental temperatures except for their thermal limit of 33°C, which is consistent with the predictions of the theory of countergradient variation. This theory suggests that the fast developmental rate and higher embryonic growth rate of fish from the northern population is likely driven by their short breeding season selecting for faster development rates at the lower temperatures consistent with higher latitudes (Taylor, 1986).

We also observed a difference in the breadth of the TPC for developmental rate between the pure crosses, with the pure southern cross having about 1°C larger thermal breadth than the pure northern cross. It has been hypothesized that if generalist-specialist trade-offs or other environmental effects do not constrain temperature adaptation, then populations adapted to warmer temperatures would have a larger thermal breadth (Knies et al., 2009). Alternatively, differences in thermal breadth between species have been suggested to be driven by environments with more thermal variability (Dowd et al., 2015). However, both populations experience similar ranges in thermal variability within their habitats both on an annual and a daily basis (Schulte, 2007), which makes this hypothesis unlikely. Interestingly, the observed patterns are consistent with "jack-of-all temperatures but master of none" hypothesis that posits the existence of a trade-off between maximal performance and thermal breadth (Angilletta, 2009; Huey & Hertz, 1984; Huey & Kingsolver, 1989), which is a form of generalist-specialist trade-off. However, the observed difference in thermal breadth between the two populations is small and may, at least in part, be a result of the way that thermal breadth is calculated. Consistent with best practices in the field, we computed thermal breadth at a set percentage of the maximum performance (in this case, 80% of maximum developmental rate, P_{max}). However, the two populations differ substantially in P_{max}, but only across a narrow thermal range (27°C–30°C; Figure 3; Supporting Information S1: Table 1). At other temperatures, the developmental rates are quite similar between the two populations. Thus, if we, for example, computed thermal breadth at a common

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performance level, rather than at a common percentage of maximum performance, thermal breadth would not differ between the crosses. This highlights an important consideration when analyzing data from TPCs. Seemingly mundane decisions about the definition of curve features can result in substantially different conclusions. Instead, we encourage consideration of the complete shape of the curve when drawing conclusions about the effects of adaptation or acclimation on TPCs.

If we consider survival to hatch instead of developmental rate, a different picture emerges. Thermal breadth for survival was found to be ~3°C–4°C smaller for the pure southern cross than the pure northern cross. This is because fish from the pure northern cross maintain high survival (>50%) across a broad thermal range (15°C–30°C), whereas fish from the southern cross have generally lower survival (<50%), and only maintain reasonably high survival from 24°C to 33°C (~50%), suggesting a narrower thermal breadth in southern fish for this critical fitness-related trait.

4.2 | Maternal and paternal effects on development rate and survival

To understand the implications of maternal and paternal effects on the shape of the TPC, we also examined the TPC for the reciprocal crosses of both populations. We predicted the reciprocal crosses would show an intermediate-shaped TPC for all phenotypes. However, we found that the TPC of the reciprocal cross with a southern mother was not different from that of the pure southern cross (having a similar T_{opt} and T_{br}) for developmental rate. This suggests strong southern maternal effects on the shape of the TPC. On the other hand, the reciprocal cross with a northern mother had an intermediate T_{opt} and P_{max}, which suggests that southern alleles from the male parent may be playing a role in both the horizontal (T_{opt}) and vertical (P_{max}) shift in the TPCs. However, the T_{br} of this cross was more similar to that of their female parent, suggesting a maternal effect for developmental rate.

Another noticeable finding from our data is the strong genotype effect on embryonic survival. Unlike developmental rate, both reciprocal crosses displayed intermediate phenotypes for survival (i.e., T_{opt}, T_{br}, and P_{max}) in support of our prediction. Furthermore, we found that crosses with a southern parent (maternal and/or paternal) displayed a low survival at colder acclimation temperatures (15°–21°C). However, this incubation temperature did not always result in the death of the embryos, as we could still observe a heartbeat even though the embryos did not hatch. It is likely that under low-temperature conditions the *F. heteroclitus* embryos went into a state of developmental arrest similar to diapause to await conditions more suitable for hatching. Diapause is a common occurrence during the development of many species of killifish (Podrabsky et al., 2010; Polačik & Vrtílek, 2023), and diapause is particularly well studied in the annual killifishes, such as *Austrofundulus limnaeus*. In these species, fish may undergo up to three consecutive periods of diapause at specific morphological stages (diapause I, II, or III), which enables them to survive the dry season until they receive an appropriate hatching cue such as rewetting by the rainy season (Podrabsky et al., 2001; Wourms, 1972). Non-annual killifish such as *Fundulus heteroclitus* do not undergo developmental arrest but instead display a postponement of hatching of variable duration (ranging from a few days to several months), which is similar to diapause III (Thompson et al., 2017). However, gene expression profiles in non-annual killifish during these developmental delays differ from those observed in diapause III in annual killifishes, suggesting that it is not a "true" diapause state (Thompson et al., 2017).

