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# Effects of reliance on stored sperm on reproduction in the sailfin molly *Poecilia latipinna*

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**Abstract**

The impacts of relying on stored sperm were evaluated in the sailfin molly, *Poecilia latipinna*. Females reliant on stored sperm had fewer offspring compared to remated females, but offspring size and short-term growth rate did not differ. Thus, females may use stored sperm in cases such as previous mating with a preferred male, lack of access to mating opportunities during a reproductive cycle, or to maximize egg fertilization. Females do not compensate for producing fewer offspring however, by allocating more resources to offspring relative to their size or initial growth.

**KEYWORDS**
Poeciliidae, reproduction, sperm storage

Life-history theory predicts that natural selection will maximize lifetime reproductive success within the limitations of an organism's specific strategies, including balancing current and future reproduction with growth, maintenance and so on (Reznick, 1996; Rodd & Reznick, 1997; Stearns, 1992). In particular, maternal investment across an organism's reproductive lifetime may vary depending on environmental and social factors (D'Amore *et al*., 2015; McGinley *et al*., 1987; Rios-Cardenas *et al*., 2013). For example, there may be trade-offs between offspring number and estimates of offspring quality, such as offspring size, growth or behaviour (Ghalambor *et al*., 2007; Reznick, 1996; Rios-Cardenas *et al*., 2013; Schluter *et al*., 1991; Zera & Harshman, 2001).

An important factor that could influence lifetime reproduction and potentially maternal investment in many organisms is the use of sperm storage. Sperm storage occurs across many groups, including all major groups of vertebrates (Birkhead & Møller, 1993; Holt & Fazeli, 2016). Sperm storage provides insurance against not finding a reproductive partner and can allow females to optimize the timing of their reproductive cycle (Birkhead & Møller, 1993). Sperm storage

may also allow dispersing females to avoid a mate-finding Allee effect (Jiménez-Franco *et al*., 2020) and allow for cryptic female choice (Albo *et al*., 2013; Birkhead, 1998; Eberhard, 1996). Females, *e.g*., may preferentially store sperm from preferred males (Devigili *et al*., 2016; Evans *et al*., 2003; Firman *et al*., 2017; Gasparini & Evans, 2018; Gasparini *et al*., 2020).

Poecilid fishes have been well studied regarding the use of sperm storage as part of their reproductive strategy (Constantz, 1984; Evans & Magurran, 2001; Evans & Pilastro, 2011; Greven, 2011; Hubbs, 1964; Turner, 1937; Zadmajid *et al*., 2019). Stored sperm may remain viable for over a year after insemination, and females of many poeciliid species have specialized sperm storage pockets in their reproductive tract (Constantz, 1984, 1989; Greven, 2011; Houde, 1997; Kobayashi & Iwamatsu, 2002; Olivera-Tlahuel *et al*., 2017; Uribe *et al*., 2010); nonetheless, sperm viability (Gasparini & Evans, 2013) and brood sizes decline over time (Clark, 1951; Greven, 2011). Sperm storage and the resulting sperm competition have likely been the driving mechanism of evolutionary pressures on males to produce sperm with divergent strategies, with

some sperm adapted for immediate fertilization and other for long-term storage (Cardozo et al., 2020; Fitzpatrick, 2020; Girndt et al., 2012). Despite the adaptations associated with sperm storage, fresh sperm are more likely to be used more often to father offspring (Constantz, 1989; Gasparini et al., 2018; Reznick & Miles, 1989). Although sperm storage in poeciliid fishes has been relatively well studied, no studies have focused on how females may adjust short-term reproductive output when only stored sperm are available.

Sailfin mollies, Poecilia latipinna (Lesueur 1821) are found in salt marshes and specialized freshwater habitats throughout the southeastern USA (Lee et al., 1980; Simanek, 1978; Trexler, 1986). Female preference has been shown for various male traits, including size and courtship display, and for mate copying (Gabor & Page, 2003; Maclaren et al., 2004; Ptacek & Travis, 1997; Schlupp & Ryan, 1997; Witte & Ryan, 2002). Females are generally lecithotrophic or weakly placentotrophic (Constantz, 1989; Trexler, 1997) and generally produce a brood every 28–64 days (Hubbs et al., 2002; Snelson et al., 1986). Variation in interbrood interval and offspring dry weight is associated with environmental factors such as temperature, water salinity and food availability (Johnson & Bagley, 2011; Smith, 1986; Snelson et al., 1986; Trexler et al., 1990, 1997; Vasagam et al., 2005) as well as phenotypic plasticity among populations (Pires et al., 2011; Trexler, 1997). Females also show high levels of multiple paternity which can influence brood size (Girndt et al., 2012; Travis et al., 1990).

