

RESEARCH ARTICLE

Increased aggression during pregnancy comes at a higher metabolic cost

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SUMMARY

Aggressive behaviour is linked to fitness, but it is metabolically costly. Changes in metabolic demand during the reproductive cycle could constrain activity and thereby modulate behavioural phenotypes. We predicted that increased metabolic demands in late pregnancy would lead to reduced aggression and a lower metabolic cost of behaviour in the mosquitofish *Gambusia holbrooki*. Contrary to our prediction, females became more aggressive in late pregnancy, but metabolic scope (i.e. the metabolic energy available for activity and behaviour) decreased. Consequently, late-stage pregnant females spent significantly more of their available metabolic scope on aggressive behaviour. Hence, as pregnancy progressed, females showed increasingly risky behaviour by depleting metabolic resources available for activities other than fighting. We argue that the metabolic cost of behaviour, and possibly personality, is best expressed with reference to metabolic scope, rather than resting metabolic rates or concentrations of metabolites. This dependence on metabolic scope could render reproductive success sensitive to environmental changes.

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INTRODUCTION

Behavioural interactions within species are linked to fitness by facilitating successful matings (Wilson et al., 2007), and by achieving dominance in aggressive interactions to gain access to resources (Maynard-Smith, 1982; Huntingford and Turner, 1987; Briffa and Sneddon, 2007). Behaviour relies on muscle-powered movement so there is a metabolic cost (Briffa and Elwood, 2004; Copeland et al., 2011), and the metabolic investment in a fight can be proportional to fighting success (Briffa and Sneddon, 2007). Hence, metabolism can constrain behaviour, and changing metabolic demands may modify behaviour temporally. For example, metabolic demands of individual females can change dramatically during pregnancy (Neumann, 2008). Our aim was, therefore, to determine whether metabolic changes are paralleled by changes in aggressive behaviour in pregnant mosquitofish, *Gambusia holbrooki*.

Mosquitofish are small freshwater fish that are native to the south-eastern USA but were introduced to many parts of the world in the 20th century to combat mosquitos (Pyke, 2008). The species has a coercive mating system, and both males and females are extremely aggressive and establish dominance hierarchies (Wilson et al., 2007; Sinclair et al., 2011). Female mosquitofish are ideal subjects for addressing our aim because they are usually mated immediately upon reaching maturity and store sperm, so that adult females undergo several continuous pregnancy cycles and deliver several clutches per year (Pyke, 2008). We chose to study aggressive behaviour because dominance in female hierarchies influences reproductive success in *G. holbrooki*. Dominant females attract more mating attempts than subordinate individuals, and this may be driven either by male mate choice, because dominance and fecundity are linked, or by the exclusion of

subordinate females by more dominant fish in the group (Chen et al., 2011).

Many animals face a trade-off between achieving success in behavioural interactions and minimising energy expenditure. Too much investment in any one behavioural interaction can be a high-risk strategy because it leaves the individual unprepared for subsequent needs such as predator escape or prey capture (Sneddon et al., 1999), and it reduces the energy available for offspring growth. The metabolic scope of individuals is therefore an important correlate of fitness (Seebacher and Wilson, 2006; Claireaux and Lefrançois, 2007) that can modulate the severity of the trade-off between behaviour and metabolic cost. Metabolic scope is defined as the difference between maximal and resting rates of oxygen consumption, and it is a measure of the total metabolic energy available for ATP-consuming processes such as locomotion and behaviour (Fry and Hart, 1948; Claireaux and Lefrançois, 2007; Eliason et al., 2011).

Metabolic scope can become limiting as a result of unfavourable environmental conditions and multiple concurrent metabolic demands (Eliason et al., 2011). Females in late pregnancy may have reduced metabolic scope as offspring take up a greater proportion of maternal body mass. ATP is not exported outside the cells where it is produced so the metabolism of the offspring does not contribute to the cost of the mother's behaviour; however, the mother's cardiovascular system has to supply her offspring. Increased offspring metabolic demand will therefore increase demand on the maternal cardiovascular system and may thereby constrain the mother's metabolic scope (Timmerman and Chapman, 2003; Clark et al., 2011). It may be expected, therefore, that females in late pregnancy would be less aggressive relative to other females in the

population to minimise metabolic investment in behaviour. In ectotherms, this constraint would increase at higher temperatures, because the increased metabolic demand of the offspring would require a greater proportion of the mothers' cardiac output. These metabolic constraints would also render behavioural performance during pregnancy sensitive to environmental temperature changes.