4.3 | Is the breadth of the TPC for early development narrower than that observed in adults?

There has been a vigorous discussion surrounding the suggestion that the early life stages of fishes have a narrower thermal breadth than juvenile and adult life stages (Dahlke et al., 2020; Pörtner & Farrell, 2008; Pottier et al., 2022b; Rebolledo et al., 2021). A key metric often used to estimate an organism's thermal tolerance at the adult stage is the upper thermal tolerance (CT_{max}) and lower thermal tolerance (CT_{min}) (Schulte et al., 2011). However, these parameters have seldom been measured in fish embryos, and instead, thermal breadth is often measured as LT50 (median lethal temperature in static exposures) (Cowan et al., 2023). Prior meta-analyses of variation in thermal breadth across life stages (Dahlke et al., 2020) may be confounded by different combinations of metrics across life stages, such as LT50 for developmental stages and CT_{max} for adults (Pottier et al., 2022b).

Because of the extensive data available for the thermal breadth of adult *F. heteroclitus*, we can directly compare previously obtained LT50 for adult fish to that of the embryos measured here. From our data, we estimate that the upper LT50 of pure northern embryos is around 33°C as there is a sharp decline in survival at that temperature, whereas the LT50 of southern embryos is between 33°C and 36°C. In comparison, previous work on the LT50 of adult *F. heteroclitus* found that the northern population has a LT50 of 36.4°C and the southern population has a LT50 38.2°C (Fangue et al., 2006). This suggests that the embryos in both populations have a lower upper lethal temperature limit than adults, suggesting they are slightly more sensitive to increasing temperatures. As for lower thermal limits, there are no specific estimates of LT50 for adults. However, previous studies have shown that both of these populations of adult *F. heteroclitus* can acclimate to temperatures near freezing (2°C; Fangue et al., 2006). In the northern parts of the species range, water temperatures are typically close to 0°C for long periods during the winter, although temperatures below 10°C are rare in the southern parts of the species range. In our study, we found that *F. heteroclitus* embryos from pure southern parents had decreased survival below 24°C, whereas embryos from pure northern parents maintained over 50% survival at 15°C (the lowest temperature which we tested), suggesting their lower thermal limits falls somewhere below 15°C.

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These data suggest that *F. heteroclitus* embryos have a narrower thermal breadth than do adults of this species. However, this reduction in thermal breadth appears to primarily be influenced by reductions in performance at lower temperatures, whereas the maximum temperature for successful development is only slightly lower than the maximum temperature to which adult fish can be acclimated. This observation is consistent with the life history of this species. *F. heteroclitus* has a life span of 3–5 years, and thus adults must be capable of surviving through the winter. On the other hand, their breeding season is restricted to the spring and summer, and thus embryos are highly unlikely to experience cold temperatures but may experience critically high temperatures. Overall, our data provides evidence that the embryo life stages of both subspecies of *F. heteroclitus* have a narrower thermal tolerance than their subsequent adult life stages. This supports the idea that earlylife stages may have a smaller thermal window than adults (Dahlke et al., 2020). However, the implications of this observation in the context of climate change are not as simple as has been suggested as the bulk of the reduction in thermal breadth is due to limited tolerance of cold temperatures by embryos. On the other hand, climate change is likely to result in increased thermal variability (including an increased incidence of "cold snaps," as well as the more often considered increase in the frequency of heatwaves (Canning-Clode et al., 2011; Roitberg & Mangel, 2016). This emphasizes the importance of considering the life history of an organism and the timing of reproduction when attempting to integrate information about the variation in TPCs across life stages into predictions of vulnerability to climate warming.