The authors investigated how sperm storage impacted reproduction in P. latipinna during their first brood following isolation. The authors hypothesized that female P. latipinna reliant on sperm storage would have fewer offspring per brood due to sperm limitation, but that they would compensate for smaller brood sizes with relatively larger offspring. In addition, they hypothesized that sperm storage–reliant females would have increased interbrood intervals if they had delayed brood production to attempt to maximize their chance of encountering a mate (and thus, the potential to use or supplement brood production with fresh sperm).

The mollies used in this study were descendants of wild-caught mollies from the "Steve's Ditch" site in Wakulla County, Florida, USA (N 29° 58.379′, W 084° 20.700′). Prior to isolation, fish were maintained at 24°C in mixed-sex aquaria (79.4- and 119.1 L tanks). A 14:10 light/dark illumination cycle was provided by fluorescent full-spectrum tubes (NaturesSunlite) that have a spectral emission simulating natural light. The fish were fed once daily a combination of OSI (Ocean Star International brand) freshwater flakes, spirulina, freeze-dried bloodworms and freeze-dried mysis shrimp. Isolated females and fry were fed once per day to satiation (c. 0.3 g day$^{-1}$ per adult fish and c. 0.1 g day$^{-1}$ for groups of fry).

Because female mollies are sexually receptive for 2–3 days after giving birth (Farr & Travis, 1986; Snelson et al., 1986; Travis, 1989), adult females were individually placed into separate 18.9 L tanks until they had a brood. Females were randomly assigned to sperm storage or mated treatment groups, with the next female to give birth being automatically assigned into the opposite group. For the sperm storage treatment, no further access to males was provided. For the mated treatment, a large, mature, "courting" male (37–53 mm SL; Ptacek &

Travis, 1996; Travis, 1989) was added to each female's tank for 4 days to ensure the opportunity to mate and was then removed back to a group tank. Once females produced their second brood, the female's standard length was measured, and they were returned to the group tanks. If a female failed to produce a brood after 90 days, this was recorded, and the female was removed from the experiment.

Photos were taken of new broods the day after they were born (day 1). Fry were placed into a glass Petri dish with a small amount of water and photographed from above with a ruler for scale using a cell phone camera. After being photographed, fry were placed into their own 18.9 L tank. On day 7, fry were removed from their tank and photographed following the same procedure as day 1. Day 7 photographs allow an estimate of fry growth in their first week. Poeciliid juveniles typically begin feeding immediately, and growth begins right away (Snelson, 1989); nonetheless, this early time would likely be when fry growth was most influenced by maternally supplied yolk. Photos were analysed using ImageJ to determine the number and length of each fry (tip of head to tip of tail) on days 1 and 7. Although measurements of fry length do not capture all aspects of fry size (including differences in mass determined by measuring fry dry weights), this measure allowed the authors to compare fry growth over a short time period (1 week) and to maintain fish for future experiments. Fry measurements were performed blind to treatment assignment. A t-test revealed no difference in female size between the females assigned to the two treatments (t = 0.855, df = 28.7, P = 0.400).

The authors used linear models to compare interbrood interval and brood size between stored sperm and mated treatments (females that did not produce a second brood within 90 days were excluded from interbrood interval analysis) and a generalized linear model with a binary outcome to compare brood success. These models included the fixed effect of treatment and the covariate of female size. They implemented linear mixed models including the fixed effect of treatment and a random effect of female identity to compare fry size between treatments and control for covariation among fry from the same female. All statistical tests were performed using R version 4.1.4 (with RStudio 2022.07.1 build 548; R Core Team, 2022) using the packages lme4 (Bates et al., 2015), car (Fox & Weisberg, 2019) and lmerTest (Kuznetsova et al., 2017).

Statistical significance was assumed with P < 0.05.

Of the females that produced broods, females in the sperm storage treatment (n = 12) had smaller brood sizes compared to females in the mated treatment (n = 15; linear model: $F_{1,23} = 7.90$, P = 0.010), and there was no influence on female size ($F_{1,23} = 0.152$, P = 0.70). Sperm storage females had a mean of 21.33 (±13.90 S.D.) offspring, whereas mated females had a mean of 34.87 (±9.52 S.D.) offspring (Figure 1, total offspring n = 1279).

