

RESEARCH ARTICLE

Per capita sperm metabolism is density dependent

Ashley E. Potter*, Craig R. White and Dustin J. Marshall

ABSTRACT

From bacteria to metazoans, higher density populations have lower per capita metabolic rates than lower density populations. The negative covariance between population density and metabolic rate is thought to represent a form of adaptive metabolic plasticity. A relationship between density and metabolism was actually first noted 100 years ago, and was focused on spermatozoa; even then, it was postulated that adaptive plasticity drove this pattern. Since then, contemporary studies of sperm metabolism specifically assume that sperm concentration has no effect on metabolism and that sperm metabolic rates show no adaptive plasticity. We did a systematic review to estimate the relationship between sperm aerobic metabolism and sperm concentration, for 198 estimates spanning 49 species, from protostomes to humans from 88 studies. We found strong evidence that per capita metabolic rates are concentration dependent: both within and among species, sperm have lower metabolisms in dense ejaculates, but increase their metabolism when diluted. On average, a 10-fold decrease in sperm concentration increased per capita metabolic rate by 35%. Metabolic plasticity in sperm appears to be an adaptive response, whereby sperm maximize their chances of encountering eggs.

KEY WORDS: Respiratory dilution effect, Sperm respiration, Sperm oxygen consumption, Metabolic plasticity, Metabolic ecology of gametes

INTRODUCTION

Metabolism is a key driver of ecology and life history as it determines the rate at which organisms use energy and perform biological work (White et al., 2022). Metabolic rate is a highly labile trait – it varies with size, temperature and activity (Gillooly et al., 2001; Glazier, 2008). Although these classic drivers of metabolism are well recognized, it has become apparent that population density also alters metabolic rates. From bacteria to metazoans, organisms at higher densities tend to have lower metabolic rates than conspecifics at lower densities (DeLong et al., 2014; Ghedini et al., 2017; Lovass et al., 2020; Malerba et al., 2017; Marshall et al., 2022). The ultimate driver for this negative association between density and metabolism is unclear, but increased competition for resources is the most commonly proposed explanation (Amundsen et al., 2007; Auer et al., 2015; DeLong et al., 2014). Competition for resources is greater at higher population densities; individuals presumably


downregulate their metabolic rates to reduce energy expenditure in the high competition environment (DeLong and Hanson, 2009; DeLong et al., 2014; Malerba et al., 2017). Although these density-metabolism effects are ubiquitous across the tree of life, one group (or gamete type, rather) that has received relatively little attention, despite an august history of the topic, is sperm. Here, we asked: do sperm exhibit density-dependent metabolism, and if so, is the relationship the same as those in metazoans?

Studies of density-dependent regulation of metabolism in multicellular and unicellular organisms have proliferated over recent years (DeLong and Hanson, 2009; DeLong et al., 2014; Ghedini et al., 2017; Lovass et al., 2020; Malerba et al., 2017), but the idea was actually first proposed almost a century ago, and specifically in relation to sperm. In a series of papers starting in the 1920s, Gray (1928a,b,c) showed that sea urchin sperm at high concentrations had lower per capita (individual sperm) metabolic rates and suffered less senescence than sperm in lower concentrations. Gray (1928a,b,c) speculated that the total energy expenditure of the sperm during its life was largely dependent upon dilution (Gray, 1928a). Subsequent studies on the same species confirmed these observations, and the phenomenon was termed the respiratory dilution effect (RDE) (Rothschild, 1948, 1950). These authors alluded to the RDE being adaptive – that sperm are able to minimize their metabolic demands (and prolong their lifespan) when suspended in high concentrations such as those typical of the newly released ejaculate, but increase their activity and metabolism when they become diluted and free swimming (Gray, 1928a). In more modern terminology, the RDE was, in essence, suggested to be a form of adaptive metabolic plasticity: sperm decrease senescence by conserving their finite energy reserves at high densities when the chances of encountering eggs are low, but maximize their competitiveness and fertilization success by increasing their energy consumption at lower densities when the chances of encountering eggs are high.

The RDE emphasizes that sperm metabolism is quite plastic within species; in contrast, sperm competition theory has tended to focus on among-species differences in sperm metabolism and has largely overlooked the potential for intraspecific variation (Boell and Burkus, 1984; Dreanno et al., 1999; Mansour et al., 2003; Rahi et al., 2020). But, as some authors have noted, sperm competition, sperm density and sperm metabolism should be intimately linked (Chia and Bickell, 1983; Reinhardt, 2007; Reinhardt and Otti, 2012). For example, internal fertilizers store sperm from multiple males, maintaining higher sperm concentrations until sperm reach the ova, perhaps maximizing sperm longevity (Reinhardt, 2007; Simmons, 2002). If sperm metabolism is density dependent, then increases in sperm density via the addition of multiple ejaculates could reduce sperm metabolism, a finding that would counter the assumptions of traditional sperm competition theory (Parker, 1993). More generally, the interplay between rates of sperm energy expenditure and their local density remains too poorly understood, but has interesting implications for whether sperm have evolved to

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outswim or outlive each other (theory makes assumptions about both; Ball and Parker, 1996; Parker, 1993).

Additionally, a range of studies suggest that dilution in female reproductive fluid (i.e. egg secretions or ovarian fluid) can enhance whole-ejaculate sperm metabolism (Gray, 1928b; Hathaway, 1963; Ohtake, 1976), motility (Cornman, 1941; Gasparini et al., 2020; Hadlow et al., 2023; Lillie, 1913; Poli et al., 2019) and longevity (Gasparini et al., 2020; Hadlow et al., 2023; Poli et al., 2019). Whether this reproductive fluid effect is (1) driven by an enhanced resource uptake following dilution, (2) from an adaptive response to egg cues (i.e. chemotaxis) indicating a higher likelihood of encountering eggs or (3) both is unclear.

