Contrasting Latitudinal Variations in Vertebral Number and Sex Determination in Pacific Versus Atlantic Silverside Fishes

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Organisms that are distributed across spatial climate gradients often exhibit adaptive local variations in morphological and physiological traits, but to what extent such gradients shape evolutionary responses is still unclear. Given the strong natural contrast in latitudinal temperature gradients between the North-American Pacific and Atlantic coast, we asked how increases in vertebral number (VN, known as Jordan's Rule) with latitude would differ between Pacific (*Atherinops affinis*) and Atlantic Silversides (*Menidia menidia*), two ecologically equivalent and taxonomically similar fishes with similar latitudinal distributions. VN was determined from radiographs of wild-caught adults (genetic + environmental differences) and its genetic basis confirmed by rearing offspring in common garden experiments. Compared to published data on VN variation in *M. menidia* (a mean increase of 7.0 vertebrae from 32 to 46°N, VN slope = 0.42 lat⁻¹), the latitudinal VN increase in Pacific Silversides was approximately half as strong (a mean increase of 3.3 vertebrae from 28 to 43°N, VN slope = 0.23 lat⁻¹). This mimicked the strong Atlantic (1.11°C lat⁻¹) versus weak Pacific latitudinal gradient (0.40°C lat⁻¹) in median annual sea surface temperature (SST). Importantly, the relationship of VN to SST was not significantly different between the two species (average slope = −0.39 vertebrae °C⁻¹), thus suggesting a common thermal dependency of VN in silverside fishes. Our findings provide novel support for the hypothesis that temperature gradients are the ultimate cause of Jordan's Rule, even though its exact adaptive significance remains speculative. A second investigated trait, the mode of sex determination in Atlantic versus Pacific Silversides, revealed patterns that were inconsistent with our expectation: *M. menidia* displays temperature-dependent sex determination (TSD) at low latitudes, where growing seasons are long or unconstrained, but also a gradual shift to genetic sex determination (GSD) with increasing latitude due to more and more curtailed growing seasons. Sex ratios in *A. affinis*, on the other hand, were independent of latitude and rearing temperature (indicating GSD), even though growing seasons are thermally unconstrained across most of the geographical distribution of *A. affinis*. This suggests that additional factors (e.g., longevity) play an important role in shaping the mode of sex determination in silverside fishes.

F OR many organisms, average environmental conditions change predictably across their geographical distribution, e.g., with latitude, altitude, or depth. Across such environmental gradients, species commonly display systematic spatial variations in physiological and morphological traits, likely as a result of ongoing local adaptation (Endler, 1986). For example, growth capacity and vertebral number often increase systematically with latitude (Conover et al., 2009), a phenomenon that is well documented especially in fish (Jordan, 1891; Lindsey, 1975; Conover and Present, 1990; Schultz et al., 1996; Pavlov and Shadrin, 1998; Imsland et al., 2000; Chavarie et al., 2010). Most studies have interpreted these spatial trait variations as adaptations to latitudinal gradients in temperature or seasonality. What is insufficiently understood, however, is to what degree gradients actually shape a given adaptive response. This is unclear because most existing studies have examined adaptive variation in single species occurring across one latitudinal gradient. A comparative approach, on the other hand, will allow further insights by contrasting spatial trait variations between different latitudinal temperature gradients.

One suitably strong contrast, for example, exists between the two latitudinal gradients in water temperature along the north-American Atlantic versus Pacific coast (Fischer, 1960). To quantify this, we extracted long-term means (1982–2008) of coastal sea surface temperature (SST) for each coast, week of the year, and degree latitude (27–49°N) from a publicly available global dataset of *in situ* and satellite observations (http://dss.ucar.edu/datasets/ds277.0, Fig. 1). This revealed that the range of weekly temperature means is two times greater along the Atlantic coast (−0.9°C to 29.3°C) than along the Pacific coast (7.6°C to 22.0°C, ΔT_{Atl} = 30.2°C, ΔT_{Pac} = 14.4°C). Annual mean temperatures decrease almost three times faster with latitude along the Atlantic (−1.11°C per latitude) than Pacific coast (−0.40°C per latitude). In addition, the difference between the mean summer maximum and the winter temperature minimum is small and latitude-independent along the Pacific coast (2.6–6.7°C), but strong and increasing with latitude along the Atlantic coast, particularly north of Cape Hatteras ($\Delta T_{Atl\ 28.5°N}$ = 6.3°C, $\Delta T_{Atl\ 45.5°N}$ = 18.6°C, Fig. 1).

Recently, we have used these contrasting gradients to compare patterns of latitudinal growth adaptation between Atlantic Silversides (*Menidia menidia*) and Pacific topsmelt Silversides (*Atherinops affinis*), two ecologically equivalent and taxonomically similar atherinopsid species occurring over almost identical geographic ranges (Baumann and Conover, 2011). Genetic differences among populations, revealed through common garden experiments, indicated that patterns of countergradient growth adaptation correspond closely to the strength of the underlying latitudinal temperature gradient (Baumann and Conover, 2011). In this study, we expand the comparative approach to two other traits that systematically change with latitude in *M. menidia*, but are unknown to vary in *A. affinis*: vertebral number (VN) and the mode of sex determination.
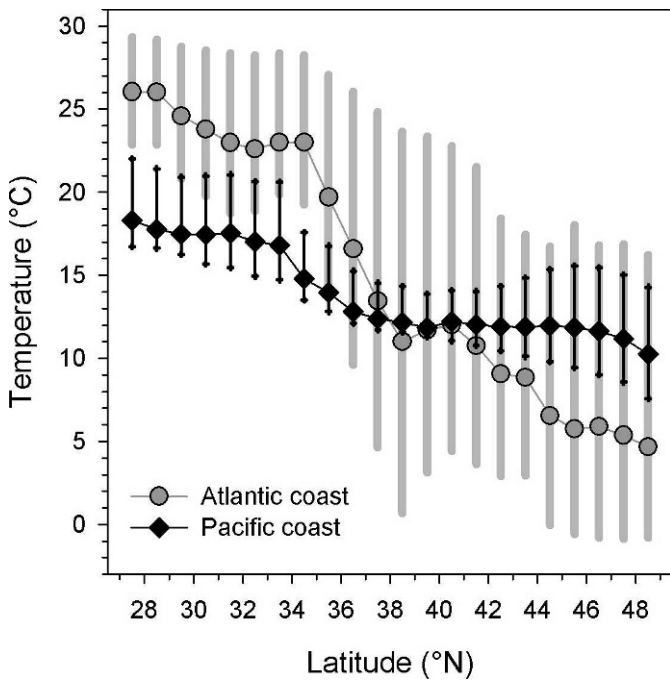
The increase in vertebral number with latitude, known as Jordan's Rule (Jordan, 1891), is common among and within fish species and hypothesized to be adaptive, for example, because more vertebrae may confer better swimming abilities in colder, more viscous waters (Billerbeck et al.,

