

## Research



**Cite this article:** Fleuren M, van Leeuwen JL, Pollux BJA. 2019 Superfetation reduces the negative effects of pregnancy on the fast-start escape performance in live-bearing fish.

*Proc. R. Soc. B* **286**: 20192245.

<http://dx.doi.org/10.1098/rspb.2019.2245>

Received: 25 September 2019

Accepted: 2 November 2019

**Subject Category:**

Morphology and biomechanics

**Subject Areas:**

biomechanics, evolution

**Keywords:**

biomechanics, C-start, evolution, Poeciliidae, viviparity, reproductive traits

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Electronic supplementary material is available online at <https://doi.org/10.6084/m9.figshare.c.4734716>.

# Superfetation reduces the negative effects of pregnancy on the fast-start escape performance in live-bearing fish

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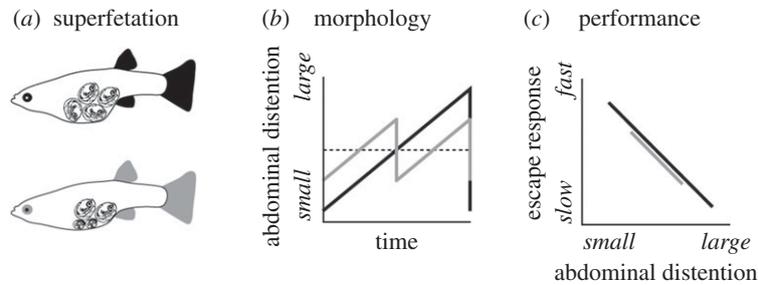
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Superfetation, the ability to simultaneously carry multiple litters of different developmental stages *in utero*, is a reproductive strategy that evolved repeatedly in viviparous animal lineages. The evolution of superfetation is hypothesized to reduce the reproductive burden and, consequently, improve the locomotor performance of the female during pregnancy. Here, we apply new computer-vision-based techniques to study changes in body shape and three-dimensional fast-start escape performance during pregnancy in three live-bearing fishes (family Poeciliidae) that exhibit different levels of superfetation. We found that superfetation correlates with a reduced abdominal distension and a more slender female body shape just before parturition. We further found that body slenderness positively correlates with maximal speeds, curvature amplitude and curvature rate, implying that superfetation improves the fast-start escape performance. Collectively, our study suggests that superfetation may have evolved in performance-demanding (e.g. high flow or high predation) environments to reduce the locomotor cost of pregnancy.

## 1. Introduction

A staggering diversity of reproductive strategies is found in viviparous animals, many of which evolved repeatedly in distantly related lineages. A prevailing idea is that (at least some of) these adaptations may have evolved to counter-balance the locomotor costs to females associated with a live-bearing mode of reproduction [1]. Live-bearing is known to carry severe locomotor costs to pregnant females in a variety of taxa, due to the internal development of embryos (e.g. [2–7]). Superfetation is a reproductive adaptation that presumably evolved to reduce the reproductive burden on females during their pregnancy [1,8–10]. Superfetation refers to the ability of females to carry multiple, overlapping litters that are each at different stages of development [11–17]. Superfetation is generally characterized by a more frequent production of litters, often in association with smaller litter sizes [8,14]. This should reduce the peak reproductive allocation (the proportion of female mass allocated to reproduction) during pregnancy and lead to a more slender female body shape, without affecting female fecundity (figure 1a) [1,8,9]. In fish, body shape is closely related with swimming performance [19]. In many fish species, fluid dynamic drag on the body plays a large role in determining the energy and power requirements of swimming [19,20]. Hence, natural selection has often resulted in a slender ‘streamlined’ low-drag body shape. If in live-bearing fish a slender body shape is associated with the better locomotor performance during pregnancy (figure 1), then superfetation might convey a selective benefit to parturient females living in performance-demanding environments, such as environments characterized by high predation risk [5,21–23] or high flow velocities [1,8,9]. However, the links between reproductive traits, morphology and locomotor performance are still insufficiently understood [24].

Pregnancy should lead to abdominal distention, but these morphological changes are rarely quantified in detail in relation to swimming performance



**Figure 1.** The hypothesized effect of pregnancy and superfetation on morphology and swimming performance in live-bearing fishes. (a) Two hypothetical live-bearing fish species that differ in the presence of superfetation: no superfetation (i.e. one brood; fish with black fins) and two overlapping broods (fish with grey fins). All other aspects of reproduction (e.g. female length, offspring developmental time, offspring size at birth, lifetime fecundity) are assumed to be equal. (b) Predicted change in female body shape through time for the two hypothetical species. An increase from 1 litter (black line) to 2 overlapping litters (grey line) results in a more frequent production of smaller litters. This decreases the amplitude around the mean reproductive burden (dashed line), effectively reducing the peak abdominal distention during pregnancy [1,8]. (c) Predicted decline in escape performance with an increase in abdominal distention: both lines have equal slopes and intercepts and differ only in the range of body thickness ( $x$ -axis). For heuristic purposes the temporal patterns in (b) and (c) are depicted as linear, because the exact relationships between body shape and time, and body shape and performance, respectively, are not known [18].

(but see [4]). The few studies that quantified the (pregnant) morphology of live-bearing fishes used two-dimensional geometric morphometrics and did not record changes over time in the same female (e.g. [25,26]). We developed a non-invasive method that allows us to directly track morphological changes in three dimensions over the pregnancy cycle [27] and combine this with high-speed video recordings of the fast-start escape response in a restriction-free, three-dimensional swim arena [28,29].

Here, we used these novel, quantitative biomechanical techniques [27,29] to study the effect of pregnancy and superfetation on morphology and swimming performance in the live-bearing fish family Poeciliidae. Superfetation has evolved at least three times independently in the Poeciliidae [1,30,31], with significant variation in the level of superfetation among species, ranging from 1 to about 14 overlapping litters [32]. We compared the effects of pregnancy between three species of Poeciliidae that differ in their level of superfetation: *Poeciliopsis turneri* (1–3 overlapping litters at different developmental stages [33]; hereafter abbreviated PT), *Heterandria formosa* (3–5 litters [11,13]; abbreviated HF) and *Phalloptychus januarius* (7–14 litters [32]; abbreviated PJ). Specifically, we tested two hypotheses: First, if superfetation is associated with an increased frequency of parturition (births), then the interbrood interval (the period between parturition events) should become shorter, and the amplitude of morphological changes in the abdomen smaller (figure 1b) under otherwise equal reproductive factors (e.g. female standard length, reproductive allocation, offspring mass, etc.). Second, if an increase in female thickness (e.g. expressed as abdominal distention) correlates with a decrease of her swimming performance, then this should be manifested in several key aspects of the fast-start escape performance (e.g. maximal speed, bending flexibility and reorientation change during the fast-start escape manoeuvre; figure 1c). The correlation between abdominal morphology and swimming performance reflects the effects of pregnancy, and consequently the potential for (reproductive) traits that affect abdominal morphology to provide an adaptive benefit to swimming performance.