To date, many modern empirical studies in sperm biology explicitly assume that sperm concentration has no effect on per capita sperm metabolic rate (Redenz, 1933) (see Z_{O_2} column in Table S1) (but see references in Chia and Bickell, 1983; Reinhardt, 2007; Reinhardt and Otti, 2012), directly contradicting the RDE hypothesis. So, we have contrasting expectations with regards to whether sperm metabolism depends on density, and if it does, the reasons for this density dependence are also unclear. In Fig. 1, we show alternative hypotheses and how we might distinguish between them. If dilution with carbohydrate-containing media increases per capita metabolic rate but dilution with carbohydrate-free media does not, then we can infer that resource limitation is driving density-dependent sperm metabolism. Otherwise, if increases occur regardless of diluent type, then plasticity in response to local biochemical conditions mediated by sperm density (e.g. carbon dioxide, bicarbonate, pH; Grahn et al., 2023), cell–cell interactions (e.g. quorum sensing; Luther and Waberski, 2019) or sperm conjugation (Higginson and Pitnick, 2011; Monclus and Fornes, 2016) could drive the effect.

To resolve the uncertainty surrounding the relationship between sperm metabolism and density, we conducted a comparative analysis. We estimated how sperm metabolism covaries with density among and within species and gained insight into the drivers of these patterns. Our analysis included 198 measures of sperm metabolism from 49 species across five phyla, encompassing a wide range of sperm concentrations (~6 orders of magnitude). We

categorized species into endotherms and ectotherms to account for the effect of thermoregulation on sperm metabolism because thermoregulation is a strong predictor of metabolic rate more generally (Glazier, 2010). We also categorized diluents as either carbohydrate-free or carbohydrate-containing media to explore the effect of resources on density-dependent metabolism. We estimated the covariance between aerobic metabolism and sperm density (including either thermoregulation or diluent type) using a linear-mixed model framework. Our estimate of metabolism focused solely on aerobic metabolism, as it was the most commonly reported metric, but we acknowledge that aerobic and anaerobic metabolism are highly interdependent metabolic processes (Ruiz-Pesini et al., 2007). Lastly, we assessed the degree of misestimation when it was assumed that sperm metabolism was independent of density.

MATERIALS AND METHODS

Comparative analysis of sperm metabolism

Protocol, registration and reporting

We followed the PRISMA (Preferred Reporting Items for Systematic reviews & Meta-Analyses; Moher et al., 2016; O’Dea et al., 2021) approach to help build our database (PRISMA Checklist; Table S2), but we used more traditional comparative analyses to test our hypothesis – a hybrid between a meta-analysis and a comparative analysis. This hybrid approach allows us to use the formality of meta-analytic search techniques but also use standard metabolic scaling approach for the analysis (see Supplementary Materials and Methods, Data extraction and effect size). This is a standard technique that has been used previously (Barneche et al., 2018; Marshall et al., 2020; Pettersen et al., 2019) but it means that we cannot include all of the steps of a formal meta-analysis.

Literature searches and study selection

We aimed to collect a comprehensive dataset using published and unpublished data to understand the relationship between sperm metabolism and sperm density across species. Studies were collected from ISI Web of Science (<http://www.webofknowledge.com/WOS>) and Google Scholar (<https://scholar.google.com/>) from 2019 to 2023. We used specific search strings based on the database (see Supplementary Materials and Methods). Studies cited within searched studies were also included in our dataset if they met the criteria. Our search strategy involved: (1) inputting the search terms into our information sources; (2) assessing titles for relevance; (3) if relevant, downloading the title, author and abstract and uploading to Rayyan QCRI (Ouzzani et al., 2016); (4) reading the abstract for relevance [(a) in title: includes sperm OR spermatozoa OR gametes AND some form of activity, energy use OR sperm quality and (b) in abstract: includes sperm OR spermatozoa OR gametes AND metabolism, respiration, oxygen consumption rate, mitochondrial respiration, energy use, sperm quality OR sperm activity]; (5) reviewing the entire paper for relevance based on criteria (Table S3); and (6) extracting the data. If the studies did not meet the criteria presented in Table S3, they were excluded from the dataset. For further information on eligibility criteria, see Supplementary Materials and Methods (Eligibility criteria) and Fig. S1. Although we attempted to collect all relevant data on sperm metabolism, we recognize that some important metabolic parameters [i.e. FAD, NAD(P)H, ATP production] and studies (e.g. Massino et al., 2021) could not be included. We used oxygen consumption rate as our measure of metabolism because it is the most widely used metabolic parameter for measuring metabolism and allowed us to include the greatest number of species and studies, increasing the reliability and generality of our findings. Overall, our dataset included 198

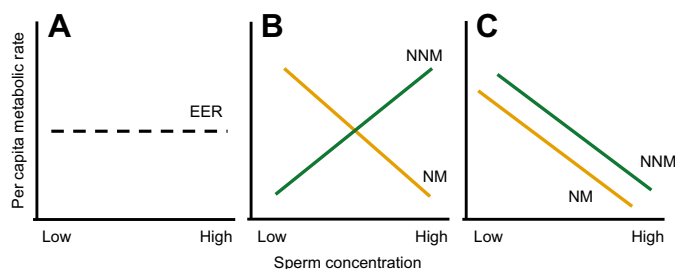


Fig. 1. Schematic showing the potential drivers of metabolic rate in spermatozoa. (A) No metabolic plasticity. If sperm rely solely on their endogenous energy reserves (EER) and show no plasticity, sperm will have the same per capita metabolism regardless of sperm concentration (black dashed line). (B) Exogenous resources mediate sperm metabolism. If the ejaculate is diluted in non-nutritive media (NNM) (e.g. seawater, carbohydrate-free Ringer’s), sperm in dense suspensions will have higher per capita metabolism because they have access to more resources (green line), whereas when the ejaculate is diluted in nutritive media (NM) (e.g. carbohydrate-containing Ringer’s, seminal fluid), sperm in diluted suspensions will have higher metabolism because they access more resources (yellow line). (C) Adaptive plasticity. If the density–metabolism relationship is driven by adaptive plasticity via cell–cell interaction (e.g. sperm conjugation), sperm in higher concentrations will have lower metabolism regardless of the type of diluent used.