Fig. 1. Long-term SST patterns along the North-American Atlantic and Pacific coasts derived from satellite and *in situ* measurements (1982–2008). Dots depict median SSTs, bars the range between minimum and maximum SSTs per year.

1997; Yamahira et al., 2006). This would increase fitness by increasing foraging success and lowering vulnerability to predators. Alternative hypotheses regarding the exact adaptive mechanism exist (Hubbs, 1922; Swain and Lindsey, 1984; Swain, 1988, 1992a, 1992b) but they ultimately invoke latitudinal temperature differences to explain Jordan's Rule. For *M. menidia*, several common garden experiments have demonstrated a strong average increase of approximately seven vertebrae from southernmost to northernmost genotypes, which has been attributed to the strong latitudinal temperature gradient that is characteristic for the Atlantic coast (Billerbeck et al., 1997; Yamahira et al., 2006). Therefore, given the much weaker temperature gradient that characterizes the Pacific coast, we hypothesized that the latitudinal increase in VN would be correspondingly smaller in *A. affinis* than in *M. menidia*. If temperature differences are indeed the ultimate cause for Jordan's Rule, then the rule's strength should vary with the strength of the underlying latitudinal gradient.

Atlantic Silversides also display a shift in the mode of sex determination that corresponds to a decrease in growing season length with increasing latitude (Conover and Heins, 1987; Conover et al., 1992; Duffy, 2010). In low latitude populations adapted to long growing seasons, temperature-dependent sex determination (TSD) produces female-biased sex ratios at low temperatures characteristic of the early breeding season and male-biased sex ratios at higher temperatures, which occur toward the end of the breeding season (Conover and Heins, 1987). Hence, females are predominantly born early in the year, reach larger body sizes, and thus achieve higher fitness through increased fertility (Conover, 1984). However, as growing seasons shorten with increasing latitude, the adaptive value of 'being-born-early' (i.e., TSD) diminishes, and temperature becomes less reliable as a cue signifying the beginning of the

growing season. Hence, TSD is gradually replaced by genetic sex determination (GSD). If the evolution of TSD requires long growing seasons to provide earlier born females with a sufficient growth advantage, then we would expect Pacific Silversides to display TSD across the entire geographic range. This is because seasonal temperature fluctuations along the Pacific coast vary little with latitude and remain above the thermal growth threshold of *A. affinis* throughout the year (10°C, Baumann and Conover, 2011).

In this study, we first present thermal reaction norms for VN and sex ratio in *A. affinis* as determined by common garden rearing of offspring from four populations at low (15°C), medium (21°C), and high temperatures (27°C). The patterns are then compared with published data from previous, identical experiments on Atlantic Silversides, *M. menidia* (i.e., Conover and Heins, 1987; Billerbeck et al., 1997; Yamahira et al., 2006).

MATERIALS AND METHODS

Study species.—*Atherinops affinis* and *M. menidia* are two atherinopsid species (New World silversides) that occur over a similarly broad latitudinal range along the Pacific and Atlantic coasts, respectively (*M. menidia*: 30–46°N [Conover and Present, 1990]; *A. affinis*: 24–45°N [O'Reilly and Horn, 2004]). Both are estuarine, schooling, omnivorous fish of equivalent trophic levels. Both are gonochoristic, multiple batch spawners laying benthic, intertidal eggs on a semilunar cycle mainly between spring and summer (Schultz, 1933; Conover and Kynard, 1984). In *M. menidia*, onset and length of the spawning season shift with latitude, while the same is not known for *A. affinis*. Both species mature and spawn at age 1, but differ in their maximum size and age: *M. menidia* is essentially an annual silverside, reaching as much as 15 cm in total length (TL) with <1% of fish reaching age 2 (Conover and Kynard, 1984), while *A. affinis* reaches up to 37 cm TL and typically lives to ages 4–5 (Emmett et al., 1991). In both species, VN is assumed to be plastic until determined at the end of the embryonic stage (McDowall, 2008). In *M. menidia*, the thermally sensitive window for sex determination is known to occur later during larval life (Conover and Kynard, 1981), and a similar window is assumed for *A. affinis*. Both species are equally suitable for experimentation and have been used in a wide range of laboratory studies (Middaugh and Shenker, 1988; Conover and Present, 1990; Anderson et al., 1995, 2009; Duffy, 2010).

***Embryo collection and laboratory rearing of* A. affinis.**— Fertilized topsmelt eggs were obtained by strip-spawning ripe adults ($n > 20$ per sex) sampled by beach-seine in spring 2008 and 2009. We chose four populations (hereafter $P1_{28°N}$, $P2_{33°N}$, $P3_{37°N}$, and $P4_{43°N}$; Table 1) from Pacific estuaries encompassing most of the latitudinal range of the species geographical distribution (O'Reilly and Horn, 2004). Two of the four locations were intentionally repeated in 2009 for inter-annual comparisons. Eggs were wrapped in moist paper towels and stored in 2 L water coolers (IgLoo) for transport to our laboratory facility (Flax Pond, Stony Brook University, Long Island, NY). Upon arrival (20–70 h post fertilization), eggs were placed in aerated 20 L plastic containers partially submerged in large (700 L) temperature-controlled seawater baths pre-set to 15, 21, and 27°C. These conditions encompass the viable thermal range of larvae of *A. affinis* (Baumann and Conover, 2011). Containers had screened holes to ensure water exchange with the

Table 1. *Atherinops affinis*. Overview of sampling sites, mean temperature conditions, and analysis in 2008 and 2009.