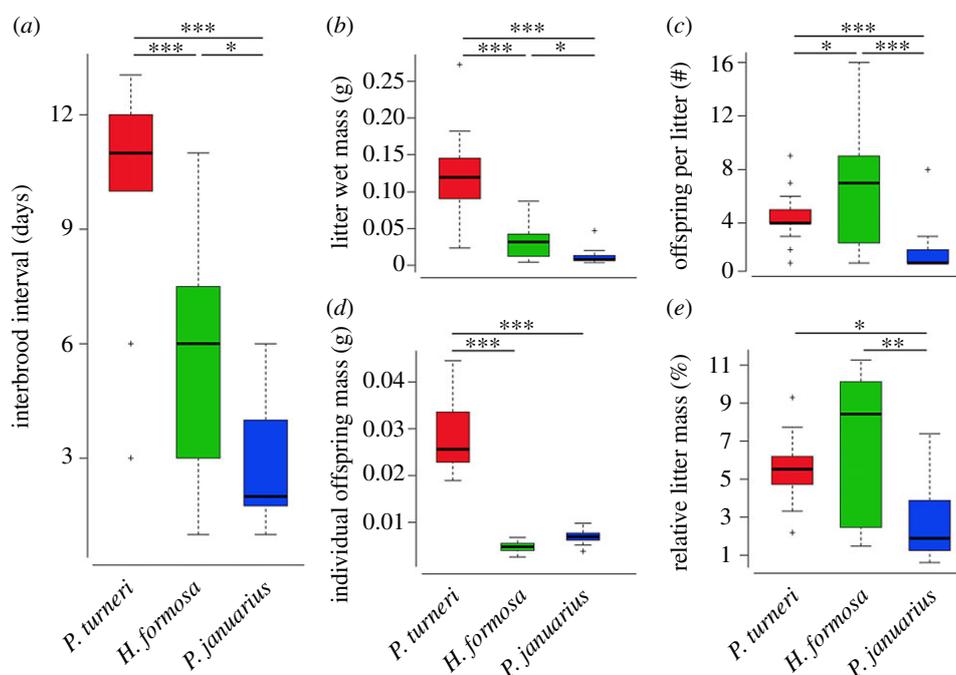
## 2. Material and methods

We compared the effects of pregnancy on morphology on three species of poeciliid fishes that differ in their level of superfetation:

*P. turneri*, *H. formosa* and *P. januarius*. For each species, female virgin fish were randomly assigned to a virgin group ( $n = 14$  individuals per species) or a pregnant treatment group ( $n = 14$  individuals per species). Virgin and pregnant fish were paired in a block for which experiments were subsequently performed on the same days, albeit in a randomized order. For *P. turneri* (bi-daily) and *H. formosa* (daily) measurements started the day following a parturition and ending after the next parturition (if the parturition occurred on a planned measurement day, an additional day of measurements was included), whereas *P. januarius* was measured for six subsequent days. This measurement scheme allowed the acquisition of a sufficient amount of data for the different species taking into account the length of their species-specific interbrood interval, while keeping handling to a minimum (electronic supplementary material, paragraph 2b).

On a measurement day, fish were first subjected to fast-start performance trials, after which they were photographed to measure three-dimensional morphology. The fast-start performance trials were performed in a cubic swimming arena in which the fish could swim freely [29], recorded with three orthogonally placed high-speed video cameras (details in electronic supplementary material, paragraph 2d), calibrated with the direct linear transformation (using the method developed by [34]). A fast-start escape manoeuvre was triggered by dropping a weight in the corner of the tank. A maximum of five responses was recorded per fish per day. For the morphological measurements, fish were photographed by three synchronized cameras [27] (electronic supplementary material, paragraph 2e,f). These pictures were then converted into a three-dimensional model of the fish using a custom-made MATLAB (v. R2013a; MathWorks) program. These models consist of 251 cross-sectional ellipses equally distributed along the anterior–posterior axis. Model segments corresponding to  $0.39 L_{SL}$  (approximately half-way along the abdomen) and  $0.78 L_{SL}$  (approximately half-way along the caudal peduncle) were used for fixed-point measurements, where  $L_{SL}$  is standard length. To correct for inter- and intraspecific size differences, longitudinal parameters were divided by  $L_{SL}$ , surface parameters by  $L_{SL}^2$  and volume by  $L_{SL}^3$ .

The effects of pregnancy and superfetation on morphology were tested for significance with linear mixed models (LMM) using *proc mixed* [35] in SAS (v. 9.3; SAS Institute) using a random intercept. The other fixed effects in the model were time (in days since the parturition that set off the measurement period) and the cumulative sum of litter wet mass born during the measurement period, interacting with species and reproductive state (pregnant or virgin). All model variables, degrees of freedom,  $t/F$ -values and  $p$ -values are shown in electronic supplementary material, table S1.



**Figure 2.** Superfotation relates to a more frequent production of smaller litters. Breeding experiments with *Poeciliopsis turneri* (PT, which carries 1–3 simultaneous overlapping litters), *Heterandria formosa* (HF, 3–5 litters) and *Phalloptychus janauarius* (PJ, 7–14 litters) show that superfotation is associated with (a) a shorter interbrood interval, indicative of more overlapping litters ( $n = 55$  interbrood intervals) and (b) smaller (i.e. less heavy) litters ( $n = 110$  litters), due to either (c) fewer or (d) smaller offspring per litter ( $n = 110$  litters). (e) When corrected for female mass, the absolute difference in litter mass between HF and PT disappears ( $n = 52$  litters). Boxplots contain the 25th–75th percentile and whiskers indicate  $\approx 99\%$  of the data, black line represents the median. LMM,  $*0.01 < p < 0.05$ ,  $**0.001 < p < 0.01$ ,  $***p < 0.001$ . (Online version in colour.)

Fast-start escape manoeuvres were tracked automatically using the Fishtracker program [36] (electronic supplementary material, paragraph 2g) for MATLAB. The time series of the centre of mass positions, body orientations and curvatures were smoothed with a Whittaker smoothing [14]. The onset of motion was deduced from the change in yaw angle of the head, the kinematics stages (stage 1 and 2) were defined by minima in the moment of inertia of the body in the yaw plane which correspond approximately with a switch of the tail motion towards the other side [29]. Stage 1 is considered most important for reorientation, stage 2 is considered most important for propulsion [29,37–39], although the role of each stage is not discrete [29,40,41]. Yaw-angle changes of the head (figure 4g) were left–right standardized; pitch-angle changes (figure 4h) were tested separately for up- and downwards responses, respectively, defined as a positive and negative pitch change from the onset of motion until the end of stage 2.