We tested the hypotheses that: (a) females in late pregnancy are less aggressive than in early pregnancy, and will win fewer fights against early-stage pregnant females; (b) the metabolic scope of late-stage pregnant females will be less than that of early-stage pregnant females, and this difference will be more pronounced at warmer temperatures; and (c) late-stage pregnant females will invest less of their metabolic scope in aggressive behaviours compared with early-stage pregnant females. We tested these ideas by firstly determining the relative success of early- and late-stage pregnant female mosquitofish (*G. holbrooki*) in dyadic aggressive encounters. We then measured the metabolic scope of each group of females at different temperatures, and finally measured the metabolic cost (oxygen consumption) of different aggressive behaviours of each group as a proportion of their metabolic scope.

MATERIALS AND METHODS

Study animals

All procedures were approved by the University of Sydney Animal Ethics Committee (approval no. L04/10-2010/3/5411). Mosquitofish (*G. holbrooki*, Girard 1859) were caught in the wild near Perpignan, France (42.698°N, 2.895°E). Mixed shoals were kept in large tanks at the Station d'Ecologie Experimentale du CNRS at Moulis, France, at densities of one fish per litre and the water from their capture site was gradually replaced with aged tap water over 2 days. Females chosen to be used in experiments were then separated into individual tanks (300×250×200 mm) and left for 4 days before experiments began. We sorted females into one group that had recently given birth (early-stage pregnant) and a second group that was at a late stage of pregnancy (late-stage pregnant) based on the shape, distension and colouration of their abdomen. To quantify differences in shape between these groups, we calculated shape factors from measurements of total length (to 0.05 cm) and mass (to 0.01 g) as $\text{shape} = \text{mass}/\text{length}^3 \times 100$ (Table 1). We re-weighed late-stage pregnant females after parturition to determine the proportion of body mass composed of offspring, and to verify that our initial groupings were accurate by comparing shape factors after birth with those of our initial early-stage pregnant group. On average, late-stage pregnant fish were significantly heavier than early-stage pregnant fish (Table 1). Hence, shape factors of early-stage pregnant fish were significantly lower than those of late-stage pregnant fish. However, after giving birth, neither mass nor shape factors of the late-stage pregnant group differed from those of the early-stage pregnant group. Note that for behavioural interactions (see below), pairs of interacting fish were length matched so that the overall mean

differences in length between the two groups (Table 1) did not affect the outcomes of aggressive encounters.

Water temperature at the capture site at the time of capture was 28–30°C, and fish were kept at that temperature throughout the experiments, except for measurements of oxygen consumption, which were also performed at 20°C.

Behavioural experiments

We staged dyadic combat bouts between early- and late-stage pregnant females ($N=18$ pairs) by placing the two fish simultaneously into an experimental tank (300×250×200 mm). Experimental fish were matched for length (mean \pm s.e.m. length: late-stage pregnant females 31.9 \pm 1.3 mm, early-stage pregnant females 32.1 \pm 1.3 mm). We recorded behaviour continuously, starting from the first interaction. We categorised behaviours into three types as follows. 'Displays' consisted of one fish turning its flank to the second fish and performing undulating movement of the whole body while stationary. There was no direct contact between fish during displays. We defined 'bites' as an escalation of aggression that usually followed displays and involved short bursts of attacks while attempting to bite the opponent. The most aggressive behaviour was 'chases' in which one fish would engage in chasing the opponent, and which could also be accompanied by bites. We recorded the incidence of each behaviour for the duration of the interaction until a winner was established, and determined whether the winner was an early- or late-stage pregnant fish. The bout ended when one fish refused to engage and avoided the other fish when approached; we defined the latter fish as the winner. In all behavioural experiments, fish were separated immediately a winner was established and fish were not injured in the experiments except for some damage to fins, which regenerate quickly in this species (Sinclair et al., 2011).