Estuary, site, location	Location	SST*(°C)	Sampling date(s)	Mean TL** (1 SD, mm)	Population acronym	Sex ratio Lab	Vertebral # Field	Vertebral # Lab
Laguna Manuela, northern arm, Baja California, MX	28.25°N 114.08°W	17.8 (16.6–21.4)	April 2009 (22–24)	171 (11)	P1$_{28°N}$	✓	✓	✓
Tijuana estuary, Oneonta Slough, California, US	32.57°N 117.13°W	17.0 (14.9–20.6)	May 2008 (19) May 2009 (11)	154 (23)	P2$_{33°N}$	✓ ✓	✓ –	✓ –
Elkhorn Slough, South Marsh, California, US	36.82°N 121.74°W	12.8 (12.1–15.3)	May 2008 (16–17) May 2009 (7–9)	112 (7)	P3$_{37°N}$	✓ ✓	✓ –	✓ ✓
Coos Bay, North Bend, Oregon, US	43.38°N 124.20°W	11.9 (10.1–14.9)	June 2008 (19)	263 (32)	P4$_{43°N}$	✓	✓	✓

* median range annual sea surface temperature at the corresponding latitude coastal ocean, based on long-term *in situ* and satellite observations (1982–2008)

** of field-collected spawning adults

baths. During both years, trials were conducted at a 15h light:9h dark photoperiod and a salinity of 30 ± 2 psu using water from saline ground wells. In 2008, lower salinity water (18 psu, different well) was adjusted upward by adding commercial sea salt (Instant Ocean). Depending on temperature, topsmelt larvae hatched 6–16 days post fertilization at approx. 6 mm in length (population-independent) and were start-fed with a mix of larval powder food (Otohime Marine Weaning Diet, size A, Reed Mariculture) and newly hatched brine shrimp nauplii (*Artemia salina*, San Francisco strain, Brine Shrimp Direct, Inc.).

Some larvae were subsequently used to establish replicated growth trials at standardized larval densities and feeding conditions (Baumann and Conover, 2011). In contrast, excess offspring were reared in 60 L containers (similarly equipped with screened holes, sitting in the same temperature-controlled baths) at high but uncontrolled food levels and similar but uncontrolled fish densities. In addition to daily rations of newly hatched brine shrimp nauplii, excess fish were also fed commercial dry food (Otohime Marine Weaning Diet, B-2, Reed Mariculture). Excess fish densities were reduced several times to avoid overcrowding. Depending on temperature, excess offspring were reared for 51–170 days until reaching an average TL ± SD of 40.5 ± 5.9 mm, at which point a random sub-sample of at least 100 specimens per temperature and population was taken and preserved in 10% buffered formaldehyde.

Vertebral number.—VNs for populations P2$_{33°N}$, P3$_{37°N}$, and P4$_{43°N}$ were determined from excess offspring reared in 2008 and 2009 (P3$_{37°N}$ only, for inter-annual comparisons). Due to limited offspring numbers for P1$_{28°N}$ in 2009, VNs were measured in fish from the controlled growth trials instead (Baumann and Conover, 2011). At least 25 random specimens per sex (see below), population, and temperature treatment were radiographed using a digital INSPEX 20i x-ray system (x-ray source: Kevex PXS10-16W [140 µA/40 kVp], detector: Varian PaxScan 4030R, VIVA software). All vertebrae between the basioccipital and urostyle were subsequently counted with the help of an image analysis program (ImagePro). In addition, a sub-sample of 30–50 adult field-collected *A. affinis*

from each of the four sampling sites was radiographed using an analog Kramex PX-20N x-ray system (10mA/80kVp) and exposure times of 2–3.2 seconds. Developed and fixed radiographs (Kodak Industrex type M) were viewed against a light board and vertebrae (basioccipital–urostyle) directly counted. VN was not size-corrected, because this trait is mainly determined during the embryonic stage (McDowall, 2008), invariant during ontogeny, and because TL and VN were statistically independent in two of four field-populations and 17 of 18 laboratory sample groups (Pearson correlations, all $P > 0.05$). The thermal plasticity of VN was assessed for each population by comparing vertebral number across the three temperature treatments, using separate ANOVAs per population (latitude) and subsequent Dunnet-T3 *post-hoc* tests to identify significantly different groups. Conversely, the population (latitude) effect on vertebral number was evaluated by separate ANOVAs per temperature and Dunnet-T3 *post-hoc* tests.

Subsequently, Pacific versus Atlantic VN patterns were analyzed using ANCOVA, with species as fixed factor and either (A) latitude or (B) median annual SST at the corresponding coastal latitudes as covariates. In addition, each ANCOVA included one interaction term, i.e., (A) species*latitude or (B) species*SST, in order to test for significant species (coast) differences in the slopes of the 'latitude versus VN' or 'SST versus VN' relationships. All statistical analyses were performed in SPSS (version 19.0 IBM®).