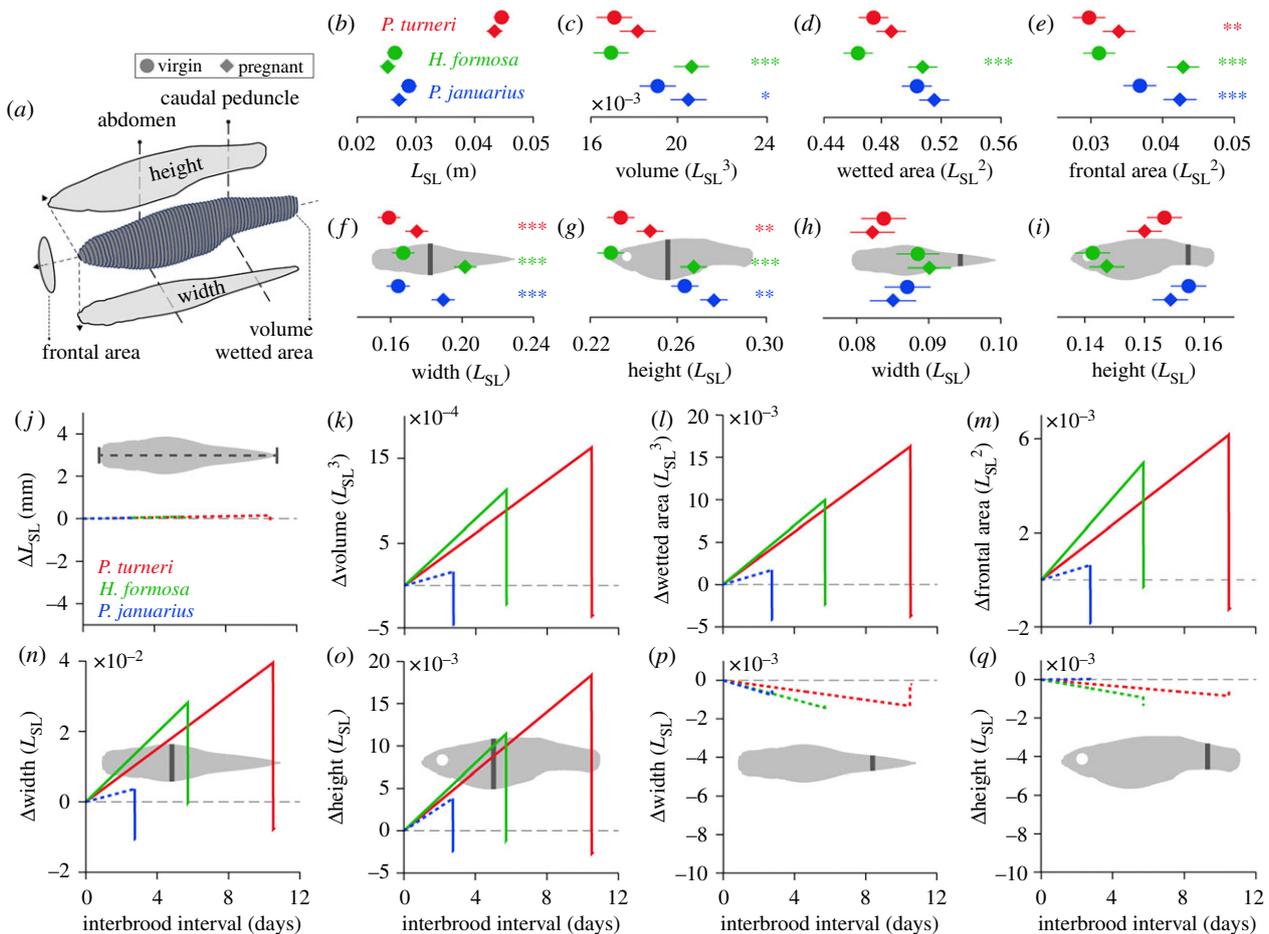
When measuring maximal performance in a laboratory setting, motivation can be a confounding variable [42]. Variation in motivation between and within individuals, e.g. through habituation to the stimulus [43], could conceal interesting effects. We resolved this by monitoring bending in the caudal peduncle (a morphology entity unaffected by pregnancy) during the fast-start response (figure 3p,q). We argue that the strength of the fast-start reflex (i.e. ‘motivation’) is reflected in the curvature and curvature rate of the caudal peduncle. We included the curvature or curvature rate of the caudal peduncle as a fixed effect in our statistical model (electronic supplementary material, table S2): high curvature (rate) corresponds with higher motivation. As a result, varying motivation between manoeuvres is (largely) filtered out. Additionally, we compared the maximum performance when the data is pooled for different abdominal width classes. The measured maximal performance provides comparable results for speed and abdominal curvature parameters, but effects are not significant for curvature rate and curvature in the peduncle (see electronic supplementary material, figure S6b,c).

Relationships between fast-start parameters and morphology were tested for significance with mixed models using *proc mixed* in SAS. Full models included interactions between species and all independent variables, but interactions were removed when this provided an equal or better fitting model ( $\Delta AIC < 2$ ). This included orientation and distance of the fish with respect to the stimulus. Repeated measures were fitted with an autoregressive covariance structure, when this model was over-specified we used compound symmetry as covariance structure. The low model likelihood in a number of escape reorientation models (indicated by an asterisk in electronic supplementary material, tables S2–S6), required measures to be regarded as independent.

### 3. Results

#### (a) Life history correlates of superfotation

Superfotation affected multiple aspects of the reproductive cycle. The expected level of superfotation was inversely related with interbrood interval, the period between parturition events: a higher level of superfotation led to shorter intervals between parturition events (figure 2a). Additionally, litter mass decreased with increasing levels of superfotation (figure 2b), due to the production of either fewer (figure 2c) or smaller (figure 2d) offspring per litter. When standardized for female length, PT and HF both had a higher relative litter mass compared to PJ (figure 2e), indicating that the assumption of the equal mean (relative) reproductive allocation (figure 1) does not apply to our study species. The implication is that inter-specific differences in morphology at the beginning of the interbrood interval (figure 1b) can be the result of both the level of superfotation and the amount of reproductive investment.



**Figure 3.** Superfation correlates with a reduced amplitude of abdominal distention during the interbrood interval. (a) Example of a three-dimensional body model of a pregnant female. For each studied species (*Poeciliopsis turneri*, red; *Heterandria formosa*, green; *Phalloptychus januaris*, blue), we created a timeseries of three-dimensional body reconstructions during pregnancy for 14 pregnant and 14 control females per species ( $n_{\text{total}} = 592$  body models). Dark grey lines in the light grey fish silhouettes in panels (f–j) and (n–q) indicate the position along the body model at which the parameters were quantified. The parameters in panels (c–i) and (k–q) were normalized for standard length ( $L_{\text{SL}}$ ) (see S2). Mean  $\pm 95\%$  confidence interval in (b)  $L_{\text{SL}}$ , (c) volume, (d) wetted surface area, (e) frontal surface area, (f) abdominal width, (g) abdominal height, (h) caudal peduncle width and (i) caudal peduncle height of virgin (circles) and pregnant (diamonds) females at the start of the interbrood interval. LMM,  $*0.01 < p < 0.05$ ,  $**0.001 < p < 0.01$ ,  $***p < 0.001$ . Pregnant fishes tend to have a significantly more distended abdominal region than their virgin conspecifics at the beginning of the interbrood interval. Changes in (j) standard length ( $L_{\text{SL}}$ ), (k) volume, (l) wetted surface area, (m) frontal surface area, (n) abdominal width, (o) abdominal height, (p) caudal peduncle width and (q) caudal peduncle height over the course of one interbrood interval are shown for each species. Dotted lines represent effects that are not significantly different from zero (electronic supplementary material, table S1). Superfation is significantly correlated with a reduced change (i.e. with a smaller amplitude) of morphological traits in the abdominal region over the course of an interbrood interval (i.e. superfation allows reduction in the variations in body distension during pregnancy). (Online version in colour.)