Oxygen consumption

We measured oxygen consumption at 20 and 30°C in each of 10 early-stage and 10 late-stage pregnant females with a fiberoptic oxygen system (Fibox 3, Presens, Regensburg, Germany) using sensor spots (Presens) attached to the insides of respirometers according to the manufacturer's instructions. For resting oxygen consumption measurements, we allowed fish to rest in a cylindrical glass respirometer (245 ml volume) placed in a darkened tank for 45–60 min. We then sealed the respirometer, making sure not to disturb the fish, and let the fish rest for a further 10–15 min before recording the decrease in oxygen over a 7–10 min period or until a steady rate of oxygen decrease was established. We sampled oxygen content of the water every second for the duration of the sampling period and determined oxygen consumption rates from the slopes of the decrease in oxygen content; the Fibox system allows oxygen consumption measurements in real-time from outside the respirometer.

Maximal rates of oxygen consumption were determined in a cylindrical glass respirometer placed on a magnetic stirrer. A magnetic stirrer within the respirometer created water flow that could be adjusted with the control on the magnetic stirrer. Turbulence and eddies within the respirometer were minimised by a central column suspended from the lid. Fish were placed into the respirometer and the speed was ramped up slowly until fish could no longer hold their position in the water column, after which we reduced the speed slightly until fish swam steadily, but occasionally had to struggle to maintain their position in the water column, i.e. fish occasionally went backwards in the water column and had to engage in burst swimming to regain their position, indicating near-

Table 1. Morphometric data for early- and late-stage pregnant females

	Early	Late	t-test
Length (cm)	3.22 \pm 0.085	3.60 \pm 0.13	$t=2.57$, $P=0.017$
Mass before birth (g)	0.66 \pm 0.083	1.31 \pm 0.084	$t=5.26$, $P<0.0001$
Mass after birth (g)		0.88 \pm 0.075	$t=2.02$, $P=0.06$
Neonates (% female mass)		32.72 \pm 3.93	
Shape factor before birth	1.87 \pm 0.048	2.82 \pm 0.19	$t=3.93$, $P<0.001$
Shape factor after birth		1.85 \pm 0.055	$t=-0.21$, $P=0.84$

Results of t-test comparing the two groups are shown.

maximal swimming speeds. Oxygen consumption was measured during 6–8 min of swimming. We calculated exercise-induced metabolic scope (Fry and Hart, 1948) as the difference between resting and swimming oxygen consumption. Oxygen consumption was measured at 20 and 30°C in the same fish in random order and with at least 24 h between measurements. Fish were kept in controlled environment rooms, and water temperature was changed by changing the settings of the rooms to the desired experimental temperature which, given the lag times, meant that it took several hours for water temperature to change.

To test the hypothesis that oxygen consumption during aggressive interactions differs between the two groups of females, we performed staged dyadic encounters between two early-stage pregnant females (i.e. early *versus* early; $N=8$ pairs) and between two late-stage pregnant females (i.e. late *versus* late; $N=8$ pairs) while measuring oxygen consumption at the same time. We used pairs of fish from the same experimental groups for this purpose to distinguish oxygen consumption between early- and late-stage pregnant females. As above, individuals making up an experimental pair were matched for length. To be able to detect the effect of particular behaviours on oxygen consumption, the behaviours had to be displayed for at least 1–2 min. Hence, we defined periods of behaviour as displays, which were often performed continuously for several minutes, chases, which involved several short and non-continuous chases and bites (as defined above) over the recording period, and finally fights, which were defined as continuous and prolonged chases and bites in which both animals engaged. Similar to above, fish were introduced simultaneously into the experimental tank, and behaviour was recorded continuously until a winner was established. Unlike above, the experimental tank was a respirometer (415 ml volume).

Rates of oxygen consumption (in $\mu\text{mol g}^{-1} \text{min}^{-1}$) were determined as the slope of the decrease in oxygen content divided by the fish body mass (single fish for metabolic scope measurements, and the combined mass of the pair for oxygen consumption during interactions) and multiplied by the volume of the container (Sinclair et al., 2006).