observations of aerobic metabolism from 88 studies conducted from 1950 to 2022, including 49 species (21 endotherms and 28 ectotherms).

Data extraction and effect size

Our data was primarily collected from original sources because there were few compilations (Jones and Murdoch, 1996) that provided information on metabolism in sperm for a range of species that were also explicit with regards to concentration. We used raw or averaged data from original sources which were extracted from tables or figures using Webplotdigitizer (v. 4.5; Rohatgi, 2020). Papers cited in these original sources were also investigated and included in our database if they followed the criteria presented in Table S3. We also included data for two species collected by the authors (see Supplemental Materials and Methods, Empirical estimates). Species were categorized by thermoregulation (endotherm and ectotherm) which largely covaries with fertilization mode in our dataset [i.e. internal=endotherms, external=ectotherms (except for *Apis mellifera* and *Loligo pealei*, both ectotherms with internal fertilization)] (Fig. S2). We also recorded information on the diluent used to dilute the sperm. Diluents were categorized as either carbohydrate-free (i.e. containing no sugar) or carbohydrate-containing (i.e. containing sugar) media. Last, we reported sperm handling (i.e. fresh, cooled or frozen–thawed), extraction method [i.e. ejaculated, extracted (male) or extracted (female)] and diluent type (i.e. activation or extender) for each species/reference in the dataset (Table S1).

We did not scale our data by their error to calculate effect size because doing so would remove the quantitative relationship that we are interested in (see Supplemental Materials and Methods, Data extraction and effect size). However, we did quantify the effect of concentration on per capita sperm metabolism by limiting our dataset to four studies that reported ‘high’ and ‘low’ sperm concentrations (~10-fold difference) and calculated a ln-transformed response ratio (LRR) (Borenstein et al., 2009). The LRR was calculated as: $LRR = \ln(X_L/X_H)$, where X_L is the mean per capita sperm metabolism at lower concentrations and X_H is the mean per capita sperm metabolism at higher concentrations. We ran a model with log response ratio as our response variable. Our results show a significant positive effect of concentration on per capita metabolic rate ($F_{1,4}=15.6$, $P=0.017$): per capita sperm metabolism was 1.2-fold higher at lower sperm concentrations. We also ran a model where we excluded studies with lower replication (<4 replicates) and found no qualitative differences in our results to those that included the entire dataset (see Results).

Empirical estimates

To expand the number of species and data points in our study, we included data that we had collected ourselves. We estimated density-dependent metabolism in two broadcast spawning invertebrates, *Galeolaria caespitosa* (a calcareous tube worm) and *Heliocidaris erythrogramma* (a sea urchin). Colonies of *G. caespitosa* were collected from the St Kilda pier (Melbourne, Australia; 37°51′ 53.964″S, 144°58′8.256″E), and placed in coolers filled with seawater and transported to Monash University. Spawning was induced using a standard method specifically for *G. caespitosa* (Marshall and Evans, 2005). The ejaculate was collected immediately after spawning using a 1.5 ml syringe, pooled together and diluted to several concentrations (10^6 – 10^8 sperm ml⁻¹) using 0.2 µm filtered seawater. Sperm density was estimated for each sperm concentration using a haemocytometer (4 replicates per dilution).

Heliocidaris erythrogramma was collected near Ricketts Point (Melbourne, Australia: 37°59′26.016″S, 145°1′39.468″E) and transported in coolers back to Monash University. Spawning was induced by injecting 5 ml of 0.5 mol l⁻¹ KCl into the peristomal membrane and then placing each sea urchin over a beaker to collect the ejaculate. A single male ejaculate was diluted to several concentrations (10^5 – 10^7 sperm ml⁻¹) using filtered seawater and sperm density was estimated as mentioned above.

Sperm metabolic rate was measured as oxygen consumption over time in an air-tight chamber. We used several 24-channel PreSens sensor dish readers (Sensor Dish Reader SDR2, PreSens, Germany) with 24-chamber 750 µl glass microplates to measure oxygen consumption over time (% O₂ h⁻¹) (White et al., 2011), where measurements were taken every minute for 30–60 min, depending on the concentration. The 750 µl glass chambers contained non-consumptive O₂ sensor spots that were calibrated with air-saturated seawater (100% AS) and water containing 2% sodium sulfide (0% AS) prior to measurements. Sperm concentration was randomized across the 24-chamber microplates and replicated 4–6 times, while the remaining four chambers were kept free of sperm and contained only filtered seawater as a control. Oxygen measurements were recorded in the dark at a constant temperature of 21°C (ambient ocean temperature), with the plates flipped on their sides to prevent the sperm from settling directly onto the sensor spot, located at the bottom of each vial. Oxygen consumption (% O₂ h⁻¹) was converted to metabolic rate (\dot{V}_{O_2} , µl O₂ h⁻¹; LoLinR package; Olito et al., 2017) using the rate of change of oxygen saturation for controls vials, oxygen capacitance of air-saturated seawater at 21°C (5.11 ml l⁻¹) (Cameron, 1986) and volume of water in the chamber (750 µl) (White et al., 2011). The rate of change was taken within 100–75% oxygen saturation for *G. caespitosa* for all concentrations (10^6 – 10^8 sperm ml⁻¹). However, for *H. erythrogramma*, oxygen declined rapidly with higher concentration such that oxygen saturation started at ~60% for higher concentrations (10^7 sperm ml⁻¹) but started at 100% for lower concentrations (10^5 – 10^6 sperm ml⁻¹), so we only used 10^5 – 10^6 sperm ml⁻¹ in the analyses to ensure our estimates of metabolism were compared across similar oxygen saturation ranges.

