

## Differences in thermal tolerance in coexisting sexual and asexual mollies (*Poecilia*, Poeciliidae, Teleostei)

C. FISCHER\*† AND I. SCHLUPP‡

\**Systematics and Evolutionary Biology, Institute of Biology and Environmental Sciences, University of Oldenburg, Carl von Ossietzky-Str. 9-11, 26129 Oldenburg, Germany and*

‡*Department of Zoology, University of Oklahoma, 730 Van Vleet Oval, Norman, OK 73019, U.S.A.*

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This study reports significant differences between the gynogenetic Amazon molly *Poecilia formosa* and one of its sperm hosts, and the sexual sailfin molly *Poecilia latipinna* in the critical temperatures at which individual fishes lost motion control. Based on these measurements, it is suggested that cold snaps occurring in winter, but not summer temperatures, can significantly change population composition of these closely related fishes by inflicting higher mortality on *P. formosa*.

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Given the two-fold advantage of asexually reproducing organisms the prevalence of sexual reproduction is still an unresolved problem in evolutionary biology. Asexual lineages theoretically should be able to replace sexuals over short periods of time (West *et al.*, 1999). Some asexual species, like the gynogenetic Amazon molly *Poecilia formosa* (Girard) depend on sperm of heterospecific males to trigger embryogenesis, but inheritance is strictly clonal (Schlupp, 2005). The two main sperm donors are the sailfin molly *Poecilia latipinna* (Le Sueur) and the Atlantic molly *Poecilia mexicana* Steindachner which gave rise to *P. formosa* in a single hybridization event between 10 000 and 100 000 years ago (Schlupp, 2005). Because of the sperm dependence, *P. formosa* cannot outcompete their sexual hosts without going extinct themselves, although *P. formosa* should have a higher population growth rate as compared to their sexual relatives because no males are produced. Indeed, sex ratios biased towards females (sexual and gynogens combined) were found by Riesch *et al.* (2008) and Heubel (2004) towards the end of the reproductive season.

†Author to whom correspondence should be addressed. Tel.: +49 441 798 3966; fax: +49 441 798 193965; email: [claus.fischer@uni-oldenburg.de](mailto:claus.fischer@uni-oldenburg.de)

This raises the question how the coexistence of the gynogenetic *P. formosa* with at least one of their hosts is accomplished. Early theoretical work highlighted the importance of behavioural decisions as mate choice and sperm allocation (Stenseth *et al.*, 1985), and argued that ecological differences are not needed for stable coexistence. But later the importance of decreased ecological similarities was stressed (Case & Taper, 1986; Kirkendall & Stenseth, 1990). The importance of abiotic factors in shaping communities was underscored in a comprehensive review by Dunson & Travis (1991). Temperature is identified as the abiotic master factor for fishes (Beitinger *et al.*, 2000) affecting virtually all biochemical, physiological and life-history activities of fishes. Probably the most dramatic effect of temperature is to act as a lethal agent. Consequently, a vast literature reports thermal tolerances of fishes, but in many cases only the upper tolerance was tested, despite the importance of low temperatures especially for tropical and subtropical species (Beitinger & Bennett, 2000).

In this present study, the upper ( $C_{Tmax}$ ) and lower ( $C_{Tmin}$ ) thermal tolerance limit of *P. formosa* and *P. latipinna* was tested in order to investigate whether differences herein could be partly responsible for the ongoing coexistence of both species. Given the hybrid origin of *P. formosa* from a fish with a rather tropical distribution and a fish with a rather subtropical range (Schlupp *et al.*, 2002), it could be expected that *P. formosa* lacks some cold tolerance compared with *P. latipinna*, and that the tolerance towards high temperatures should be somehow greater. Alternatively, heterosis due to the original hybridization could have led to superior viability as found in the hybridogenetic *Poeciliopsis*-complex (Bulger & Schultz, 1982).