Sex ratio determination.—Similar to VN, sex ratios were determined using excess laboratory offspring for P2$_{33°N}$, P3$_{37°N}$, and P4$_{43°N}$, but offspring from controlled growth trials for P1$_{28°N}$. At least 100 randomly selected specimens per temperature and population were first measured for TL to the nearest 0.1 mm (using digital calipers) and then carefully dissected at low magnification to examine the gonads. We found that in *A. affinis* >35 mm TL, female gonads are readily distinguishable from male gonads by their thicker, more three-dimensional shape; when in doubt, gonads were placed on glass slides and microscopically (100X) checked for the presence of developing oocytes.
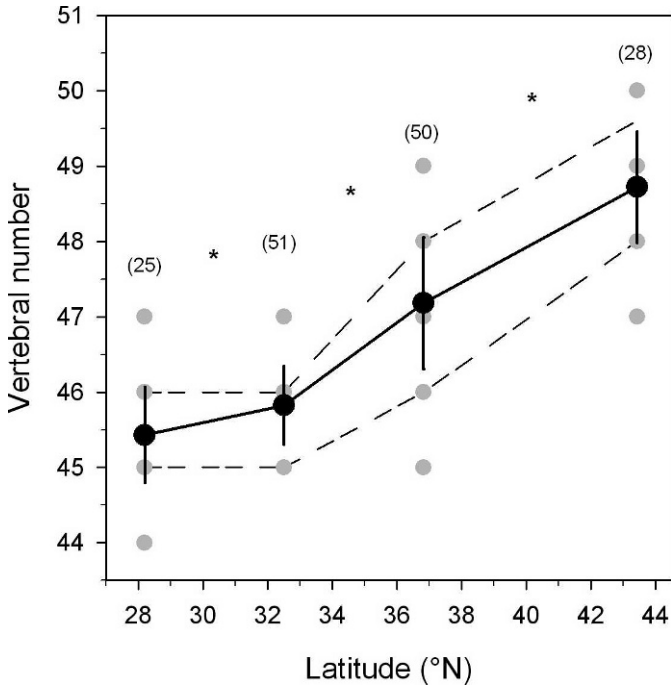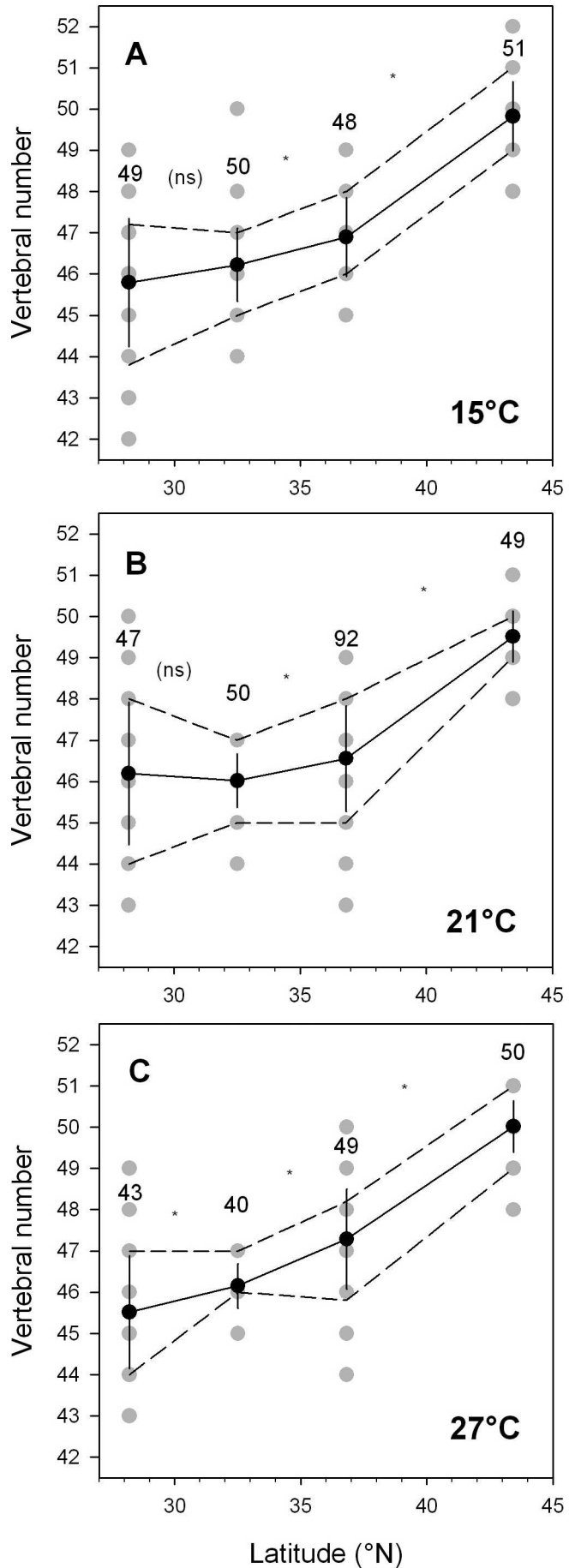
Fig. 2. *Atherinops affinis*. Mean (± SD) vertebral number of field-collected mature adults from four populations along the North-American Pacific coast (P1$_{28°N}$–P4$_{43°N}$). Gray circles denote the data range, black circles and error bars are population means ± 1 SD. Dotted lines depict 10th and 90th percentiles. Sample sizes are given in parentheses next to each population, asterisks identify significant differences ($P < 0.05$) between adjacent populations (ns = non-significant).

For each population, sex ratios were checked for significant departures from a 1:1 ratio by binomial tests, using Bonferroni-adjusted α-values of 0.05/3 (P1$_{28°N}$ and P4$_{43°N}$, 1 year × 3 temperatures per population) or 0.05/6 (P2$_{33°N}$ and P3$_{37°N}$, 2 years × 3 temperatures per population). To test for sexual dimorphism in *A. affinis* (as it is known for *M. menidia*), mean TL was compared between sexes in laboratory-reared offspring and field-collected adults using ANOVA (SPSS 19.0, IBM).

RESULTS

VNs in field collected specimens ranged from 44–50, with population means increasing significantly with latitude (ANOVA, $F_{3,153} = 132.4$, $P < 0.001$, Fig. 2) but not consistently with body size (Table 1). VNs in laboratory offspring ranged from 42–52 (Fig. 3). With the exception of P2$_{33°N}$ at 15°C and 21°C, population means at all three temperature treatments increased significantly in rank order with latitude (ANOVA, $F_{[15°C]3,197} = 139.1$, $F_{[21°C]3,237} = 97.3$, $F_{[27°C]3,181} = 185.6$, all $P < 0.001$, Fig. 3). The northernmost P4$_{43°N}$ (VN$_{field}$ = 48.7, VN$_{lab}$ = 49.8) had on