Statistical analysis

We compared length, mass and shape factors between early- and late-stage pregnant females using *t*-tests. Outcomes of aggressive encounters between early- and late-stage pregnant females were compared by chi-square test with 1 degree of freedom, where the expected value (9) was determined by the null-hypothesis that the two categories of fish would win a similar number of the 18 dyadic combats.

To determine whether the different behavioural categories (displays, bites, chases) were displayed with equal frequency by early- and late-stage pregnant females during dyadic combat bouts, we analysed the number of times each behaviour was displayed during an interaction by PERMANOVA (ePrimer, www.eprimers.org), with stage of pregnancy (early and late) as a fixed factor, and behaviour (display, bite, chase) as a random factor. We followed PERMANOVA with analyses of simple main effects comparing the levels of pregnancy (early/late) within each level of behaviour separately (by *t*-test) (Quinn and Keough, 2005). The lengths of behavioural interactions until a clear winner was determined between the different categories of fish (early *versus* late, early *versus* early, late *versus* late) were compared by one-way ANOVA.

To determine possible effects of body mass, we regressed metabolic rates over body mass; resting oxygen consumption was independent of body mass, except for early-stage pregnant females

at 20°C. However, maximum exercise-induced oxygen consumption decreased with increasing body mass for both groups. As a result, exercise-induced metabolic scope also decreased with increasing body mass (these data are summarised in supplementary material Table S1). Hence, we used mass-corrected residuals in the analyses comparing metabolic rates between early- and late-stage pregnant females; however, in the figures we show actual oxygen consumption data.

We analysed resting and maximal oxygen consumption, and metabolic scope separately by ANOVA with repeated measures, with early/late pregnancy as an independent factor and test temperature (20 and 30°C) as a repeated measure. We used Pillai's trace as the test statistic for the repeated measure. To test the hypothesis that resting and maximal rates of oxygen consumption co-varied, we performed Pearson's correlation analyses of all fish at 20 and 30°C.

We compared oxygen consumption for different categories of aggressive interactions, expressed either as metabolic rate ($\mu\text{mol O}_2 \text{ consumed g}^{-1} \text{min}^{-1}$) or as a percentage of exercised-induced metabolic scope for each group (early- and late-stage pregnant) and adjusted for body mass using equations in supplementary material Table S1, by ANOVA with stage of pregnancy as fixed factor and behaviour category as repeated measure.

We used Levene's test to check homogeneity of variances and log-transformed data in case of heterogeneity (residuals of resting and maximal rates of oxygen consumption). The truncated product method (Zaykin et al., 2002) was used to combine all the *P*-values in this study to determine whether there is a bias from multiple hypothesis testing. The truncated product method *P*-value was <0.0001 , showing that the results are not biased.

RESULTS

Late-stage pregnant females are more aggressive

In the dyadic combats between early- and late-stage pregnant females, late-stage pregnant females won significantly more fights (77.8% of all encounters) than early-stage pregnant females ($\chi^2=5.56$, $P<0.02$). During staged encounters, the different types of behaviours were displayed at different frequencies (pseudo $F_{2,102}=8.16$, $P<0.001$; Fig. 1), and 'displays' were performed more often than 'bites' or 'chases'. Stage of pregnancy had a significant effect on behaviour (pseudo $F_{1,102}=19.09$, $P<0.04$); early- and late-stage pregnant females did not differ in the frequency of displays ($t=-0.75$,

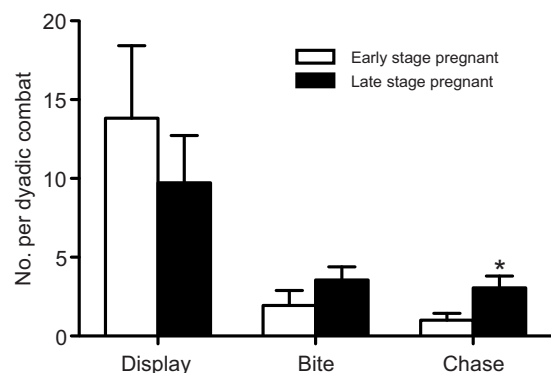


Fig. 1. Mean frequency of different behaviours displayed by early- and late-stage pregnant females per dyadic combat bout. The behaviours represent an escalation of aggression from displays to bites and chases, and late-stage pregnant females displayed significantly more chases than early-stage pregnant females (indicated by the asterisk).