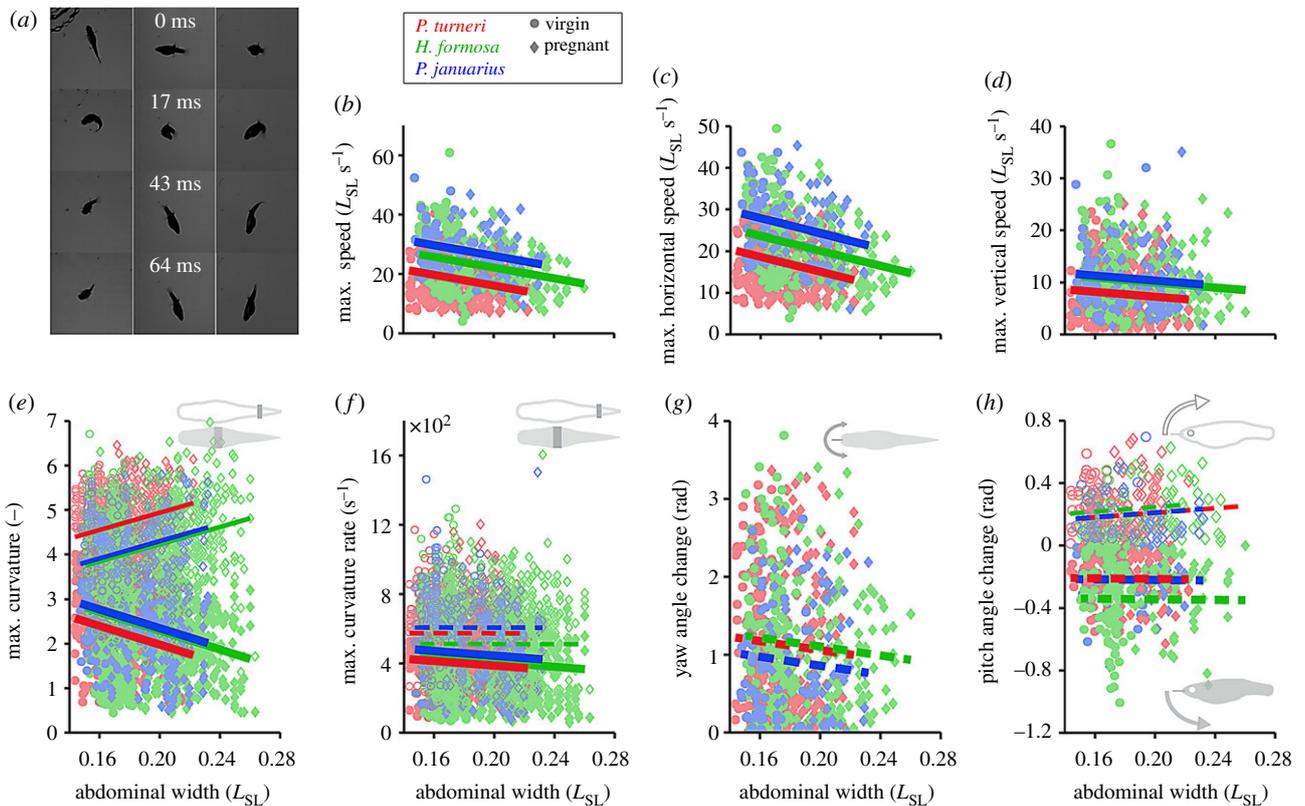
### (b) Changes in three-dimensional morphology during pregnancy

We studied changes in seven morphological traits (i.e. width and height at fixed points in the abdomen and caudal peduncle, total body volume, wetted surface area and frontal surface area; figure 3a) by creating three-dimensional body reconstructions at multiple time points during a single interbrood interval of 84 different females (14 pregnant and 14 non-pregnant control females for each of the three species). We analysed a total of 592 three-dimensional body models.

Female morphology differs among the study species in three ways: (i) the difference in female shape between pregnant and virgin females at the start of the interbrood interval (figure 3b–i), (ii) the amplitude of the morphological changes during pregnancy and (iii) the time span over which these morphological changes takes place during the interbrood interval (figure 3j–q). All these could be the result of the difference in the level of superfation between the species. Standard length ( $L_{\text{SL}}$ ) was not affected by pregnancy,

neither at the start of the interbrood interval between conspecifics (figure 3b) nor over time (figure 3j; i.e. longitudinal growth over this period was not detectable). Because of the interspecific differences in  $L_{\text{SL}}$  (figure 3b) all subsequent parameters were normalized by this trait (see S2). Differences between pregnant and virgin fish were pronounced at the start of the interbrood interval (figure 3c–g) for volume, wetted surface area, frontal surface area, abdominal width and abdominal height. Interspecific differences here can be the result of differences in the superfation level and differences in reproductive investments.

Morphological changes during pregnancy were confined to the abdomen: the morphology of the caudal peduncle was not affected by the state of pregnancy (figure 3h–i,p–q). The abdomen distended in width and height during the interbrood interval (figure 3n,o), with a significant increase in total volume (figure 3k), wetted area (figure 3l) and frontal surface area (figure 3m) in PT and HF, but not in PJ. The amplitude of the changes in these five parameters was biggest in PT, the species with the lowest level of superfation



**Figure 4.** Increasing abdominal thickness correlates with decreasing maximum speed and abdominal flexibility during fast-start escape manoeuvres. Fast-start manoeuvres were recorded for pregnant (diamonds) and virgin (circles) individuals of *Poeciliopsis turneri* (red), *Heterandria formosa* (green) and *Phalloptychus januarius* (blue). LMM,  $p < 0.05$ , solid line;  $p > 0.05$ , dashed line. (a) A fast-start manoeuvre by *H. formosa* displaying strong three-dimensional motion as recorded by the bottom view camera (left column) and the side view cameras (middle, right columns). See electronic supplementary material, movie S1 for more examples. (b–f) An increase in abdominal distention (expressed in standard lengths ( $L_{SL}$ )) correlates with a decrease in maximum speed (b), maximum horizontal speed (c) and maximum vertical speed (d). The abdominal distention increase is further correlated with a decrease in maximum curvature (e) and maximum curvature rate (f) in the abdominal region (thick lines and closed symbols, as indicated by the grey silhouettes;  $n = 808$ ). At the same time, an increase in abdominal distention correlates with an increase in maximum curvature in the caudal peduncle (thin lines and open symbols, as indicated by the open silhouettes). Thus, an increase in abdominal width is associated with decreased escape speed and flexibility, and females may (partially) compensate by increasing the curvature in the caudal peduncle. Reorientation angles for yaw and pitch (g and h, respectively) are not significantly correlated with abdominal width ( $n = 696$ ). (Online version in colour.)

(figure 3*k–o*). In PJ, the species with the highest superfetation level, the abdomen did not significantly distend during the short interbrood interval (though parturition did cause a noticeable decline in abdominal thickness; figure 3*k–o*).

### (c) Linking morphology to three-dimensional fast-start performance

Next, we evaluated how a change in body shape affected the swimming performance of females. We focused on the fast-start escape response, because it is known to be an important determinant of survival of the Poeciliidae during predator–prey interactions [44,45]. When startled, fish exhibit a fast-start escape response (also called C-start), a manoeuvre to rapidly reorient in three-dimensional space and propel away from eminent danger (figure 4*a*; electronic supplementary material, movie S1) [29,37,46]. We used three high-speed video cameras to record a total of 2173 escape manoeuvres, of which 1942 were of sufficient length and quality to include in the analysis. Subsequently, manoeuvres were subdivided into three categories: (i) complete manoeuvres (i.e. manoeuvres in which a complete kinematic stage 2 was detected). Here, the fish completes one full tail beat cycle to propel itself forward [29,37]. These manoeuvres were used

in all analyses. (ii) Manoeuvres in which a stage 1 but no stage 2 was detected. Here, the fish completes the initial reorientation, but the subsequent tail beat is not detected. These manoeuvres are only included in analyses that specifically focus on stage 1. (iii) Manoeuvres in which neither stage 1 nor stage 2 are detected. These manoeuvres were not used in further analyses. Refer to electronic supplementary material, paragraph 2g for details on the detection criteria for stage 1 and stage 2 completion. The five morphological traits in the abdomen (i.e. abdominal width, body volume, wetted area, frontal area and abdominal height) are, by definition, strongly correlated with each other (figure 3) [27]. Consequently, we found that each of these traits showed very similar relationships with the key fast-start escape parameters. We present here one of these relationships in figure 4*b–h*: the relationship between abdominal width ( $x$ -axes) and the fast-start escape parameters ( $y$ -axes). Abdominal width is particularly interesting from a kinematic and biomechanical perspective, because presumably it directly influences axial bending in the lateral direction. We show the relationships of the other four morphological traits with each of the key fast-start escape parameters in the electronic supplementary material, figures S1–S4 (for body volume, wetted area, frontal area and abdominal height, respectively).