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Fig. 3. *Atherinops affinis*. Latitudinal variation in vertebral number in offspring from four populations (P1$_{28°N}$–P4$_{43°N}$) reared at three temperatures in common garden experiments (A: 15°C, B: 21°C, C: 27°C). Black circles and error bars represent population means ± 1 SD. Dotted lines depict 10th and 90th percentiles. Sample sizes are given in parentheses, asterisks identify significant differences ($P < 0.05$) between adjacent populations (ns = non-significant).
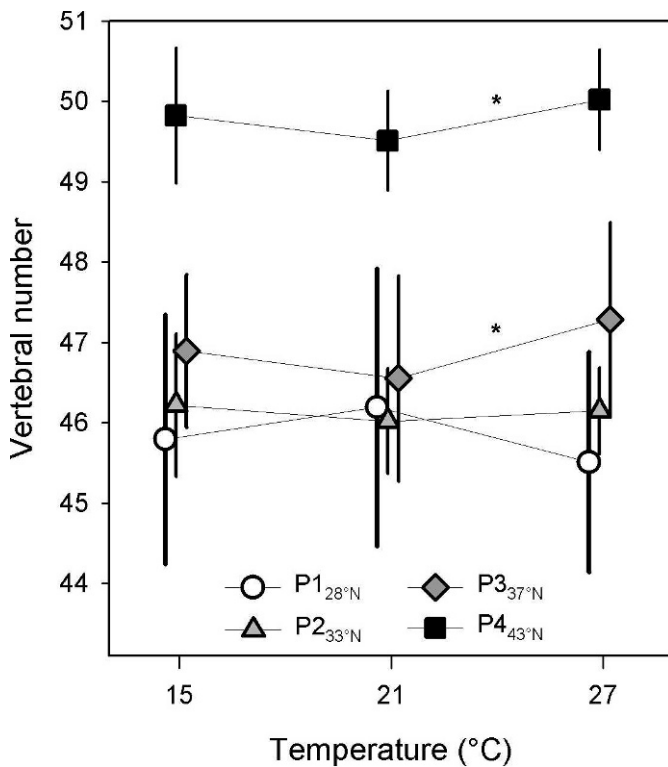
Fig. 4. *Atherinops affinis.* Mean ($\pm$ SD) number of vertebrae for juveniles of four populations (P1$_{28°N}$–P4$_{43°N}$) reared at 15, 21, and 27°C. Asterisks denote significant differences ($P < 0.05$) between adjacent temperature treatments.

average 3.3–4.0 vertebrae more than the southernmost P1$_{28°N}$ population (VN$_{field}$ = 45.4, VN$_{lab}$ = 45.8). Field VNs were generally less variable than those of experimental offspring (mean SD[VN]$_{field}$ = 0.69, mean SD[VN]$_{lab}$ = 1.06, Figs. 2, 3). There was no evidence for sex-dependent differences in VN, neither in field nor in laboratory fish (ANOVA, $F_{[field]1,100}$ = 0.3, P = 0.6, $F_{[lab]1,435}$ = 0.5, P = 0.5), and no evidence for inter-annual effects, based on a 2008 versus 2009 comparison of P3$_{37°N}$ juveniles reared at 21°C (ANOVA, $F_{1,91}$ = 0.6, P = 0.4). The plasticity of VN was small, given that within populations temperature-specific VNs differed little, and significant VN differences only occurred in two populations between 21°C and 27°C (ANOVA, $F[P3_{37°N}]_{2,188}$ = 6.2, P = 0.003, $F[P4_{43°N}]_{2,149}$ = 6.6, P = 0.002, Fig. 4). Contrary to expectation, intermediate temperatures never produced intermediate vertebral counts (Fig. 4).

Published average VNs for two contiguous Atlantic Silverside species, *M. peninsulae* and *M. menidia*, ranged between 34–38 and 37–44, respectively (Billerbeck et al., 1997; Yamahira et al., 2006; Fig. 5A). In *M. peninsulae*, VN increased on average by 3.7 vertebrae over five degrees of latitude corresponding to a slope of 0.66 vertebrae °latitude^{-1} (r^2 = 0.94). In *M. menidia*, between 32.4–46.2°N, VN increased on average by 7.0 vertebrae, corresponding to a slope of 0.42 vertebrae °latitude^{-1} (r^2 = 0.96). In *A. affinis*, a much smaller slope of 0.23 vertebrae °latitude^{-1} was found (r^2 = 0.97), owing to the small average increase of 3.3 vertebrae over almost 15° of latitude. The slopes of *A. affinis* and *M. menidia* were significantly different, given the significant species*latitude interaction in the ANCOVA (Table 2A, Fig. 5B). Furthermore, VNs of both *M. menidia* and *A. affinis* decreased significantly with increasing SST at

population origins (ANCOVA, Table 2B, Fig. 6). However, the species*SST interaction was not significant (P = 0.81), indicating a common temperature-dependency of VN across species/coasts (Fig. 6).

Sex ratios of *A. affinis* did not change consistently with latitude or temperature in either of the two experimental years (Fig. 7). After adjusting significance levels to multiple comparisons (Bonferroni), none of the 18 groups showed significant departures from a 1:1 sex ratio ($P_{min–max}$ = 0.02–0.93). A tentative trend for increasing female proportions with temperature, as perhaps interpretable in P2$_{33°N}$ and P3$_{37°N}$ populations during the first experimental year, was not apparent anymore during the second year (Fig. 7). There were also no significant TL differences between sexes, neither in field collected adults (ANOVA, $F_{1,100}$ = 2.3, P = 0.13) nor in laboratory-reared offspring (ANOVA per population, temperature, and year, $F_{[min]1,150}$ = 0.002, P = 0.96, $F_{[max]1,115}$ = 3.6, P = 0.06), with the exception of P2$_{33°N}$ at 27°C in 2009 (Mean $\pm$ SD males = 41.3 $\pm$ 4.9 mm, mean $\pm$ SD females = 43.9 $\pm$ 3.9 mm, P = 0.002).

DISCUSSION

By conducting two sets of common garden experiments on Pacific Topsmelt silversides, we sought to determine whether the species displays genetic latitudinal variations in vertebral number and sex ratios and whether differences between Pacific versus Atlantic Silverside patterns reflect their contrasting latitudinal temperature gradients. We found that vertebral number increased in rank order with latitude in both field and common garden-reared *A. affinis*, which indicates a genetic basis to the pattern consistent with the classic eco-geographic law in fishes known as Jordan's rule (Jordan, 1891). This rule must have adaptive significance, because other genetic effects (drift, founder, or bottleneck) generally fail to produce strong correlations between trait variations and environmental gradients (Endler, 1986; Yamahira et al., 2006).