4.4 | Does embryonic heart rate reflect observed differences in development rate across populations?

One of the most striking differences we observed in embryonic development between northern and southern killifish was the faster developmental rate in embryos from pure northern crosses. This difference has been observed in several studies (DiMichele & Westerman, 1997; McKenzie et al., 2017), and here we asked whether this increased developmental rate was associated with an increased heart rate. As expected, developmental temperature had a strong effect on embryonic heart rate, as demonstrated by a strong positive correlation with temperature. However, the effect of cross-type was less evident, with only the reciprocal northern cross having a significantly different slope of the heart rate to temperature relationship relative to all other crosses. The limited differences in heart rate between pure northern and southern fish at any temperature suggest that the faster developmental rate of the pure northern fish is unlikely to be supported by increased aerobic metabolism. This suggests that differences in developmental rate may not be due to differences in aerobic metabolism. However, other metrics of cardiac performance (such as stroke volume), which were not measured here, could result in differences in cardiac output. Previous work by DiMichele and Westerman (1997) supports this hypothesis, that developmental rate may not be driven by differences in aerobic metabolism. They found that differences in developmental rate between northern and southern killifish were most evident before blastulation. Before the mid-blastula transition, *F. heteroclitus* embryos are resistant to cyanide (Wilde & Crawford, 1966), suggesting that metabolism of these early stages is reliant primarily on glycolytic pathways. By contrast, as embryos develop past the mid-blastula transition, they become increasingly reliant on aerobic metabolism (Mendelsohn & Gitlin, 2008). Once aerobic metabolism commences, northern and southern embryos do not develop at different rates (DiMichele & Westerman, 1997). However, the early advantage of rapid early cleavage in northern embryos that is supported by anaerobic metabolism cannot be overcome, and thus northern embryos hatch earlier.

To the extent that heart rate is a proxy for rates of aerobic metabolism, our data suggest that this may not differ between *F. heteroclitus* embryos from different populations at later stages of development. However, we only measured heart rate at a single time-point late in development and did not directly measure metabolic rate. Examination of Q_{10} patterns between crosses also provides insight into the thermal sensitivity of heart rate in embryos. We found that colder temperatures (<24°C) resulted in Q_{10} values between 3 and 4, whereas between 24°C and 33°C Q_{10} values remained between 1.5 and 2, suggesting that embryo heart rate has a much higher sensitivity at colder temperatures. Indeed, this pattern is especially true for crosses with southern mothers. Increases in Q_{10} at low temperatures are often observed, and recent developments using macromolecular rate theory suggest that increased Q_{10} is a result of underlying biochemical processes and is evidence that the temperature is below the optimum for the biological process being studied (Alster et al., 2016).

4.5 | Effects of temperature during early development on larval phenotypes across populations

Previous studies have detected differences in larval phenotypes among populations of *F. heteroclitus* (Marteinsdottir & Able, 1992). Consistent with these previous studies, we found that larvae from pure northern parents were smaller than those from southern parents, regardless of developmental temperature. These contrasting phenotypes between the pure crosses may, at least in part, be explained by differences in egg size, as southern fish produce eggs with a larger egg diameter (Marteinsdottir & Able, 1988) and larval size at hatch is correlated with egg size in *F. heteroclitus* (Marteinsdottir & Able, 1992). In addition, embryos of the southern population have more oil droplets within their yolk-sacs, which may lead to differences in energy allocation, usage, and growth (Morin & Able, 1983). The differences in egg size and larval size between populations have been suggested to be driven by local adaptation of life history strategies (Taylor, 1986). For example, the shorter breeding season in the north may impose selection for a higher clutch size because of the reduced opportunities for multiple rounds of reproduction, and this may result in a trade-off

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in egg size and, therefore, in larval size (Taylor, 1986). When comparing the weight of larvae from both reciprocal crosses, we found that they were similar to that of the pure cross with the same mother, suggesting maternal (possibly egg size) effects. On the other hand, both reciprocal crosses were intermediate in length, suggesting that maternal effects cannot be solely responsible for this phenotypic difference between the pure crosses.