Misestimation

Papers typically reported metabolism as a standardized rate (i.e. $Z_{O_2} = \mu\text{l O}_2 10^8 \text{ sperm ml}^{-1} \text{ h}^{-1}$) meaning that they transformed the metabolic rate to what it would be based on a standardized sperm concentration (i.e. 10^8 or 10^9 sperm ml⁻¹) (Redenz, 1933) instead of reporting the rate that the actual sperm concentration respiration was measured at (i.e. 3.2×10^8 sperm ml⁻¹). To report sperm metabolism at a standardized concentration, most studies [67% or $n=62$ (species=38)] used a linear transformation to extrapolate metabolic rates from the concentration under which it was measured to the standardized value: this approach explicitly assumes that sperm metabolic rate is unaffected by sperm density. Hence, using a linear transformation introduces systematic biases if the relationship between sperm concentration and sperm metabolism is actually nonlinear (which it is; see Results). For example, when a study reported a standardised metabolic rate of $1.34 \mu\text{l O}_2 10^8 \text{ sperm ml}^{-1} \text{ h}^{-1}$ but the methods reported that the actual sperm concentration used during the metabolism measurement was 3×10^8 sperm ml⁻¹, we were able to back-calculate the estimate to get to the actual metabolic rate that was measured. To back-calculate, we first divided the reported metabolic rate provided ($1.34 \mu\text{l O}_2 \text{ h}^{-1}$) by the standardised sperm concentration (10^8 sperm ml⁻¹), then multiplied that value by

the actual sperm density reported in the methods of the paper ($1.34 \times 10^{-8} \mu\text{l O}_2 \text{ h}^{-1} \times 3 \times 10^8 \text{ sperm ml}^{-1} = 4.02 \mu\text{l O}_2 \text{ h}^{-1}$ for $3 \times 10^8 \text{ sperm ml}^{-1}$). This approach represents the same linear transformation as was used by the original author (Redenz, 1933). Data from other papers [28% or $n=31$ (species=33)] did not need to be transformed because metabolic rate was reported at the actual sperm density that was used. A subset of papers [$n=20$ (species=16)] could not be included in the analyses because they did not report the actual sperm density and only reported a standardized metabolic rate.

We wanted to understand the extent to which papers overestimated or underestimated sperm metabolism, so we calculated the percent misestimation for papers that reported standardized sperm metabolism, using the following equation:

$$R = aC^\beta, \quad (1)$$

where R is the metabolic rate, a is the coefficient, C is the sperm concentration and β is the density–metabolism exponent for sperm. First, we rearranged Eqn 1 to solve for a :

$$a = R/C^\beta, \quad (2)$$

where we used the actual sperm concentration used in the experiment (C), actual metabolic rate (R) and the density–metabolism exponent found for sperm across species ($\beta=0.87$; see Results). Then we used a from Eqn 2 and plugged it into Eqn 1 with the standardized sperm concentration as C , to find the ‘actual’ metabolic rate, i.e. metabolic rate that was supposed to be measured if the standardized concentration was actually used. Next, we used the ‘actual’ metabolic rate to find the percent misestimation using this equation:

$$\text{Percent misestimation} = \frac{\text{Standardized metabolic rate}}{\text{‘Actual’ metabolic rate}} \times 100. \quad (3)$$

Finally, we calculated the fold change (i.e. increase or decrease) in sperm concentration using the actual sperm concentration used in the experiment and the standardized reported sperm concentration:

$$\text{Fold change in concentration} = \frac{\text{Actual concentration}}{\text{Standardized concentration}}. \quad (4)$$

Statistical analyses

All analyses were done in R (v. 4.2.2, <https://www.r-project.org/>), and the assumptions of a linear model were checked using Q-Q plots, histograms and boxplots of residuals. We constructed a tree from the Open Tree of Life (OTL) using the `rotl` R package to account for phylogenetic non-independence (Michonneau et al., 2016). We first explored our data using a series of phylogenetically controlled linear mixed-effects (random intercept) models with species as a random effect and sperm concentration and thermoregulation (two levels: endotherm, ectotherm) or diluent (two levels: carbohydrate-containing, carbohydrate-rich) as fixed effects using the `phyr::pglmm` R package (Ives, 2018). Thermoregulation was included as a factor because it is a strong predictor of metabolic rate (Glazier, 2010). When we compared models with and without phylogeny, we found no difference in model fit ($\chi^2=0.15$, $P=0.70$, $\Delta\text{AIC}<2$) and the results were quantitatively and qualitatively similar. Therefore, we decided to use a linear mixed-effects model (using the `lmer` R package) with species as a random effect. We used a multistep approach for our statistical analyses where we evaluated the overall significance of full model effects using analysis of deviance tests based on χ^2 distributions and Akaike’s information criterion (AIC; Akaike, 1973) and then reduced models in which interactions were not significant ($P>0.05$). We then used a Wald test to determine whether the coefficient was significantly different from 1. We combined both empirical observations and data compiled from external sources for this analysis.