There was no significant effect of treatment on fry length at day 1 (linear mixed model, $F_{1,22.8} = 0.763$, P = 0.391) or on day 7 [$F_{1,21.6} = 1.51$, P = 0.234; day 1: sperm storage (mean ± S.D.) 10.59 ± 1.01 mm; mated 10.54 ± 0.88 mm, day 7 sperm storage 11.41 ± 1.25 mm; mated 11.10 ± 1.17 mm). In addition, the authors did not find a difference in interbrood interval between sperm storage
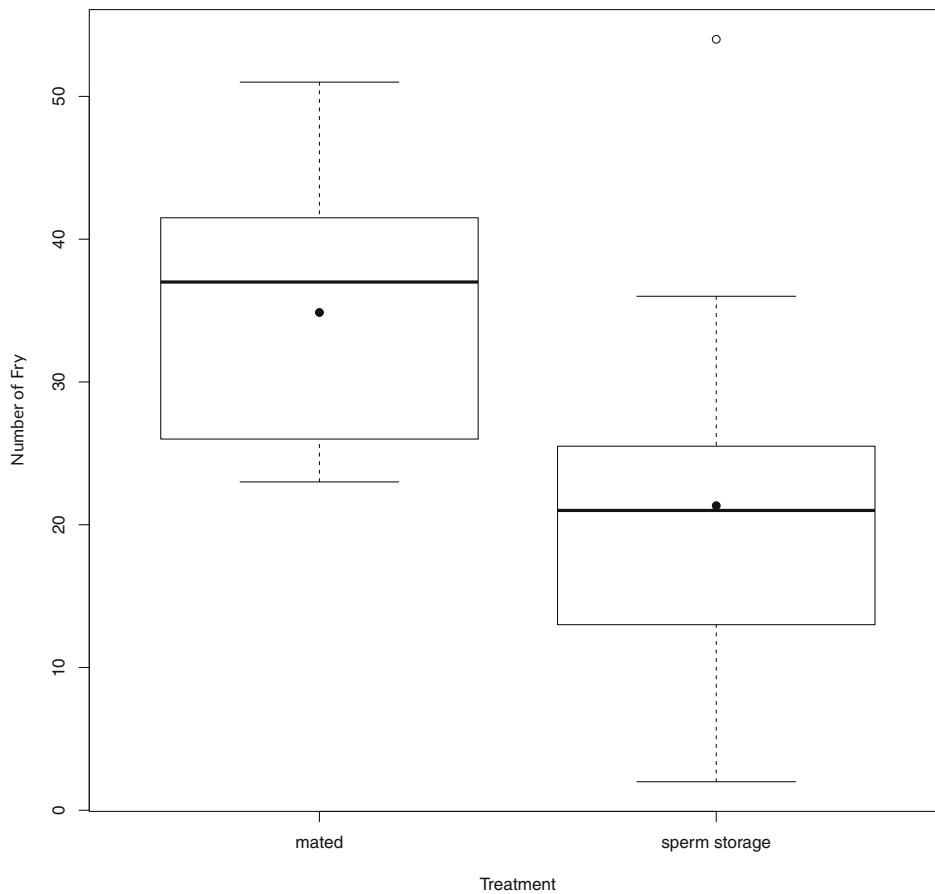
and mated females (linear model: $F_{1,23} = 0.768$, $P = 0.390$; female size $F_{1,23} = 3.97$, $P = 0.0583$) with sperm storage females having an interbrood interval of 38.3 (±14.17 S.D.) and mated females having a mean interbrood interval of 34.5 days (±9.88 S.D.).

After isolation and initial brood production, 15 females in the mated treatment produced a brood, whereas one did not produce a brood (after the initial brood produced once isolated). For females in the sperm storage treatment, 12 successfully produced a brood, whereas an additional 4 did not produce a brood. Although this result suggests a trend in the likelihood of producing a brood, the authors did not find a significant difference in brood success when controlling for female size ($X^2 = 1.90$, $df = 1$, $P = 0.168$; female size: $X^2 = 0.049$, $df = 1$, $P = 0.83$).

The authors found that female *P. latipinna* that relied on stored sperm during an initial reproductive cycle had smaller broods compared to females that were able to mate. This decrease could be due to sperm degradation within the female's reproductive tract over time (Gasparini *et al*., 2018), or the inability to use all available stored sperm for a given clutch. Mated females, however, had the ability to rely not only on fresh sperm but also on stored sperm from previous matings (Greven, 2011). Of the 10 smallest broods produced overall, 9 were from females in the sperm storage treatment, as were all broods with fewer than 20 fry (although the largest brood was with a female in the sperm storage treatment). Despite having smaller broods, females using stored sperm did not produce larger offspring or delay brood

production. The result suggests that females do not (or cannot) delay brood production in an effort to secure additional matings, at least during their first reproductive cycle using stored sperm.