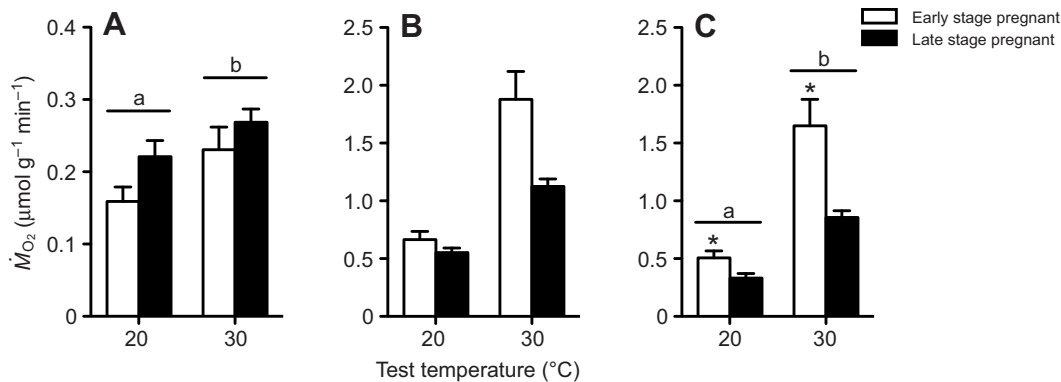


Fig. 2. (A) Resting and (B) maximum exercise-induced oxygen consumption rates (\dot{M}_{O_2}) and (C) aerobic scope (maximum–resting) of females in early and late pregnancy. Different lowercase letters indicate significant differences between test temperatures, and asterisks indicate significant differences between stages of pregnancy. There was an interaction between test temperature and stage of pregnancy for maximum rates of oxygen consumption (B, $P < 0.01$). Note that all statistical results are for mass-corrected data, but raw data are shown in the figure.

$P = 0.46$) and bites ($t = 1.27$, $P = 0.21$), but late-stage pregnancy females exhibited a greater number of chases ($t = 2.35$, $P < 0.03$; Fig. 1).

Late-stage pregnant females have lower metabolic scope

Resting oxygen consumption rate increased with increasing test temperature ($F_{1,18} = 6.63$, $P < 0.02$), but stage of pregnancy did not have a significant effect on resting metabolic rates ($F_{1,18} = 3.74$, $P = 0.069$; Fig. 2A). Stage of pregnancy and test temperature interacted to determine maximal rates of oxygen consumption ($F_{1,18} = 38.91$, $P < 0.0001$), which were considerably higher in early-stage pregnant females at 30°C than in late-stage pregnant females (Fig. 2B). As a consequence, aerobic scope was lower in late-stage pregnant females and exhibited a smaller increase with temperature (interaction $F_{1,18} = 110.53$, $P < 0.0001$; Fig. 2C). Resting and maximum rates of oxygen consumption were not correlated at 20°C ($r = 0.24$, $P = 0.30$) or at 30°C ($r = 0.16$, $P = 0.51$; Fig. 3).

Late-stage pregnant females invest more of their metabolic scope in fighting

Escalating aggressive interactions from displays to chases and ultimately to fighting significantly increased rates of oxygen consumption ($F_{2,8} = 21.13$, $P < 0.002$), but there were no differences between early- and late-stage pregnant females ($F_{1,9} = 0.157$, $P = 0.70$; Fig. 4A). However, when expressed as a percentage of exercise-induced metabolic scope, late-stage pregnant females had significantly greater metabolic costs for aggressive interactions

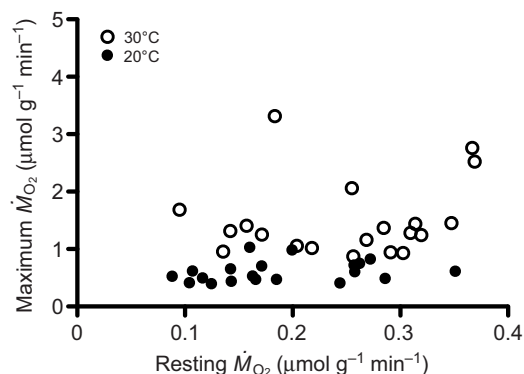


Fig. 3. There were no correlations between resting and maximum rates of oxygen consumption at 20 or 30°C.