RESULTS

Comparative analysis of sperm metabolism

We found that the relationship between sperm concentration and sperm metabolism is nonlinear – sperm have lower per capita (i.e. individual) metabolisms at higher densities relative to sperm at lower densities (Fig. 2). In more formal statistical terms, the scaling exponent for per capita metabolism and concentration is -0.13 (Fig. 2A), meaning that the scaling exponent for ejaculate-level (i.e. sample of multiple sperm) metabolism and concentration is 0.87 (Table 1), which was significantly different from 1 (Wald test, $t_{198}=3.25$, $P=0.001$; Fig. 2B). Additionally, there was no evidence that the relationship between sperm concentration and metabolism

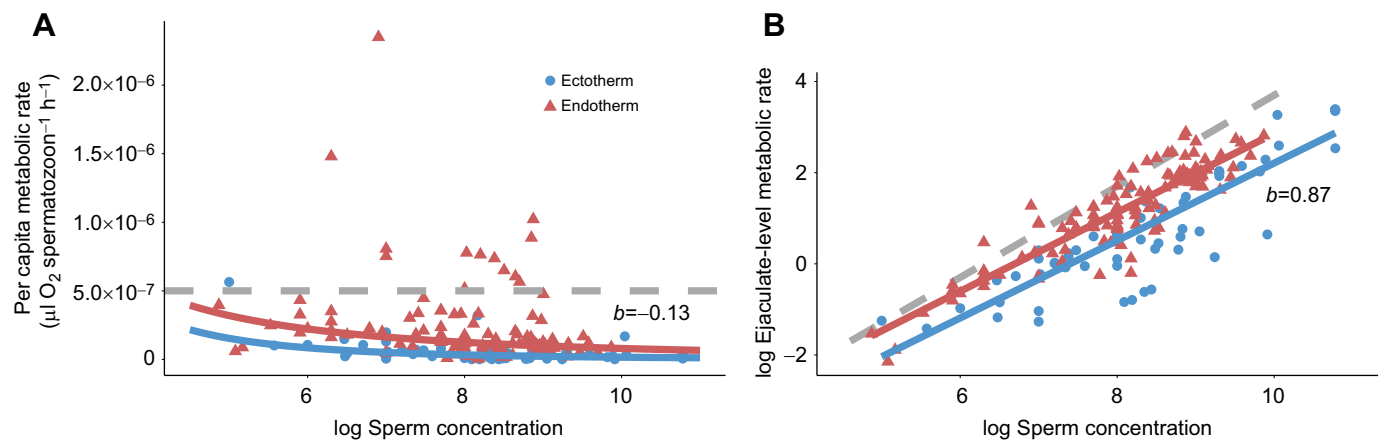


Fig. 2. Among-species relationships between density-dependent per capita and ejaculate-level metabolic rates. Fitted lines represent line of best fit based on a linear mixed-effects model using all the data in the dataset. (A) Relationship between per capita metabolic rate ($\mu\text{l O}_2 \text{ spermatozoon}^{-1} \text{ h}^{-1}$) and \log_{10} sperm concentration (sperm ml^{-1}) ($b=-0.13$). The y-axis is on a non-log scale for ease of view. The two outliers did not change the density–metabolism relationship, so they were left in. (B) Relationship between \log_{10} ejaculate-level metabolic rate ($\mu\text{l O}_2 \text{ h}^{-1}$) and \log_{10} sperm concentration ($b=0.87$). Lines within plots do not significantly differ from each other. Each point represents an observation from a single species for 49 species total (endotherms: red triangles, $n=123$; ectotherms: blue circles, $n=75$). Grey dotted lines represent a linear relationship (or no density-dependence) between sperm metabolism and concentration. Data were analysed in a natural log framework but are displayed on a \log_{10} scale for ease of view.

Table 1. Linear mixed-effects model for the relationship between aerobic metabolism, sperm concentration (density) and thermoregulation (endotherm and ectotherms)

Predictor	Coefficient (\pm s.e.)	χ^2	CI	<i>P</i>
Intercept	-14.1 (0.67)	432.7	-15.41(-12.79)	<0.001
Concentration (Conc)	0.87 (0.03)	625.5	0.81-0.93	<0.001
Thermoregulation (Thermo)	-0.76 (0.15)	24.5	-1.05(-0.47)	<0.001
Model selection		d.f.	logLik	AIC
Conc+Thermo Conc×Thermo		3	-282.0	575.9
Conc+Thermo		2	-282.3	574.6

We included species as a random effect. We reduced our models by removing terms that were not significant and testing the model fit using Akaike's information criterion (AIC). Bold terms are significant in the model. logLik=log-likelihood.

differed significantly within different species (Fig. 3): for 11 species for which we have multiple measures of metabolism across a range of concentrations (\sim 100-fold difference), the species \times concentration interaction was not significant (Δ AIC=0.3, $P=0.12$). However, this nonsignificant interaction needs to be interpreted with caution considering that the sample size for most species was small ($n=4$ to 28).