In all three species, an increase in abdominal distention is significantly correlated with a decline in maximal speed (LMM,  $p < 0.0001$ ; figure 4b), as well as the maximal speed in both the horizontal ( $p < 0.0001$ ; figure 4c) and vertical ( $p = 0.0075$ ; figure 4d) planes. An increase in abdominal distention is furthermore correlated with a decrease in lateral body curvature. Specifically, in the abdominal region both maximal curvature (LMM,  $p < 0.0001$ ; figure 4e) and maximal curvature rate ( $p < 0.0001$ ; figure 4f) decline as the abdomen thickens. By contrast, in the caudal peduncle, the maximum curvature correlated positively with abdominal distention ( $p < 0.0001$ ; figure 4e), whereas there was no clear correlation between abdominal distention and maximum curvature rate in the caudal peduncle ( $p = 0.2349$ ; figure 4f). This indicates that with increasing abdominal width, fish increase the bending in their caudal peduncle, but not the rate with which they bend it. However, there is a distinction between the two stages of the fast-start manoeuvre. Specifically, during the 'propulsive' stage 2 of the fast-start manoeuvre, we observed a positive correlation between abdominal width and maximal curvature rate in the caudal peduncle (electronic supplementary material, figure S5d): during stage 2, thicker fish appear to bend their caudal peduncle faster. These results indicate that fast-start performance, kinematics and flexural stiffness all are affected by pregnancy-related changes in morphology. Furthermore, pregnant females may (partially) compensate some of these effects by kinematic changes, namely by an increase in caudal peduncle curvature (figure 4e).

#### (d) Linking morphology to three-dimensional fast-start reorientation

The fast-start manoeuvre allows fishes to rapidly reorient themselves in three-dimensional space (i.e. change their three-dimensional angle as defined in §2): the unpredictable character of this reorientation makes it hard for predators to anticipate a prey's future position. The observed effects of abdominal thickness on curvature (rate) could reduce the scope of possible reorientation angles of pregnant fishes, making them not only slower but also more predictable for predators. However, we found that the increasing abdominal distention and concomitant changes in maximum curvature (rate) did not affect the overall reorientation of the fish as measured at the end of stage 2. Neither the change in yaw angle (LMM,  $p = 0.1271$ ; figure 4g), which roughly equates to left–right rotation, nor the change in pitch angle (upwards pitch:  $p = 0.2702$ , downwards pitch:  $p = 0.8661$ ; figure 4h), which roughly equates to up–down rotation, were affected by abdominal width. So, despite a negative correlation between abdominal thickness and abdominal curvature, overall reorientation appeared to be unaffected by abdominal distention. This could be the result of the positive correlation between abdominal distention and caudal peduncle curvature, which could serve as a kinematic compensation mechanism to the decreased abdominal flexibility.

However, there is an effect of abdominal distention on reorientation during the kinematic stage 1 of the fast start. Abdominal distention correlates with a reduction in reorientation in the yaw plane during stage 1 (LMM,  $p = 0.0027$ ; figure 5a), but there is no clear correlation with reorientation in the yaw plane during stage 2 ( $p = 0.2262$ ; figure 5b). Correlation between abdominal distention and reorientation in the pitch plane is relatively complex: the effect during stage 1 is

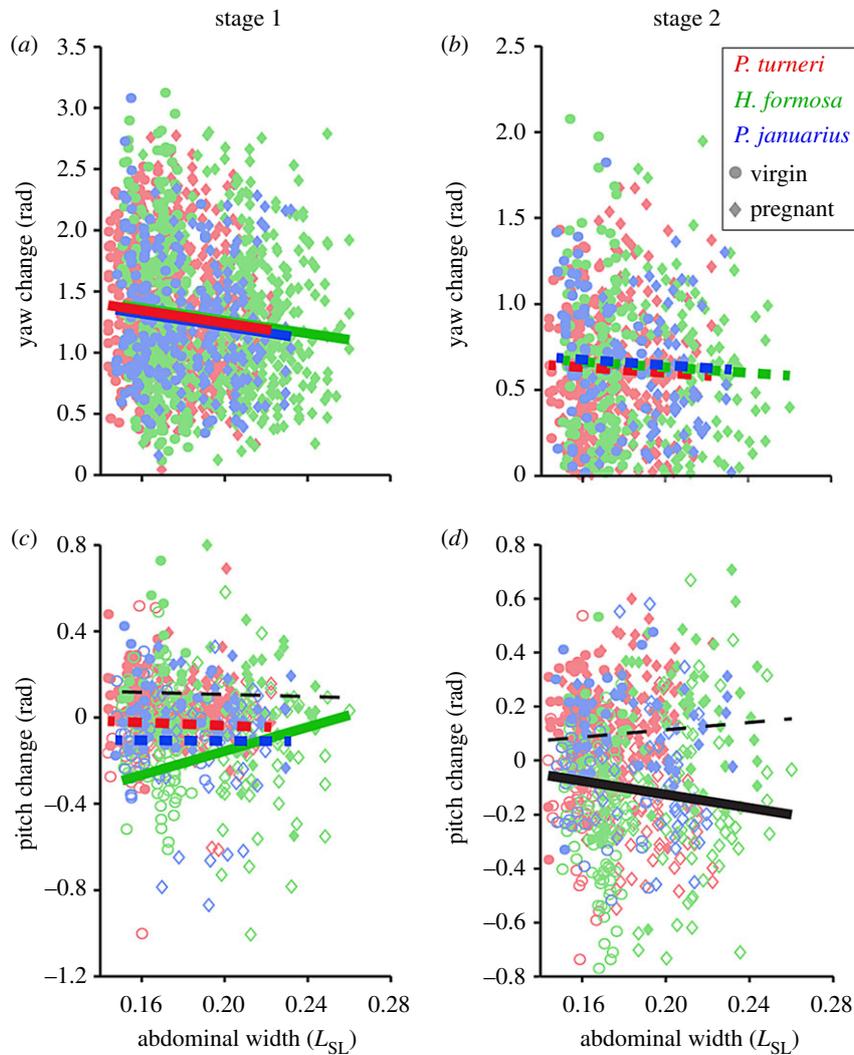
species-dependent, with the only significant (but positive) correlation being that with downwards pitch in HF (figure 5c; electronic supplementary material, table S2). Downwards pitch change during stage 2 is negatively correlated with abdominal width increase (figure 5d). These results indicate that increasing abdominal width has complex effects on the three-dimensional reorientation.