Surprisingly, the actual adaptive mechanisms responsible for Jordan's rule remain speculative (McDowall, 2008). One view holds that variations in vertebral numbers could have evolved as a by-product of selection on body size, given that both traits are often correlated between populations, and maximum body size tends to increase with latitude in many species (Bergmann's rule, Bergmann, 1847; Lindsey's pleomerism, Lindsey, 1975). In Atlantic Silversides, one mechanism could therefore be size-selective winter mortality, which is stronger and more biased against smaller individuals in higher latitudes, thereby indirectly producing a gradient in vertebral numbers (Billerbeck et al., 1997; Yamahira et al., 2006). But Jordan's rule also applies to species, where winter mortality is unlikely to be a major selection agent, e.g., to Pacific Silversides, which probably grow almost year-round due to mild winter temperatures that are rarely below the species thermal growth threshold (approx. 10°C, Baumann and Conover, 2011). One plausible alternative is that variations in vertebral number might be directly adaptive because they affect swimming performance, and hence foraging success and predation vulnerability of fish larvae, in waters of different temperature-dependent viscosities. Due to methodological obstacles, mainly the difficulty to separate temperature- from viscosity-related effects on larval swimming performance, this has yet to be rigorously tested (but see: Swain, 1986, 1992a). However, Swain (1988, 1992a, 1992b) demonstrated
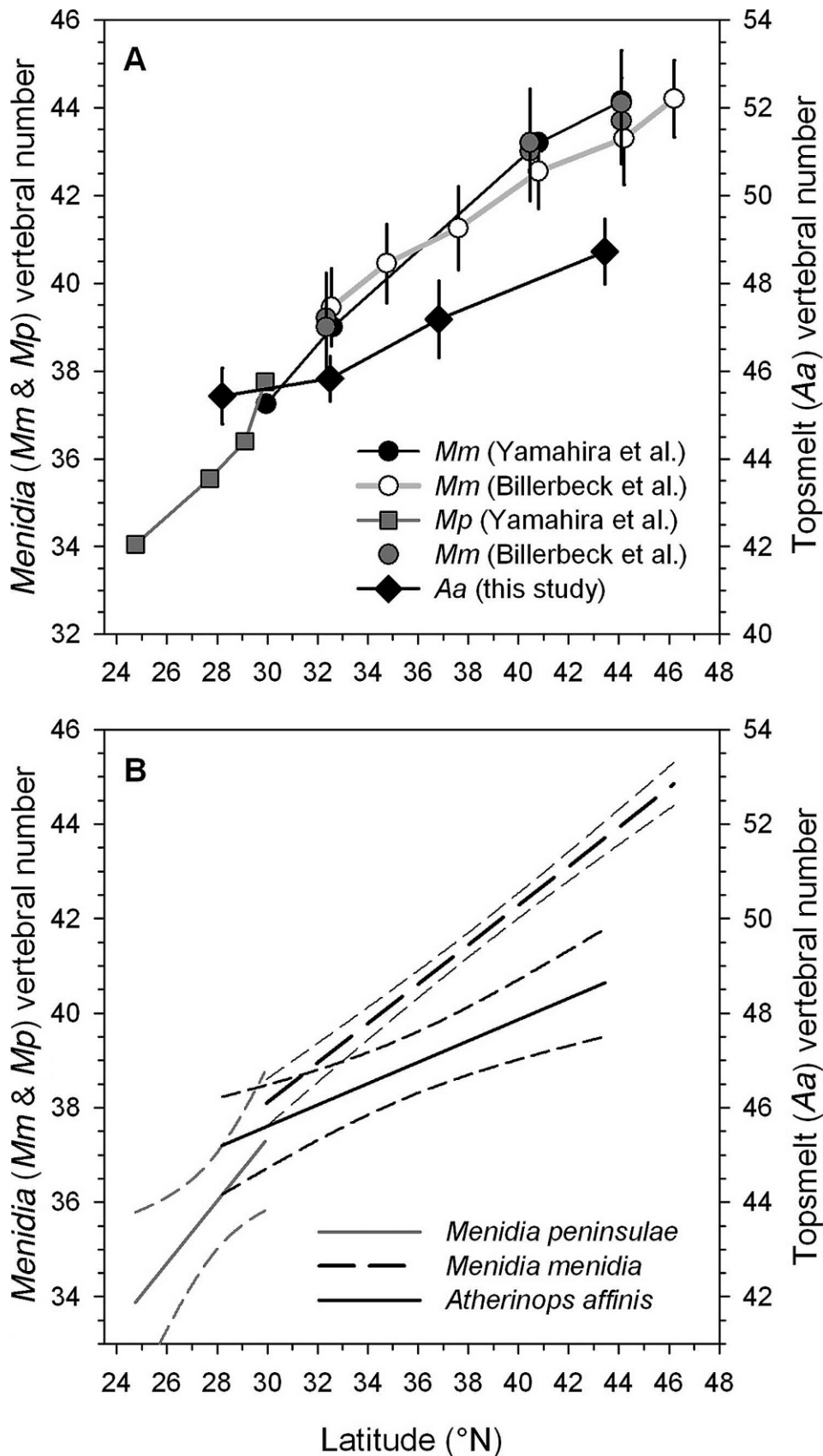
Fig. 5. Latitudinal variation in vertebral number in field-caught Atlantic versus Pacific Silverside populations. (A) Population means ± 1 SD for *Atherinops affinis* (*Aa*, this study), *Menidia menidia* (*Mm*, compiled from Billerbeck et al., 1997 [white circles: material collected 1986, gray circles: material collected 1988/89]; Yamahira et al., 2006), and *M. peninsulae* (*Mp*, compiled from Yamahira et al., 2006). (B) Least squares regression lines and 95% confidence intervals for *Aa* (b95%$_{Aa}$ = 0.17–0.30), *Mm* (b95%$_{Mm}$ = 0.37–0.47), and *Mp* (b95%$_{Mp}$ = 0.14–1.18), based on the data shown in panel A.