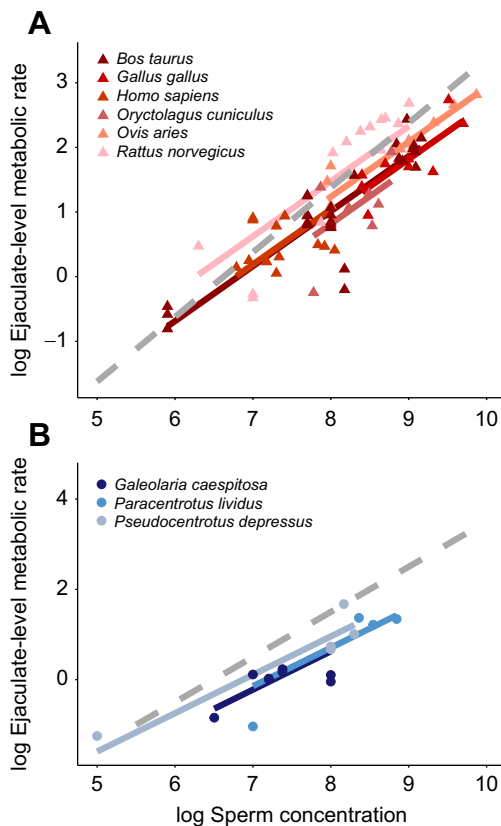


Fig. 3. Within-species relationships between sperm concentration and ejaculate-level metabolic rate. Fitted lines represent line of best fit based on a phylogenetic linear mixed-effects model using a limited dataset for species for which we had multiple measures of sperm concentration (nine species). Plots show the relationship between ejaculate-level metabolic rate ($\mu\text{l O}_2 \text{ h}^{-1}$) and sperm concentration on \log_{10} - \log_{10} axes for (A) endotherms and (B) ectotherms. Each data point represents a single observation of metabolic rate for a given species. Slopes did not differ among species, so we fit the same slope to each species ($b=0.87$). Dotted lines show the expected relationship if density (concentration)-dependent metabolic plasticity was not observed ($b=1$). Data were analysed in a natural log framework but are displayed on a \log_{10} scale for ease of view. Data for *Galeolaria caespitosa* were collected empirically and the remaining species were collected from other sources. (Note: 9 out of the 12 species were plotted for ease of view.)

We found that density effects were similar among endotherms and ectotherms but there was a significant difference in the oxygen consumption rate between sperm of endotherms and ectotherms (Table 1): sperm of endotherms have \sim 2-fold higher metabolisms than sperm of ectotherms (Fig. 2). The results did not change when we excluded studies with lower replication (<4 replicates) ($\chi^2_{\text{Thermoregulation}\times\text{Concentration}}=0.02$, d.f.=1, $P=0.69$; $\chi^2_{\text{Concentration}}=429.4$, d.f.=1, $P=<0.001$, coefficient \pm s.e.= 0.83 ± 0.04 ; $\chi^2_{\text{Thermoregulation}}=10.1$, d.f.=1, $P=0.001$). As endotherms were exclusively internal fertilizers and all but two of the ectotherms were external fertilizers, disentangling endothermy effects from fertilization mode effects is not possible (see Fig. S2). We found that density effects were similar

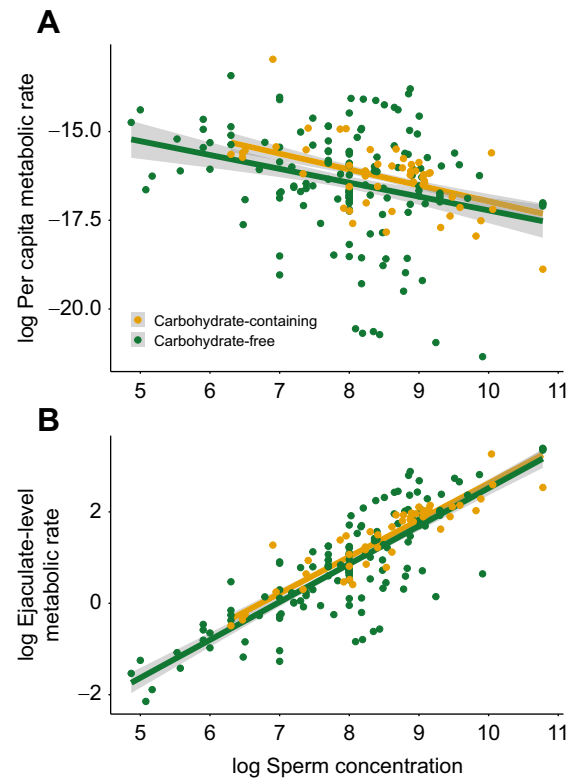


Fig. 4. Among-species relationships between density-dependent per capita and ejaculate-level metabolic rates based on the diluent. Plot shows the relationship between (A) per capita metabolic rate and sperm concentration (sperm ml^{-1}) and (B) ejaculate-level metabolic rate ($\mu\text{l O}_2 \text{ h}^{-1}$) and sperm concentration (sperm ml^{-1}) on \log_{10} - \log_{10} axes separated by diluent (i.e. carbohydrate-containing and carbohydrate-free). Fitted lines and confidence intervals represent line of best fit based on a linear mixed-effects model. Each point represents an observation from a single species for 49 species. Colours indicate different diluents (green, carbohydrate-free media, $N=148$; yellow, carbohydrate-containing media, $N=50$; see Supplementary Materials and Methods for more information on diluent classification).

Table 2. Linear mixed-effects model for the relationship between aerobic metabolism, sperm concentration (density) and diluent (carbohydrate-containing or carbohydrate-free media)

Predictor	Coefficient (\pm s.e.)	χ^2	CI	<i>P</i>
Intercept	-13.7 (0.96)	327.0	-15.58(-11.82)	<0.001
Concentration (Conc)	0.85 (0.05)	501.5	0.75-0.95	<0.001
Diluent	-0.06 (0.10)	0.68	-0.26-0.14	0.41
Model Selection		d.f.	logLik	AIC
Conc+Diluent Conc×Diluent		3	-291.1	594.2
Conc+Diluent		2	-292.3	594.6

We included species as a random effect. Model selection: we reduced our models by removing terms that were not significant and testing the model fit using AIC. Numbers in bold indicate terms that are significant in the model.

among fertilization modes ($\chi^2_{\text{Fertilization mode} \times \text{Concentration}}=0.72$, d.f.=1, $P=0.40$) and our main result remained the same ($\chi^2_{\text{Concentration}}=620.8$, d.f.=1, $P<0.001$, coefficient \pm s.e.= 0.86 ± 0.03).