Several approaches can be taken to determine critical temperatures. The critical thermal method (Beitinger *et al.*, 2000) that uses quick changes of water temperature was chosen here due to the rapid cooling events that were detected in the field (unpubl. data) connected with cold snaps frequently occurring in Texas. Furthermore, it involves fewer fishes and leads to no lethality if conservative endpoints, that, however, disable individuals to escape lethal conditions are chosen (Beitinger *et al.*, 2000), Becker & Genoway (1979) and Lutterschmidt & Hutchison (1997) discuss the different endpoints frequently used. The temperature change used was  $1^{\circ}\text{C min}^{-1}$  as recommended in some of the literature (Lutterschmidt & Hutchison, 1997; Mora & Maya, 2006), although Becker & Genoway (1979) and Beitinger *et al.* (2000) recommended increasing the temperature by only  $0.3^{\circ}\text{C}$ .

The test fishes were collected with seines in 2006 and 2007 from three different sites in the Guadalupe River basin in central Texas. They were housed prior to testing in separate mixed groups in large stock tanks (*c.* 1000 l) in a greenhouse at the Aquatic Research Facility of the University of Oklahoma. Two collection sites were located in the San Marcos River, with one of them a few kilometres downstream of the spring at County Road 101 (CO 101;  $29^{\circ}51'26''\text{ N}$ ;  $97^{\circ}53'48''\text{ W}$ ) and the other near Martindale (SMA;  $29^{\circ}51'29''\text{ N}$ ;  $97^{\circ}51'51''\text{ W}$ ). The third site was the springhead of the Comal River in New Braunfels (COM;  $29^{\circ}42'46''\text{ N}$ ;  $98^{\circ}08'10''\text{ W}$ ). For more information regarding the sites see Heubel (2004). All *P. formosa* individuals of this study originated from SMA and all *P. latipinna* individuals from CO 101 or COM.

Two collection sites for *P. latipinna* were used to control for possible effects of higher thermal stability near a springhead.

Before the experiments, 10 *P. formosa* [mean  $\pm$  s.d. standard length ( $L_S$ ) =  $37 \pm 5$  mm, mean  $\pm$  s.d. mass =  $1.43 \pm 0.38$  g] and 20 *P. latipinna* ( $L_S \pm$  s.d. for CO 101 =  $48 \pm 3$  mm, mass  $\pm$  s.d. =  $3.00 \pm 0.54$  g and for COM =  $37 \pm 3$  mm,  $1.58 \pm 0.17$  g; each 10 individuals) were transferred individually into 2 l tanks in which temperature was controlled *via* the air-conditioning of the room. The illumination cycle was 12L:12D. The diet consisted of commercial flake food and frozen chironomid larvae. In order to keep water quality stable, water was partially changed at least every second day during the whole study.

In the room, fishes were allowed to habituate to the tanks, or recover from a previous experiment, and acclimate to the current acclimation temperature ( $T_{acc}$ ) for 3 days. The same fishes were used to test  $C_{Tmin}$  with two different  $T_{acc}$  ( $18.5$  and  $26.0^\circ$  C) as well as  $C_{Tmax}$  at  $T_{acc}$  of  $26.0^\circ$  C. The temperature of  $18.5^\circ$  C lies near the mean daily maximum air temperatures in December to February ( $17.4$ ,  $16.2$  and  $18.8^\circ$  C), and the higher temperature represents roughly the mean summer air temperatures of the period 1971 to 2000 in San Marcos ( $27.5^\circ$  C in June; NOAA, 2003).

After acclimation was complete, one single randomly selected test fish at a time was transferred to a 2 l test tank with fresh water and an overflow. The temperature in that tank was monitored by a centigrade thermometer (Cole-Parmer Instruments Co., Model 8502-20; www.coleparmer.com), and the water circulated by an air stone. The experiment started when fishes appeared to swim calmly. The decrease or increase of the water temperature was achieved by manually adding  $3\text{--}4^\circ$  C cold or  $48\text{--}50^\circ$  C hot water. In order to control the accuracy of this process, the temperature was recorded every 30 s. Regression analysis of these records yielded mean  $\pm$  s.d. decreases of  $-0.977 \pm 0.022^\circ$  C  $\text{min}^{-1}$  and  $-0.999 \pm 0.014^\circ$  C  $\text{min}^{-1}$  and an increase of  $0.995 \pm 0.091^\circ$  C  $\text{min}^{-1}$ .