Within a given developmental temperature, most crosses had a similar YSV at hatch. This is surprising given the clear effects of cross-type on larval length. This suggests that there may be differences in growth efficiency or energy allocation among crosses. In particular, larvae from reciprocal crosses with a northern mother tended to have lower YSV at hatch at most developmental temperatures. This is suggestive of an inability to match energetic demand to the more limited energy supply provided by the smaller and less energy-dense yolk contributed by the northern mother.

Larval phenotypes such as hatchling size and YSV were also strongly influenced by incubation temperatures during embryogenesis, as is the case in many fish species (Jordaan et al., 2006; Pepin et al., 1997; Politis et al., 2017). We found that decreasing temperatures resulted in larvae hatching at a longer length for all crosses and higher weight at hatch for both maternal northern crosses. A negative relationship between length at hatch and developmental temperature that has been found in many species of developing fish (Brown et al., 2011; Jay et al., 2020; Martell et al., 2005; Ojanguren & Braña, 2003) and this phenomenon could possibly be explained by longer incubation periods at lower temperatures resulting in a more advanced developmental stage and larger size at hatch, which aligns well with our findings (Ojanguren & Braña, 2003). We also observed differences in larval weight at hatch across temperatures, which could be the result of differences in yolk-sac absorption efficiency driven by differences in metabolic rates (Kamler, 2008). In crosses with a northern mother, YSV and larvae weight follow the same positive relationship such that larvae that hatched at warmer temperatures also had larger YSV. In contrast, crosses with a southern mother had no clear relationship between larval weight and YSV at hatch. This suggests that northern and southern embryos may be utilizing yolk differently across different developmental temperatures. To further assess this possibility, we estimated embryonic growth rates by dividing size at hatch by days to hatch at each temperature (Figure 6). Maximum growth rate (P_{max}) was highest around ~29°C in the crosses with a northern mother highlighting potential differences in yolk-utilization. The possibility of differences in yolk utilization efficiency, energy allocation, or usage across populations is thus a fruitful avenue for future work.

5 | CONCLUSIONS

In summary, we provide evidence that TPC shape varies between latitudinally distinct populations of *F. heteroclitus*, in patterns consistent with both local adaptation (i.e., T_{opt} for development rate, survival, and embryonic growth rate) and countergradient variation (i.e., in P_{max} for developmental rate). As well, we found evidence that embryos from two subspecies of *F. heteroclitus* have a narrower thermal breadth than their adult life stages, which partially supports the hypothesis that embryos are a particularly thermally sensitive life stage (Dahlke et al., 2020; Rebolledo et al., 2021). However, the implications of this observation in the context of climate change are less clear, as the bulk of the difference in the thermal breadth between life stages was due to declines in embryonic performance at lower, rather than higher, temperatures. Lastly, we also found that the pure northern population develops faster, hatch larger, and has higher survival across most developmental temperatures. This emphasizes the importance of including information about the thermal biology of early-life stages and sufficient developmental incubation temperatures to help make clear predictions about how organisms are likely to cope with changing environments.

AUTHOR CONTRIBUTIONS

Tessa S. Blanchard and Patricia M. Schulte conceived the ideas and designed the experiments. Tessa S. Blanchard, Ariel K. Shatsky, and Madison L. Earhart collected the data. Tessa S. Blanchard and Ariel K. Shatsky analyzed all the data. Tessa S. Blanchard and Patricia M. Schulte drafted the manuscript. All authors contributed to edits and final publication.

ACKNOWLEDGMENTS

The authors acknowledge the contributions of Beatrice Rost-Komiya for assistance with fish collection and Daniel Laronde for assistance with embryo care. We also acknowledge Madison Earhart for the design of Figure 1 and the graphical abstract. The work for this project was supported by a Canada Research Chair in Natural Sciences (CRC-2021-00040) and Engineering Research Council of Canada (NSERC) Discovery grant (RGPIN-2017-04613) to P. M. S and an NSERC Postgraduate Fellowship (Doctoral) to T. S. B.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data from this work can be found on Borealis, the Canadian Data Repository: https://doi.org/10.5683/SP3/FAKO55.

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How to cite this article: Blanchard, T. S., Earhart, M. L., Shatsky, A. K., & Schulte, P. M. (2024). Intraspecific variation in thermal performance curves for early development in *Fundulus heteroclitus*. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, 1–18. https://doi.org/10.1002/jez.2827

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