## 4. Discussion

Our results provide a mechanistic explanation for the locomotion performance decline during pregnancy. Using a large sample size and the latest techniques from morphology and biomechanics research, we show that abdominal distention affects not only performance-influencing traits, but also kinematics and flexibility, with different effects in the horizontal and vertical planes. By implementing these techniques in a comparative evolutionary framework, we demonstrate one adaptive potential of superfetation in live-bearing fishes: superfetation reduces the amplitude of changes in the reproductive burden and concomitant reductions in performance.

The hypothesized benefit of superfetation (figure 1) lies in an increased frequency of parturitions coinciding with a reduction of the amplitude of morphological changes [1,8]. This should lead to a less slender body at the beginning of the pregnancy, yet at the same time to a more slender body at the end of a pregnancy (figure 1b). We indeed find that superfetation is associated with shorter interbrood intervals and litters of smaller mass (figure 2b). Furthermore, despite interspecific differences in relative litter mass (figure 2e) and, consequently, female body shape at the beginning of the interbrood interval (figure 3b–l), we find that superfetation is significantly associated with a reduction of the amplitude of morphological changes (figure 3k–o). We argue that this may represent a general phenomenon (i.e. that species with a higher level of superfetation will tend to show a relatively smaller increase in abdominal thickness, total body volume and wetted and frontal surface area during their interbrood interval). Moreover, female survival rates are generally lower at the end of pregnancy [22], where a performance decline could bring late-stage females within the range of hunting speeds of predators from which non-pregnant or early-stage females could more easily escape [6]. It is therefore likely that the benefits of a more slender body at the end of the interbrood interval outweigh the potential costs at the beginning of pregnancy.

The morphological benefit of superfetation can potentially provide an adaptive advantage if a more slender (abdominal) morphology leads to an improved locomotor performance and if this, in turn, translates to higher rates of survival. We show that certain aspects of the fast-start performance of thick fish are impaired compared with more slender individuals (figure 4); that is, fish with a more slender abdomen perform better. Fast-start escape responses are three-dimensional manoeuvres that fish use to avoid predatory strikes [28,29,38,47], and are a key factor in their survival probability during predator encounters [44,45]. Specifically, the survival probability depends on the prey's ability to rapidly swim away (here: maximum speed) and the ability to rapidly bend and rotate during stage 1 of the fast start (here: maximum curvature rate) [45]. We find that abdominal distention both reduces the ability of fish to



**Figure 5.** The effect of abdominal width on reorientation angles, split by kinematic stages. Representation of LMM for the relationship between abdominal width (expressed in standard lengths ( $L_{SL}$ )) and reorientation angles. Solid lines indicate significant relationships (LMM,  $p < 0.05$ ), otherwise dashed lines are used. Circles: virgins; diamonds: pregnant fish; for *Poeciliopsis turneri* (red), *Heterandria formosa* (green) and *Phalloptychus januaris* (blue).  $n = 1627$  data points (a,c) or  $n = 696$  data points (b,d) from 84 individuals. (a–b) Yaw-angle change during stage 1 (a) and stage 2 (b) of the fast start. (c–d) Pitch-angle change during stage 1 (c) and stage 2 (d) of the fast start. Open symbols, thick lines: downwards pitch at end of stage 2; closed symbols, thin lines: upwards pitch at end of stage 2. Black line: model without species-specific slope and intercept provided a fit equal to or better than other, more complicated models ( $\Delta AIC < 2$ ). (Online version in colour.)

swim away, and the ability of fish to rapidly rotate and reorient themselves (figures 4b–d,f and 5a,c). In our study, we did not study the effect of superfetation on survival; however, a reduced fast-start performance is known to correlate with a reduced survival during predator attacks [44,45] and selection for improved escape performance under high predation conditions could favour the evolution of superfetation. Our findings may, for example, provide a mechanistic explanation for the observed higher levels of superfetation in natural populations of the poeciliid *Phallosceros harpagos* that are characterized by a high predation pressure [48]. Such a potential adaptive benefit of superfetation does not conflict with other hypothesized benefits [9], such as facilitating multiple paternity [30,49], or as a bet-hedging strategy [50,51], which can be multifaceted and vary between species, populations and/or ecological contexts.

Interestingly, reorientation during the complete fast-start manoeuvre was not correlated with an increasing abdominal thickness (figure 4g,h). However, if we break up the manoeuvre into two kinematic stages, we observe a negative correlation between abdominal width and reorientation in the yaw plane at the end of stage 1 (figure 5a). Although

this stage can also contribute to propulsion [40,52], it is the most important stage for reorientation of the fish [29,39]. These findings suggest that the maximum reorientation that fishes can achieve, usually a full  $180^\circ$  turn or more, may be reducing over the period of pregnancy. Additionally, these findings show that some aspects of the fast-start manoeuvre merit analysis by its different kinematic stages.

Acquisition and analysis of large sample sizes (e.g. over 2000 recorded events here) are facilitated by the recent availability of automated tracking software (e.g. [36,39,53]) and relatively affordable high-speed video cameras, which can be combined to record morphology and performance in three dimensions. Here, we have shown that these three-dimensional recordings have added value (figures 4b–d,f and 5). Three-dimensional recordings allow capturing more natural behaviour compared to traditional two-dimensional fast-start set-ups in which fish are restricted to a shallow volume of water. Furthermore, we show that the effects of pregnancy, or the scale of these effects, differ between horizontal and vertical planes. Fish achieve the highest maximal speed in the horizontal plane, but despite this, there is still a small but significant decline in maximal vertical speed

with increased abdominal distension (figure 4c,d). Also, abdominal width correlates differently with yaw and pitch reorientation during the fast-start manoeuvre when split up into its kinematic stages (figure 5). This novel approach opens possibilities to comparatively study a broad range of evolutionary questions in fish and other aquatic species in a controlled laboratory setting that mirrors the natural freedom of movement of fishes, including the study of other modes of swimming. Whereas the fast-start escape manoeuvre is the default mode of escaping predators, poeciliid fish swim routinely by using undulating body and caudal fin (BCF) motion. Body drag plays a more important role in BCF swimming compared to fast-start manoeuvres [19,20]. Two important influencers of body drag, fish shape and body surface area, are affected by pregnancy [27,54] and superfetation (this study). Therefore, we suggest that this drag reduction associated with superfetation may benefit fishes inhabiting fast-flowing waters [1,8,14], a hypothesis for which experimental evidence is currently indecisive [25,26,55].

Superfetation has evolved at least three times independently in the Poeciliidae [1,30,31]. To study its potential adaptive advantage, we applied computer-vision based techniques to quantify changes in body shape and fast-start escape performance during pregnancy in three live-bearing fish species. We showed that, independent of species-specific differences in female body shape prior to the pregnancy (in virgins), superfetation reduces the amplitude of morphological changes during pregnancy, effectively reducing a female's peak abdominal distension. We furthermore demonstrated that a slender female body shape improves fast-start escape

performance during pregnancy in terms of speed, abdominal curvature and abdominal curvature rate. Our results suggest that superfetation may (at least partly) alleviate the negative effects of pregnancy on the locomotor ability of females and may provide insights into the conditions under which this trait evolves. Finally, our research approach can be applied to study other reproductive traits that alter abdominal morphology during pregnancy in female fish (e.g. matrotrophy [1,27]).

**Ethics.** All procedures were approved by the Animal Ethics Committee of Wageningen University and Research (permit number 2013103).