Table 2. Results of Two ANCOVAs Testing for Effects of Species on VN (Fixed Factor: *Menidia menidia, Atherinops affinis*) with Covariates (A) Latitude or (B) Median Annual SST. Interaction terms tested for significant species differences in slopes of the (A) 'VN–latitude' or (B) 'VN–SST' relationships.

		Type III SS	df	F	P
A	Species	19.1	1	77.8	<0.001***
	Latitude	69.8	1	284.4	<0.001***
	Species*latitude	4.7	1	19.3	<0.001***
B	Species	14.5	1	27.1	<0.001***
	SST	31.1	1	58.0	<0.001***
	Species*SST	1.8	1	3.4	0.81

vertebral-number-selective predation by sunfish *Lepomis gibbosus* on larvae of stickleback *Gasterosteus aculeatus* and peamouth *Mylocheilus caurinus*, which supports the general hypothesis that VN variations are adaptive via influencing swimming performance. However, Swain (1992a, 1992b) also reported that selection was even stronger for a related trait, i.e., the ratio of abdominal to caudal vertebrae, which points to a more complex interaction between swimming mechanics, vertebral phenotype, and resulting fitness. Selection for certain vertebral phenotypes via swimming performance may only occur during the brief period of high early larval mortality (Swain, 1988, 1992a), since temperature-related changes in water viscosity are likely relevant only to small organisms that experience a low Reynolds number (typically Re < 100, Müller et al., 2000).
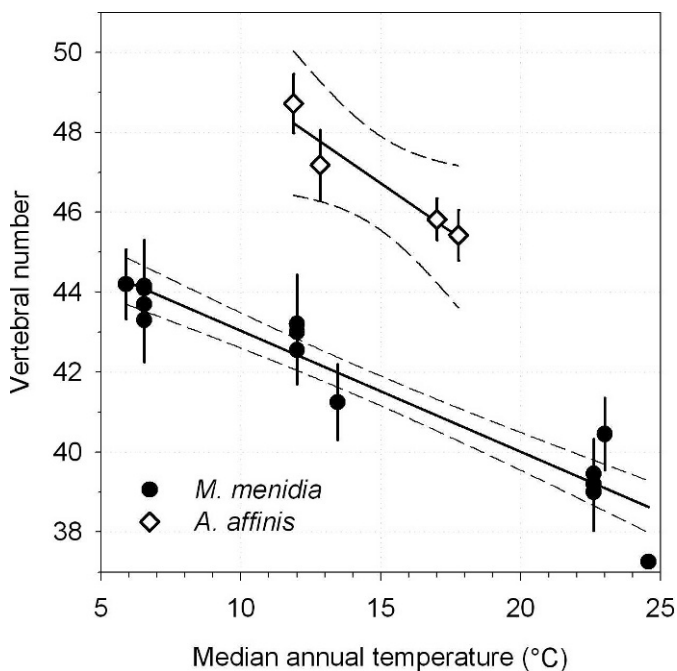

Fig. 6. Relationship between temperature and vertebral number (VN) for Atlantic (*Menidia menidia*, black circles) and Pacific Silverside fishes (*Atherinops affinis*, empty diamonds). Mean VNs of field caught, mature individuals were regressed against median annual SSTs at coastal latitudes corresponding to the origin of each population (extracted from a long-term data set of satellite and *in situ* observations, publicly available at http://dss.ucar.edu/datasets/ds277.0). Black dashed lines depict 95% confidence intervals. The slopes of the two relationships (*M. menidia*: VN = 46.1−0.30*SST, r² = 0.91; *A. affinis*: VN = 54.0−0.48*SST, r² = 0.91) were not significantly different (ANCOVA, *P* = 0.81, Table 2).

Irrespective of the exact adaptive mechanism, our comparative approach resulted in novel support for the major role of temperature in mediating Jordan's rule. This follows from recognizing how closely Pacific versus Atlantic latitudinal gradients in vertebral number scale with relative temperature change. In Atlantic Silversides (*M. menidia* + *M. peninsulae* combined), vertebral number increased by approximately half a vertebra per degree latitude, which was associated with an average temperature decline of $-1.11°C$ per degree latitude. Given that the Pacific coastal temperature gradient is less than half as steep ($-0.40°C$ per latitude), we predicted and found a less than half as steep vertebral number gradient in Pacific Silversides. We thus observed statistically comparable rates of decreasing vertebral number with increasing ambient temperature across the two species, which suggests the existence of a common relationship at least for ecologically equivalent and taxonomically similar species such as Atlantic and Pacific Silversides (here: -0.39 vertebrae $°C^{-1}$). Other fish groups that would lend themselves to our comparative approach include estuarine killifish (Fundulidae, e.g., *Fundulus parvipinnis*, Bernardi and Talley, 2000) or southern hemisphere galaxiids; particularly in the latter, vertebral number variations have already been extensively documented (McDowall, 2003a, 2003b, 2008). To advance this framework, comparative approaches should be expanded, e.g., by contrasting three or more equivalent species/thermal gradients or in form of a meta-analysis of published cross-taxa examples of Jordan's rule.