Individual trials in the cooling experiments were terminated when the motion of the pectoral fins ceased and did not start again by disturbance of the fish with a glass rod. Initial loss of motion control, identifiable by violent escape responses and brief turns to the side, was used as a criterion in the warming experiment (Becker & Genoway, 1979). The temperature at that time was recorded as  $C_{Tmin}$  or  $C_{Tmax}$  for each test fish in this study. After recovery each fish was weighed, but only very slight differences in mass of individual fishes were detected during the duration of the whole study, indicating no differences in fitness between the three consecutive experiments.

For all statistical test, SPSS (12) (www.spss.com) was used. A repeated measures ANOVA (rmANOVA) with the temperatures at which the individual trials were stopped in each experiment (*e.g.*  $C_{Tmin}$  and  $C_{Tmax}$  combined) as dependent variables and collection site as a factor was conducted. As no linear association between  $L_S$  and mass with either  $C_{Tmin}$  or  $C_{Tmax}$  was observed during graphical exploration, and inclusion of these covariates did not yield significant results for them or changed the overall results, the use of  $L_S$  or mass as covariate was omitted. Thus, Dunnett's T3 *post hoc* test was conducted to determine differences between collection sites.

Because there was no significant difference in the rmANOVA between the two different *P. latipinna* populations used, the differences between *P. latipinna* and *P. formosa* in each treatment were analysed afterwards with independent *t*-tests. Furthermore, it was tested with *t*-tests if acclimation temperatures affected the  $C_{TMin}$  for the two cooling experiments, and if there was a difference between the effects of the acclimation temperatures between both species using the differences between the two  $C_{TMin}$  values. The *P*-values of these *post hoc* *t*-tests were adjusted by using the sequential Bonferroni procedure (with  $c = 6$  and  $\alpha = 0.05$ ).

The assumptions for all statistical tests conducted were met. There was a statistically significant interaction between the  $C_{TMin}$  and the  $C_{TMax}$  values with collection site (Wilks'  $\lambda$ ,  $n = 30$ ,  $P < 0.001$ ). *Post hoc* comparisons revealed no significant differences between the two different *P. latipinna* collection sites ( $P > 0.05$ ), but between those and the *P. formosa* (both  $P < 0.001$ ), indicating a species effect but not a site effect. In both cooling experiments, the sexual *P. latipinna* tolerated significantly lower  $C_{TMin}$  than *P. formosa* (*t*-test,  $n = 30$ ,  $P < 0.001$  and  $\alpha = 0.008$  for  $T_{acc}$  of  $18.5^\circ\text{C}$ ,  $n = 30$ ,  $P < 0.001$  and  $\alpha = 0.01$  for  $T_{acc}$  of  $26^\circ\text{C}$ , respectively). The measured  $C_{TMin}$  (mean  $\pm$  s.d.) for *P. latipinna* were  $5.96 \pm 0.44^\circ\text{C}$  ( $T_{acc}$  of  $18.5^\circ\text{C}$ ) and  $7.90 \pm 0.46^\circ\text{C}$  ( $T_{acc}$  of  $26^\circ\text{C}$ ). *Poecilia formosa* exhibited  $C_{TMin}$  (mean  $\pm$  s.d.) of  $8.56 \pm 0.90^\circ\text{C}$  ( $T_{acc}$  of  $18.5^\circ\text{C}$ ) and  $9.30 \pm 0.37^\circ\text{C}$  ( $T_{acc}$  of  $26^\circ\text{C}$ ). The mean  $\pm$  s.e. difference between them was  $2.60 \pm 0.24^\circ\text{C}$  for the lower acclimation temperature and  $1.40 \pm 0.17^\circ\text{C}$  for the higher one. Furthermore, in both species a higher acclimation temperature was associated with a significantly higher  $C_{TMin}$ . *Poecilia latipinna* showed an increase (mean  $\pm$  s.d.) of  $1.95 \pm 0.69^\circ\text{C}$  (*t*-test,  $n = 20$ ,  $P < 0.001$  and  $\alpha = 0.0125$ ). In *P. formosa*,  $C_{TMin}$  increased (mean  $\pm$  s.d.) only by  $0.75 \pm 0.87^\circ\text{C}$  for the higher acclimation temperature, but this was still significant (*t*-test,  $n = 10$ ,  $P < 0.05$  and  $\alpha = 0.05$ ). Consequently, the influence of  $T_{acc}$  on the  $C_{TMin}$  was significantly lower in *P. formosa* (*t*-test,  $n = 30$ ,  $P < 0.001$  and  $\alpha = 0.017$ ). In the warming experiment *P. formosa* reached an only slightly, but statistically significant higher  $C_{TMax}$  (mean  $\pm$  s.d.) of  $40.43 \pm 0.44^\circ\text{C}$  as compared to *P. latipinna* with  $39.89 \pm 0.38^\circ\text{C}$  (*t*-test,  $n = 30$ ,  $P < 0.01$  and  $\alpha = 0.025$  for  $26^\circ\text{C}$   $T_{acc}$ ).