**Data accessibility.** Data available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.r8n434d> [56].

**Authors' contributions.** M.F., J.L.V.L. and B.J.A.P. conceptualized the experiment, M.F. and J.L.V.L. designed the experimental methodology (hardware and software), M.F. performed the experiments and analysed the data, M.F. wrote a first draft of the manuscript and finalized the manuscript with comments from J.L.V.L. and B.J.A.P. All authors gave final approval for publication and agree to be held accountable for the work performed therein.

**Competing interests.** We declare we have no competing interests.

**Funding.** This study was supported by NWO-ALW Open Program grant no. 821.02.024 to J.L.V.L. and B.J.A.P. and NWO-VIDI grant no. 864.14.008 to B.J.A.P.

**Acknowledgements.** We gratefully acknowledge Michael Kempkes (*H. formosa*) and David N. Reznick (*P. turneri* and *P. januarius*) for providing the breeding stocks. Furthermore, we thank Elsa M. Quicazan-Rubio, Remco P. M. Pieters, Cees J. Voesenek and Cajo J. F. ter Braak with their help during various stages of the experiments and analyses, the staff of Carus-ARF for their expert advice and help with husbandry and three anonymous reviewers for their helpful comments on the manuscript.

## References

- Pollux BJA, Pires MN, Banet AI, Reznick DN. 2009 Evolution of placentas in the fish family Poeciliidae: an empirical study of macroevolution. *Annu. Rev. Ecol. Evol. Syst.* **40**, 271–289. (doi:10.1146/annurev.ecolsys.110308.120209)
- Shine R. 1980 'Costs' of reproduction in reptiles. *Oecologia* **46**, 92–100. (doi:10.1007/BF00346972)
- Shaffer LR, Formanowicz DR. 1996 A cost of viviparity and parental care in scorpions: reduced sprint speed and behavioural compensation. *Anim. Behav.* **51**, 1017–1023. (doi:10.1006/anbe.1996.0104)
- Plaut I. 2002 Does pregnancy affect swimming performance of female mosquitofish, *Gambusia affinis*? *Funct. Ecol.* **16**, 290–295. (doi:10.1046/j.1365-2435.2002.00638.x)
- Ghalambor CK, Reznick DN, Walker JA. 2004 Constraints on adaptive evolution: the functional trade-off between reproduction and fast-start swimming performance in the Trinidadian guppy (*Poecilia reticulata*). *Am. Nat.* **164**, 38–50. (doi:10.1086/421412)
- Noren SR, Redfern JV, Edwards EF. 2011 Pregnancy is a drag: hydrodynamics, kinematics and performance in pre- and post-parturition bottlenose dolphins (*Tursiops truncatus*). *J. Exp. Biol.* **214**, 4151–4159. (doi:10.1242/jeb.059121)
- Banet AI, Svendsen JC, Eng KJ, Reznick DN. 2016 Linking reproduction, locomotion, and habitat use in the Trinidadian guppy (*Poecilia reticulata*). *Oecologia* **181**, 87–96. (doi:10.1007/s00442-015-3542-9)
- Thibault RE, Schultz RJ. 1978 Reproductive adaptations among viviparous fishes (Cyprinodontiformes: Poeciliidae). *Evolution* **32**, 320–333. (doi:10.1111/j.1558-5646.1978.tb00648.x)
- Zúñiga-Vega JJ, Macías-García C, Johnson JB. 2010 Hypotheses to explain the evolution of superfetation in viviparous fishes. In *Viviparous fishes II* (eds M Uribe, H Grier), pp. 241–253. Homestead, FL: New Life Publications.
- Pires MN, Arendt JD, Reznick DN. 2010 The evolution of placentas and superfetation in the fish genus *Poecilia* (Cyprinodontiformes: Poeciliidae: subgenera *Micropoecilia* and *Acanthophaelus*). *Biol. J. Linn. Soc.* **99**, 784–796. (doi:10.1111/j.1095-8312.2010.01391.x)
- Turner CL. 1937 Reproductive cycles and superfetation in Poeciliid fishes. *Biol. Bull.* **72**, 145–164. (doi:10.2307/1537249)
- Turner CL. 1940 Superfetation in viviparous cyprinodont fishes. *Copeia* **1940**, 88–91. (doi:10.2307/1439048)
- Scrimshaw N. 1944 Superfetation in Poeciliid fishes. *Copeia* **3**, 180–183. (doi:10.2307/1437814)
- Reznick DN, Miles DB. 1989 Review of life history patterns in Poeciliid fishes. In *Ecology and evolution of livebearing fishes (Poeciliidae)* (eds GK Meffe, FF Snelson), pp. 125–148. Englewood Cliffs, NJ: Prentice Hall.
- Gunn JS, Thresher RE. 1991 Viviparity and the reproductive ecology of clinid fishes (Clinidae) from temperate Australian waters. *Environ. Biol. Fishes* **31**, 323–344. (doi:10.1007/BF00002357)
- Reznick DN, Meredith R, Collette BB. 2007 Independent evolution of complex life history adaptations in two families of fishes, live-bearing halfbeaks (Zenarchopteridae, Beloniformes) and Poeciliidae (Cyprinodontiformes). *Evolution* **61**, 2570–2583. (doi:10.1111/j.1558-5646.2007.00207.x)
- Roellig K, Menzies BR, Hildebrandt TB, Goeritz F. 2011 The concept of superfetation: a critical review on a 'myth' in mammalian reproduction. *Biol. Rev.* **86**, 77–95. (doi:10.1111/j.1469-185X.2010.00135.x)
- Shrader M, Travis J. 2005 Population differences in pre- and post-fertilization offspring provisioning in the least killifish, *Heterandria formosa*. *Copeia* **2005**, 649–656. (doi:10.1643/CE-04-230R)
- Webb PW. 1984 Form and function in fish swimming. *Sci. Am.* **251**, 72–82. (doi:10.1038/scientificamerican0784-72)
- Videler JJ. 1993 *Fish swimming*, 1st edn. London, UK: Chapman & Hall.
- Plath M, Riesch R, Culumber Z, Streit B, Tobler M. 2011 Giant water bug (*Belostoma sp.*) predation on