Local adaptations in vertebral number are generally considered an example of cogradient variation (CoGV, Conover and Schultz, 1995). CoGV occurs when genotypes that shift trait expression in a particular direction are found in environments that shift trait expression in the same direction, thereby accentuating phenotypic variation across a gradient (Conover et al., 2009). In Pacific Silversides, however, we observed that phenotypic differences in wild vertebral numbers were similar or even slightly smaller than those in common garden environments, which represent the genetic component of phenotypic differences. The negligible influence of the environment on vertebral number was also indicated by its very low thermal plasticity, with little decrease from 15°C to 21°C (consistent with CoGV expectation) and even a small increase from 21°C to 27°C (inconsistent with CoGV expectation). However, such U-shaped reaction norms are commonly reported in the literature (Fowler, 1970; Lindsey, 1988) and emerged from experiments on a number of genera including *Oncorhynchus* (Seymour, 1959), *Macropodus* (Lindsey, 1954), *Channa* (Itazawa, 1959), and even *Menidia* (Yamahira et al., 2006).
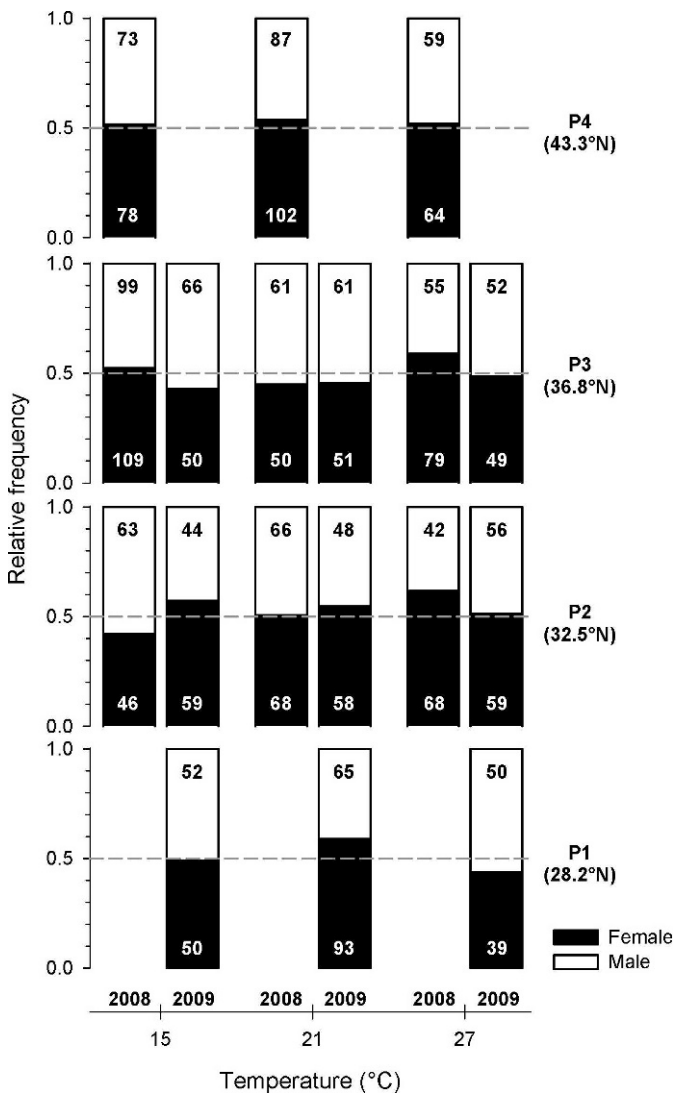
Fig. 7. *Atherinops affinis*. Sex ratios of juveniles from four spatially distinct populations (P1$_{28°N}$–P4$_{43°N}$) reared from the egg stage at three temperatures in 2008 and 2009. Absolute numbers of males (black letters) and females (white letters) are given in each bar.

In the latter, however, the latitudinal slope in vertebral number is indeed steeper in wild than common garden-reared fish (unlike *A. affinis*), thus indicating CoGV. Given that vertebral number is likely thermo-sensitive only during the embryonic stage (Lindsey, 1975; Pavlov and Shadrin, 1998; McDowall, 2008), detection of CoGV may often be hindered by logistical constraints to start experiments soon enough after fertilization. In our case, first generation offspring of *A. affinis* might have experienced temperature variations during transport (20–70 h post fertilization), which represented only a small fraction of the egg stage, but might have introduced additional variation. Alternatively, the observed higher variability in vertebral number in common garden versus field specimens may reflect natural selection for optimal genotypes.

With respect to sex ratio, we found no consistent temperature- or population-specific differences in *A. affinis*, suggesting that this species has genetic (GSD) not temperature-dependent sex determination (TSD) across its geographic distribution. While an invariant mode of sex determination was expected along the Pacific coast (given the lack of a

latitudinal seasonality gradient), our expectation was to encounter TSD. This was because Pacific winter temperatures likely allow for year-round offspring growth of *A. affinis* (provided adequate food resources, Baumann and Conover, 2011), which could give females born earlier in the spawning season (spring–summer, Schultz, 1933) a growth advantage and thus result in higher fecundity at the time of maturity. Under similar environmental conditions, TSD has evolved in low latitude populations of the Atlantic Silverside *M. menidia* (Conover and Heins, 1987). Hence, finding GSD instead TSD in *A. affinis* suggests that long growing seasons do not necessarily confer an adaptive advantage of TSD in silverside populations, but that other conditions have to be met.

For example, for TSD to evolve, developing larvae may need to experience a sufficiently strong temperature signal that acts as a reliable proxy for the season of the year (Conover and Heins, 1987; Yamahira and Conover, 2003). While this may generally be the case along the North-American Atlantic coast, the seasonal temperature fluctuations along the Pacific coast are much smaller. Therefore, thermal cues might simply be too weak or unreliable for TSD to evolve in *A. affinis*. The importance of seasonal cues for the evolution of TSD is also supported by evidence from *M. peninsulae*, a southern congener of *M. menidia*. Interestingly, *M. peninsulae* shows a shift from TSD (at 30°N) to GSD with decreasing latitude (25–29°N), despite continuous growing seasons (Yamahira and Conover, 2003). This has been attributed to a corresponding shift in reproductive mode in *M. peninsulae*, from annual (at 30°N) to semi-annual spawning (25–29°N). Because reproduction in southern populations of *M. peninsulae* occurs throughout much of the year, larvae experiencing low temperatures could be born in spring or fall. Hence, the beginning or end of the growing season is poorly defined by temperature (Yamahira and Conover, 2003). This renders thermal cues inadequate proxies for the season of the year and therefore favors the evolution of GSD over TSD.

Alternatively, TSD may have a lower adaptive value for Pacific than Atlantic Silversides because of the species life history differences. The shorter life span and semelparity of *M. menidia* plausibly magnify the importance of first year growth. In longer-lived, iteroparous species like *A. affinis*, fitness is likely more a function of cumulative growth and fertility over the entire life span, hence decreasing the impact of first year growth. The role of longevity in the evolution of TSD is supported by recent evidence for TSD in California grunion, *Leuresthes tenuis*, a shorter-lived Pacific atherinopsid than *A. affinis* (Brown, 2010).

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