Thus, the present study revealed significant differences in the response to temperature stress, especially in lower critical temperatures of *P. formosa* and *P. latipinna*. The species difference is biologically relevant, because conditions that might lead to differential mortality due to low temperatures seem to occur in nature in high enough frequency underscoring the importance of rare extreme events. For example in December 2006, a situation mimicking the present experiment was recorded near SMA. At this site, water decreased relatively fast from higher temperatures to  $8^\circ\text{C}$  and sometimes below this temperature to a minimum of  $4^\circ\text{C}$ . For *P. formosa*, with its measured  $C_{TMin} > 8^\circ\text{C}$  this should induce temperature related mortality, while *P. latipinna* with a  $C_{TMin} < 6^\circ\text{C}$  (both  $T_{acc}$   $18.5^\circ\text{C}$ ) should suffer less. Thus, the frequency of *P. formosa* could be reduced during the winter to a level allowing coexistence with sexual host species (Schlupp, 2005). Obviously, the laboratory setup prevented behavioural regulation such as habitat selection (Bennett & Judd, 1992). Some

studies, however, showed agreement of laboratory derived tolerance values with distributions in the field and *vice versa* (Dunham *et al.*, 2003; Huff *et al.*, 2005).

As other studies showed that a gain of heat tolerance is accompanied by an even higher loss of cold tolerance (Fangue & Bennett, 2003), the smaller difference between both  $C_{T_{min}}$  of *P. formosa* could be due to slower acclimation as compared to *P. latipinna*. But, *P. formosa* could also have a broad spectrum of temperatures associated with nearly the same  $C_{T_{min}}$  as it was shown for the mummichog *Fundulus heteroclitus* (L.) (Fangue *et al.*, 2006). Hence, the time for acclimation and the associated changes in  $C_{T_{min}}$  and  $C_{T_{max}}$  are further important variables not tested here, which should be addressed in future studies concerned with temperature effects on fish communities.

The difference between both species in  $C_{T_{max}}$  of  $0.54 \pm 0.16^\circ \text{C}$  was small. Maximum water temperature recorded in 2001 was  $30.4^\circ \text{C}$ , and temperatures in March 2007 were  $<30^\circ$  (Heubel, 2004). As a conservative endpoint was chosen (Becker & Genoway, 1979) and only a relatively low acclimation temperature was used, it is possible that both species can exhibit even higher  $C_{T_{max}}$ . Thus, it is unlikely that adult females of both species experience mortality due to high water temperatures. Nevertheless, feeding behaviour along with growth declines significantly at high temperatures well before temperatures become lethal (Selong *et al.*, 2001). The difference between  $C_{T_{max}}$  and temperatures occurring in the field, however, should probably be great enough to prevent the occurrence of reduced fitness due to high temperatures for both species.

As temperature was found to have different effects on both species regarding the susceptibility to mortality addressed here, it is tempting to speculate that there might be considerable non-lethal ecological differences as well. If the differences found here indicate also different thermal optima in both species, this could result in differences in reproductive timing, could reduce niche overlap through different activity patterns or different microhabitat usage (Laha & Mattingly, 2006). Furthermore, such differences could result in different growth optima and related susceptibility to food stress at different times (Selong *et al.*, 2001; M. Tobler & I. Schlupp, unpubl. data). Additionally, different optima could have an indirect lethal effect by potentially influencing the outcome of interactions with predators, if predators consume fishes at temperatures near the limits of the coexisting species.

Future studies will have to investigate critical temperatures of males and juveniles of both species, and in *P. mexicana* to elucidate the 'whole picture'. Furthermore, time series of life-history patterns will shed light on cryptic differences in reproductive timing and habitat use.

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