Life-history theory predicts that selection will favour organisms that maximize lifetime reproductive success (Reznick, 1996; Stearns, 1992). That females in this study did not initially vary offspring size or short-term growth relative to offspring number may be interpreted in several ways. Physiological constraints that limit reproductive cycle length or stored sperm use may be beneficial overall, even if not within the short single brood cycle observed here. Females may benefit from reserving energy towards maintenance or future reproduction rather than increasing offspring size (Ghalambor *et al*., 2007; Schluter *et al*., 1991; Zera & Harshman, 2001). In addition, variation in environmental factors such as temperature, water salinity and food availability (Johnson & Bagley, 2011; Smith, 1986; Snelson *et al*., 1986; Trexler *et al*., 1990; Vasagam *et al*., 2005) may play a role in regulating reproductive trade-offs that do not occur when held constant (as in this study). For example, Trexler (1997) previously found that matrotrophy may decrease the offspring size-number trade-off, but this was most obvious in larger, food-restricted females. Without food restriction, *P. latipinna* females may have primarily used lecithotrophic provisioning, with the majority of yolk provisioning occurring prior to fertilization so adjustment, even though possible, may not be detected until the female has had additional reproductive cycles. In addition, although maternal investment impacts offspring size, number

and behaviour based on environmental factors such as salinity, temperature and food levels (Alcaraz & García-Berthou, 2007; D'Amore *et al.*, 2015; Trexler, 1997; Trexler *et al.*, 1990; Vasagam *et al.*, 2005), females may not have the physiological plasticity available to adjust reproduction when these environmental factors are constant. In addition, it may be rare that females lack access to mates; thus selection on the use of only stored sperm may be very weak, especially compared to selection that balances how females use both fresh stored sperm within a reproductive cycle. Finally, long-term brood production may show alterations in reproductive allocation not captured during a female's first reproductive cycle.

Alternatively, individual females may show plastic responses within the sperm storage treatment. If reproductive output is limited, some females may have dedicated extra energy towards growth and maintenance (Heino & Kaitala, 1999; Marshall & Uller, 2007), ensuring that future reproduction could be maximized, whereas other females may have delayed reproduction, resulting in no brood production within the timing of this study. Thus, the impacts of sperm storage may not be apparent until females have relied on stored sperm for several reproductive cycles. The (non-significant) trend in this study for differences in failure to produce a brood may be related to this plasticity, and future work with a larger sample pool could determine whether failure to produce a brood under certain conditions might be advantageous. *P. latipinna* from different populations might also allocate resources differently in cases of sperm storage and mated conditions. Differences in resource allocation have been shown for a wide array of variables, including average size, mate preferences, interbrood interval and rates of multiple paternity (Farr *et al.*, 1986; Girndt *et al.*, 2012; Hubbs *et al.*, 2002; Ptacek & Travis, 1996; Trexler *et al.*, 1990; Trexler, 1997). Given the wide environmental variation where *P. latipinna* live, different strategies may be selected at different rates among the populations, an area for future research. Although the authors did not observe differences in short-term fry growth rate, changes in time to maturity could also differ (Rios-Cardenas *et al.*, 2013).

As females in this study were haphazardly paired with males, the potential impacts of male size and condition on brood size are of interest. Male mollies show mating preferences and may adjust sperm allocation based on female condition (Aspbury, 2007; Aspbury & Gabor, 2004; Gabor, 1999; Schlupp & Ryan, 1997). Although this study did not show brood size differences related to female size and all females were presented with males within their receptive period, there remains the possibility that some aspect of male size, mating status or interaction with female size or condition could influence brood size or lack of brood production overall.

This study shows that females differing in access to fresh sperm through recent mating produced broods of very different sizes without differences in the size of individual offspring in their first reproductive cycle. Although this study was constrained relative to sample sizes and duration, it demonstrates the importance of sperm storage and highlights the need for longer-term investigations into how sperm storage impacts female investment. Future studies should address whether females that rely on sperm storage for a reproductive cycle change their reproductive output in subsequent broods, *e.g.*, by increasing fry number. It is possible that mollies from other populations might allocate resources differently in cases of sperm storage. Finally, continued research into sperm storage, sperm degradation and cryptic female choice within *P. latipinna* could inform our understanding of maternal provisioning under conditions of sperm limitation.

## AUTHOR CONTRIBUTIONS

Shala Hankison, Breanna Fry, Sandra D. Otap and Jenell M. Betts designed the study. Shala Hankison designed the statistical approach, and Shala Hankison and Eric J. Gangloff analysed the data. Eric J. Gangloff provided the R-Markdown document and prepared the data for archiving. Shala Hankison, Breanna Fry, Alena Arnold, A.J. Lashway, Jenell M. Betts, Sandra D. Otap and Katherine Walter were involved in data collection. Shala Hankison, Alena Arnold, A.J. Lashway, Katherine Walter, Makenna Y. Juergens, Eric J. Gangloff and Alax Crawford participated in discussions on data interpretation and on writing the manuscript.

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## ETHICS STATEMENT

The care and use of the experimental fish was carried out according to IACUC protocol #05-2019-03, #05-2020-03 and #05-2021-03 through Ohio Wesleyan University, USA.

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