($F_{1,9} = 9.00$, $P < 0.02$; Fig. 4B). The time taken until a clear winner was established did not differ between the different categories of pairs ($F_{2,30} = 0.011$, $P = 0.99$; late versus late 432.1 ± 56.5 s, early versus early 453.6 ± 45.5 s, early versus late 448.1 ± 91.8 s).

DISCUSSION

Contrary to our predictions, females in late pregnancy are more aggressive than those in early pregnancy and are more successful at winning fights. Nonetheless, late-stage pregnant females have lower metabolic scopes than early-stage pregnant females, as we predicted. The associated trade-off is that late-stage pregnant females also invested more of their available metabolic scope in aggressive interactions.

Resting metabolic rates did not differ between early- and late-stage pregnant females, which indicates that there is no association between behavioural phenotype and resting metabolic rates (cf. Careau et al., 2008; Biro and Stamps, 2010). We argue that resting metabolic rate should be a poor predictor of behaviour, because it represents a cost of cellular maintenance, particularly in ectotherms where there is no metabolic heat production. Resting and maximal metabolic rates are not regulated by the same mechanisms; the former is driven mainly by protein synthesis rates and membrane-bound ATPases (Hulbert and Else, 2000), while the latter is determined by mitochondrial maximal capacities that are controlled by transcriptional regulators, and energy-sensing mechanisms (Lin et al., 2005; Hardie, 2008; Walter and Seebacher, 2009).

Any behaviour or activity will use ATP at a rate that lies somewhere between resting and maximal rates. During recovery from strenuous activity such as fighting, animals will have limited capacity for activity while ATP levels are low and acid–base balance and substrate concentrations are restored to resting levels (Wang et al., 1994). However, the greater the metabolic scope, the faster the recovery from exercise (Brill, 1996) and the less likely it will be that behaviour is constrained by maximal metabolic capacities (Eliason et al., 2011; Killen et al., 2012). Hence, metabolic scope is the more appropriate reference point for the energetic cost of behaviour, and maybe for personality because it can constrain behaviour and activity (Briffa and Elwood, 2004; Briffa and Sneddon, 2007). During the recovery period after fighting, females will therefore be particularly constrained in responding to situations that require expenditure of ATP, such as locomotor activity necessary to escape predators and foraging (Sneddon et al., 1999), and this constraint would be increased during late pregnancy when the metabolic investment in fighting is greater.

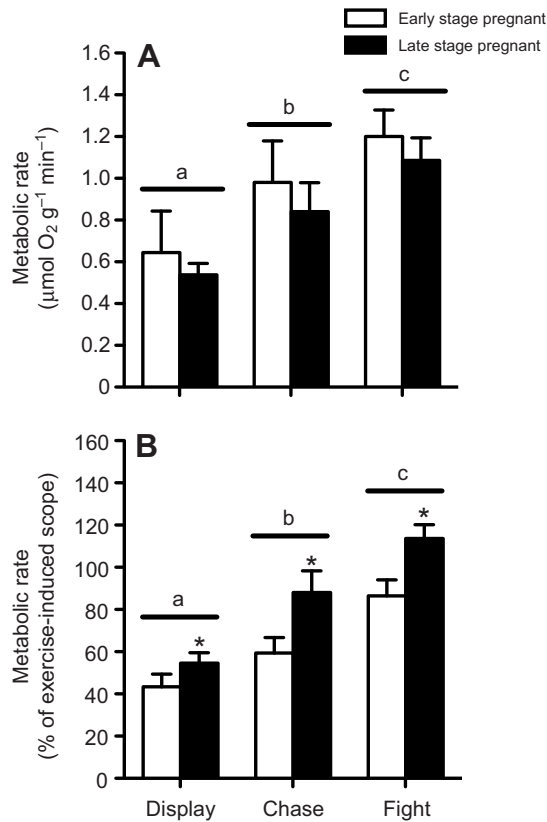


Fig. 4. Metabolic cost of behaviour. Rates of oxygen consumption increased significantly as fish escalated aggressive behaviour from displays to chases and fighting (A), but there were no differences between early- and late-stage pregnant females. However, late-stage pregnant females invested a significantly larger percentage of exercise-induced metabolic scope in aggressive interactions (B). Horizontal bars with different lowercase letters indicate significant differences between behaviours, and asterisks indicate significant differences between groups. Note that all statistical results are for mass-corrected data, but raw data are shown in the figure.