- a cave fish (*Poecilia mexicana*): effects of female body size and gestational state. *Evol. Ecol. Res.* **13**, 133–144. (doi:10.1111/j.1365-2311.2007.00892.x)
22. Laidlaw CT, Condon JM, Belk MC. 2014 Viability costs of reproduction and behavioral compensation in western mosquitofish (*Gambusia*). *PLoS ONE* **9**, e110524. (doi:10.1371/journal.pone.0110524)
  23. Belk MC, Tuckfield RC. 2010 Changing costs of reproduction: age-based differences in reproductive allocation and escape performance in a livebearing fish. *Oikos* **119**, 163–169. (doi:10.1111/j.1600-0706.2009.17742.x)
  24. Orr TJ, Garland T. 2017 Complex reproductive traits and whole-organism performance. *Integr. Comp. Biol.* **57**, 407–422. (doi:10.1093/icb/ix052)
  25. Zúñiga-Vega JJ, Reznick DN, Johnson JB. 2007 Habitat predicts reproductive superfetation and body shape in the livebearing fish *Poeciliopsis turubarensis*. *Oikos* **116**, 995–1005. (doi:10.1111/j.2007.0030-1299.15763.x)
  26. Frías-Alvarez P, Zúñiga-Vega JJ. 2016 Superfetation in live-bearing fishes is not always the result of a morphological constraint. *Oecologia* **181**, 645–658. (doi:10.1007/s00442-015-3477-1)
  27. Fleuren M, Quicazan-Rubio EM, van Leeuwen JL, Pollux BJA. 2018 Why do placentas evolve? First evidence for a morphological advantage during pregnancy in live-bearing fish. *PLoS ONE* **13**, e0195976. (doi:10.1371/journal.pone.0195976)
  28. Nair A, Azatian G, McHenry MJ. 2015 The kinematics of directional control in the fast start of zebrafish larvae. *J. Exp. Biol.* **218**, 3996–4004. (doi:10.1242/jeb.126292)
  29. Fleuren M, van Leeuwen JL, Quicazan-Rubio EM, Pieters RPM, Pollux BJA, Voeseck CJ. 2018 Three-dimensional analysis of the fast-start escape response of the least killifish, *Heterandria formosa*. *J. Exp. Biol.* **221**, jeb168609. (doi:10.1242/jeb.168609)
  30. Pollux BJA, Meredith RW, Springer MS, Garland T, Reznick DN. 2014 The evolution of the placenta drives a shift in sexual selection in livebearing fish. *Nature* **513**, 233–236. (doi:10.1038/nature13451)
  31. Furness AI, Pollux BJA, Meredith RW, Springer MS, Reznick DN. 2019 How conflict shapes evolution in poeciliid fishes. *Nat. Commun.* **10**, 1–12. (doi:10.1038/s41467-019-11307-5)
  32. Pollux BJA, Reznick DN. 2011 Matrotrophy limits a female's ability to adaptively adjust offspring size and fecundity in fluctuating environments. *Funct. Ecol.* **25**, 747–756. (doi:10.1111/j.1365-2435.2011.01831.x)
  33. Banet AI, Au AG, Reznick DN. 2010 Is mom in charge? Implications of resource provisioning on the evolution of the placenta. *Evolution* **64**, 3172–3182. (doi:10.1111/j.1558-5646.2010.01059.x)
  34. Hedrick TL. 2008 Software techniques for two- and three-dimensional kinematic measurements of biological and biomimetic systems. *Bioinspir. Biomim.* **3**, 034001. (doi:10.1088/1748-3182/3/3/034001)
  35. Singer JD. 1998 Using SAS PROC MIXED to fit multilevel models, hierarchical models, and individual growth models. *J. Educ. Behav. Stat.* **23**, 323–355. (doi:10.3102/10769986023004323)
  36. Voeseck CJ, Pieters RPM, Van Leeuwen JL. 2016 Automated reconstruction of three-dimensional fish motion, forces, and torques. *PLoS ONE* **11**, e0146682. (doi:10.1371/journal.pone.0146682)
  37. Weihs D. 1973 The mechanism of rapid starting of slender fish. *Biorheology* **10**, 343–350. (doi:10.3233/BIR-1973-10308)
  38. Domenici P, Blake RW. 1993 Escape trajectories in Angelfish (*Pterophyllum eimekei*). *J. Exp. Biol.* **177**, 253–272.
  39. Voeseck CJ, Pieters RPM, Muijres FT, Van Leeuwen JL. 2019 Reorientation and propulsion in fast-starting zebrafish larvae: an inverse dynamics analysis. *J. Exp. Biol.* **222**, jeb203091. (doi:10.1242/jeb.203091)
  40. Wakeling JM. 2006 Fast-start mechanics. In *Fish biomechanics* (eds RE Shadwick, GV Lauder), pp. 333–368. San Diego, CA: Elsevier Academic Press.
  41. Tytell ED, Lauder GV. 2002 The C-start escape response of *Polypterus senegalus*: bilateral muscle activity and variation during stage 1 and 2. *J. Exp. Biol.* **205**, 2591–2603.
  42. Losos J, Creer D, Schulte J. 2002 Cautionary comments on the measurement of maximum locomotor capabilities. *J. Zool.* **258**, 57–61. (doi:10.1017/S0952836902001206)
  43. Oufiero CE, Garland T. 2009 Repeatability and correlation of swimming performances and size over varying time-scales in the guppy (*Poecilia reticulata*). *Funct. Ecol.* **23**, 969–978. (doi:10.1111/j.1365-2435.2009.01571.x)
  44. Katzir G, Camhi JM. 1993 Escape response of black mollies (*Poecilia sphenops*) to predatory dives of a pied kingfisher (*Ceryle rudis*). *Copeia* **1993**, 549–553. (doi:10.2307/1447160)
  45. Walker JA, Ghalambor CK, Griset OL, McKenney D, Reznick DN. 2005 Do faster starts increase the probability of evading predators? *Funct. Ecol.* **19**, 808–815. (doi:10.1111/j.1365-2435.2005.01033.x)
  46. Domenici P, Blake RW. 1997 The kinematics and performance of fish fast-start swimming. *J. Exp. Biol.* **200**, 1165–1178.
  47. Kasapi MA, Domenici P, Blake RW, Harper D. 1993 The kinematics and performance of escape responses of the knifefish *Xenomystus nigri*. *Can. J. Zool.* **71**, 189–195. (doi:10.1139/z93-026)
  48. Gorini-Pacheco B, Zandonà E, Mazzoni R. 2018 Predation effects on matrotrophy, superfetation and other life history traits in *Phalloceros harpagos*. *Ecol. Freshw. Fish* **27**, 442–452. (doi:10.1111/eff.12359)
  49. Macías-García C, González-Zuarth CA. 2005 Reproductive behaviour in viviparous fish and intersexual conflict. In *Viviparous fishes* (eds MC Uribe, HJ Grier), pp. 289–302. Homestead, FL: New Life Publications.
  50. Burley N. 1980 Clutch overlap and clutch size: alternative and complementary reproductive tactics. *Am. Nat.* **115**, 223–246. (doi:10.1086/283556)
  51. Travis J, Farr JA, Henrich S, Cheong RT. 1987 Testing theories of clutch overlap with the reproductive ecology of *Heterandria Formosa*. *Ecology* **68**, 611–623. (doi:10.2307/1938466)
  52. Tytell ED, Lauder GV. 2008 Hydrodynamics of the escape response in bluegill sunfish, *Lepomis macrochirus*. *J. Exp. Biol.* **211**, 3359–3369. (doi:10.1242/jeb.020917)
  53. Butail S, Paley DA. 2012 Three-dimensional reconstruction of the fast-start swimming kinematics of densely schooling fish. *J. R. Soc. Interface* **9**, 77–88. (doi:10.1098/rsif.2011.0113)
  54. Quicazan-Rubio EM, van Leeuwen JL, van Manen K, Stamhuis EJ, Fleuren M, Pollux BJA. 2019 Coasting in live-bearing fish: the drag penalty of being pregnant. *J. R. Soc. Interface* **16**, 20180714. (doi:10.1098/rsif.2018.0714)
  55. Zúñiga-Vega JJ, Olivera-Tlahuel C, Molina-Moctezuma A. 2017 Superfetation increases total fecundity in a viviparous fish regardless of the ecological context. *Acta Oecol.* **84**, 48–56. (doi:10.1016/j.actao.2017.08.002)
  56. Fleuren M, van Leeuwen JL, Pollux BJA. 2019 Data from: Superfetation reduces the negative effects of pregnancy on the fast-start escape performance in live-bearing fish. Dryad Digital Repository. (doi:10.5061/dryad.r8n434d)