We compared the density-dependent metabolism of sperm diluted with different media types (i.e. carbohydrate-containing versus carbohydrate-free) and found no significant interaction or main effect of diluent (Table 2) (Fig. 4): sperm experienced the same decline in per capita density-dependent metabolism regardless of the diluent used (Fig. 4A). We also analysed the effects of methodological differences in sperm handling (i.e. storing, extraction, diluent) and found that none of these methods altered the relationship between density and metabolism (Table S1). We reduced our dataset to only freshly collected sperm (excluding frozen-thawed or cooled sperm) and found that our results remained unchanged ($\chi^2_{\text{Thermoregulation} \times \text{Concentration}}=1.98$, d.f.=1, $P=0.16$; $\chi^2_{\text{Concentration}}=492.2$, d.f.=1, $P<0.001$, coefficient \pm s.e.= 0.87 ± 0.04 ; $\chi^2_{\text{Thermoregulation}}=25.3$, d.f.=1, $P<0.001$). We also analysed the density-dependent effects of different extraction methods (i.e. ejaculated or extracted from female) on sperm metabolism and found no significant difference ($\chi^2_{\text{Extraction method} \times \text{Concentration}}=0.59$, d.f.=1, $P=0.44$). Similarly, diluent job (i.e. activating or extending)

had no effect on density-dependent metabolism of sperm ($\chi^2_{\text{Diluent Job} \times \text{Concentration}}=1.40$, d.f.=1, $P=0.24$) but the use of an extender diluent did increase oxygen consumption slightly ($\chi^2_{\text{Diluent Job}}=6.86$, d.f.=1, $P=0.01$).

Misestimation

We found that assuming a linear relationship between sperm concentration and metabolism introduced systematic misestimates of sperm metabolic rate. The direction and magnitude of the misestimation depended on the concentration (C) at which respiration was measured relative to the standardized concentration reported. When the actual sperm concentration was lower than the standardized concentration, linear transformations systematically overestimated metabolic rate at the standardized concentration (Fig. 5). When the actual sperm concentration was higher than the standardized concentration, linear transformations systematically underestimated metabolism at the standardized concentration. The relationship between the degree of misestimation follows the form of $y=x^{-0.13}$, where y is the degree of misestimation (%) and x is the log-fold difference between the actual sperm concentration used in the experiment and the standardized reported sperm concentration (Fig. 5).

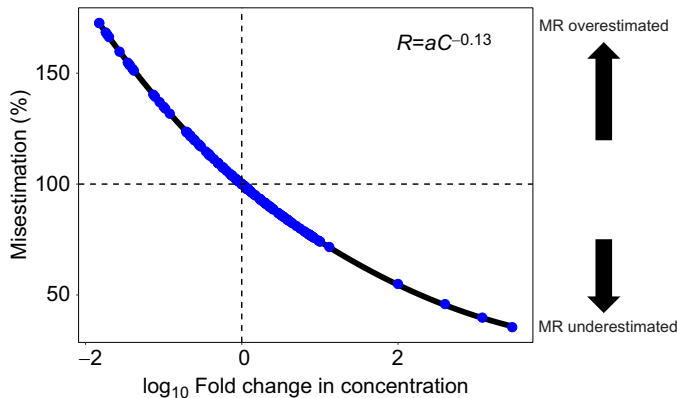


Fig. 5. Percent misestimation of sperm metabolism based on the fold change in reported sperm concentration relative to the actual sperm concentration. Misestimation was calculated as the proportion of the adjusted standardized sperm metabolism (adjusted for the actual scaling relationship, $b=0.87$) and the standardized sperm metabolism reported from literature. Sperm concentration ratio was calculated as the proportion of the actual sperm concentration and the standardized sperm concentration on a log scale. Misestimation was calculated using Eqn 1, where R is the respiration rate, a is the coefficient and C is the concentration. Dashed lines represent the boundaries for misestimation: the point of intersection, no misestimation; right of vertical line, standard sperm concentration is less than the actual sperm concentration; left of vertical line, standard sperm concentration is greater than the actual sperm concentration; above horizontal line, sperm metabolic rate is overestimated; below the horizontal line, sperm metabolic rate is underestimated.

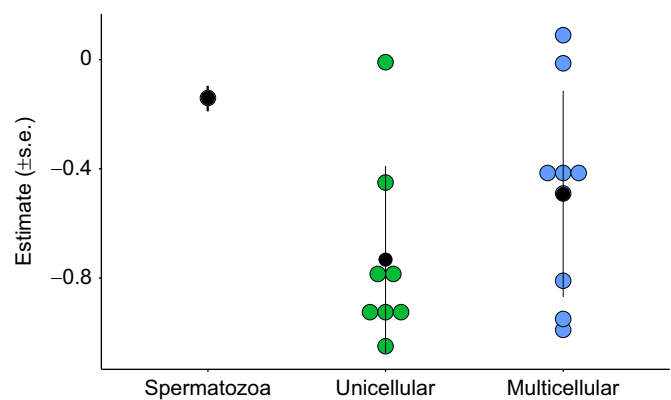


Fig. 6. Comparing mean scaling exponents for density-dependent metabolism. Mean estimate of the scaling exponent for density-dependent metabolism for sperm compared with estimates from unicellular and multicellular species taken from several published sources (DeLong et al., 2014; Ghedini et al., 2017; Malerba et al., 2017; Marshall et al., 2022). The mean estimate of scaling of metabolism with density is shallower in sperm ($b=-0.13$) than most estimates for unicellular species ($b=-1.05$ to -0.009) and multicellular species ($b=-3.05$ to 0.09); note: one extreme scaling of -3.05 from the eusocial insect *Zacryptocerus pusillus* was removed from the plot for ease of view. Black dots are summary means (\pm s.e.m.) for each group.