The ‘asset-protection principle’ predicts that the larger the current reproductive asset, the more important it becomes to protect it (Clark, 1994). This theory was formulated in the context of predator–prey interactions and it predicts that females in late pregnancy (i.e. with a greater asset) are less willing to take risks in the presence of predators. The trade-off of this strategy is that late-stage pregnant females will also obtain less food and risk starvation (Clark, 1994). Similar predictions can be made for aggressive interactions. Hence, females in late pregnancy should be less willing to engage in aggressive interactions to minimise their metabolic investment. However, we found the opposite response.

It is possible that even though the probability of losing fitness increases with a more aggressive strategy because of the greater metabolic investment in fighting during late pregnancy, the advantages gained by being dominant over other females outweighs this cost. Increased aggression as pregnancy progresses may be advantageous for mosquitofish because dominance increases access to resources and thereby increases fitness in poeciliid fish where lack of food can lead to fewer offspring, or to offspring of poorer quality (Reznick et al., 1996). The increased aggression in late-stage pregnant females could be associated with increased motivation, driven by greater need, to access resources. Additionally, in zebrafish, dominant females can suppress reproduction in

subordinates (Gerlach, 2006) so that increased aggression during pregnancy may improve offspring fitness by reducing density-dependent competition.

Changes in aggressive behaviour could represent a correlated response to hormonal changes that occur during pregnancy (White et al., 2002). In rats, increases in estradiol and testosterone during late pregnancy increase maternal aggression (Albert et al., 1992). Brain oxytocin levels during late pregnancy increase maternal aggression, particularly in response to stressful stimuli (Neumann, 2008). The neural processes that dictate social behaviour are highly conserved among vertebrates, and all classes, including fish, possess homologous forebrain and midbrain areas and hormone receptors (Goodson, 2005). Hence, it is not unlikely that similar hormonal changes to those in mammals will also determine aggressive behaviour in late-stage pregnant fish. Both sexes of *G. holbrooki* are highly aggressive (Chen et al., 2011; Pyke, 2008), so intraspecific interactions could be a stressor during late pregnancy, stimulating an oxytocin-mediated increase in aggression.

Consistent behavioural differences between individuals are defined as personality if they occur across different contexts (Briffa and Weiss, 2010). Our data show that during pregnancy, aggressive behaviour changes relative to other individuals, which may indicate that personality may not be fixed within individuals, although this needs to be verified for different contexts in addition to aggression. Whether behaviour is stable within individuals over time is an important question because it can explain fitness differentials within populations (West and Graziano, 1989; Briffa and Weiss, 2010; Webster and Ward, 2011); stability can optimise behavioural responses in constant environments, or result in behavioural mismatches in novel situations and changing environments.

In our mosquitofish, maximum rates of oxygen consumption increased faster than resting rates between 20 and 30°C, so that metabolic scope was greater at the higher temperature. This finding emphasises the importance of interpreting activity and behaviour relative to metabolic scope. Acclimation to different temperatures may change the thermal sensitivity of metabolic scope, although the direction of the response should remain the same unless there is a decrease in metabolism as a result of damage by high temperatures. There will be a temperature beyond which metabolic scope will decrease as a result of cardiovascular constraints (Steinhausen et al., 2008) and damage to proteins and membranes. Hence, both increasing and decreasing environmental temperatures can decrease metabolic scope and thereby affect behaviour. Increasing environmental temperatures have been implicated in modulating metabolic scope at high temperatures, and thereby affect migration success in salmon (Eliason et al., 2011; Clark et al., 2011) and community structure in reef fish (Nilsson et al., 2009). We have shown that behavioural interactions are likely to be affected if metabolic scope is reduced, particularly in late-stage pregnant females that utilise a larger proportion of their metabolic scope for behavioural interactions.

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