DISCUSSION

Our comparative analysis combined with empirical observations suggest that density affects sperm metabolism across a wide range of taxa. We found a negative relationship between density and per capita metabolism – sperm at higher densities have lower per capita metabolic rates than sperm at lower densities. We show that the observed patterns are more likely caused by density-mediated changes to the sperm environment (e.g. pH, [CO₂]) than resource limitation. Our results show that patterns of density-dependent metabolism extend beyond diploid metazoan phases into haploid phases (i.e. gametes) – both haploid and diploid phases can detect and modify their metabolism in response to their own density. However, when we compared our estimate of density-dependent metabolism with estimates found in whole organisms, we found that the relationship for sperm is shallower than most other estimates (DeLong et al., 2014; Ghedini et al., 2017; Malerba et al., 2017; Marshall et al., 2022) (Fig. 6). Our results indicate that sperm metabolism is highly plastic, similar to whole organisms, and shows greater variability than is appreciated.

Our results show a shallow, negative relationship between per capita metabolic rate and density ($b = -0.13$; Fig. 2A) both within and among species – sperm at lower densities have slightly higher individual metabolic rates than sperm at high densities. We find no support for resource limitation driving this pattern in that sperm diluted with carbohydrate-containing media had the same density-dependent metabolic response as sperm diluted with carbohydrate-free media (Fig. 4), similar to the hypothesis shown in Fig. 1C. Therefore, we interpret the results much the same way as the progenitors of the RDE, perceiving them as evidence for adaptive plasticity in sperm metabolism (Gray, 1928a; Rothschild, 1950, 1956). According to this hypothesis, sperm in dense suspensions have lower per capita metabolisms and longer lifespans than sperm in dilute suspensions (Chia and Bickell, 1983; Gray, 1928a; Levitan et al., 1991; Ohtake, 1976; Rothschild, 1956). Sperm remain energetically inactive within the dense matrix of the ejaculate, allowing them to conserve their limited energy reserves. Once diluted and free swimming, sperm increase their metabolism and expend energy to outcompete other sperm (i.e. within- or between-ejaculate competition) to fertilize eggs. For example, some internal fertilizers can store diluted sperm from multiple males at high densities in sperm storage organs before they fertilize eggs, increasing the risk of sperm competition (Simmons, 2002). In storage, sperm have decreased senescence, increased longevity and lower metabolic demands (Reinhardt, 2007; Ribou and Reinhardt, 2012). Once sperm reach the site of fertilization, they will rapidly increase their energy use, through greater motility and speed, to outcompete rival sperm for access to the ova (Simmons, 2002). Selection would not favour sperm that increased their metabolism while in storage, as this would increase senescence, decrease lifespan and sperm would likely expire before successfully reaching the ova. Similarly, having a reduced metabolism in a dilute suspension would mean that sperm may be too sluggish to compete for fertilizations. A large number of studies have demonstrated that sperm show adaptive changes in behaviour in response to local conditions (Alvarez et al., 2012; Babcock et al., 2014; Bahat and Eisenbach, 2006; Evans et al., 2012; Friedrich and Jülicher, 2007; Hadlow et al., 2023; Levitan et al., 1991); our results imply that this adaptive plasticity extends to metabolism.

Although our results indicate that density-dependent metabolism is not driven by resources, we still cannot rule out the possibility that resources play a role in the RDE. Increasing evidence suggests that female reproductive fluid (FRF) – i.e. fluid from the female

reproductive tract or eggs – helps maintain sperm function (e.g. longevity, motility, physiology) across species (Gasparini et al., 2020). Seminal work from Gray (1928b) showed that metabolism and longevity increased in the presence of FRF (i.e. egg secretions) (Gray, 1928b). A more recent study by Hadlow et al. (2023) showed that age-related declines in sperm motility were somewhat alleviated by the addition of FRF (Hadlow et al., 2023). If FRF provides sperm with more per capita resources, this could explain higher per capita metabolisms at lower densities. An interesting addition to this research would be to investigate the unexplored effects of FRF on density-dependent sperm metabolism by diluting sperm with FRF and measuring metabolism at different concentrations in both internal and external fertilizers.

Our finding that the relationship between density and metabolism was the same regardless of diluent used (resource rich versus resource limited) implies that something other than resource availability is mediating metabolism. We, along with Gray, favour the hypothesis that the accumulation of metabolic products drives the relationship between sperm density and metabolism. Specifically, oxygen saturation, intracellular pH, carbon dioxide tension (Chia and Bickell, 1983), ion concentrations (e.g. bicarbonate and calcium; Pereira et al., 2017) and cell-to-cell communication (i.e. quorum sensing, Luther and Waberski, 2019; and sperm conjugation, Higginson and Pitnick, 2011; Monclus and Fornes, 2016) have all been suggested as cues, but some seem more likely than others. It is possible that oxygen limitation at high sperm concentrations depresses metabolic rate, but more than half of our estimates from published studies were at oxygen saturation levels that were greater than 50% [i.e. above most mammalian sperm critical P_{O_2} levels (Marshall et al., 2013); see Table S4]. In our own empirical data, we do see evidence of a relationship between metabolic rate and oxygen saturation – metabolic rate (\dot{V}_{O_2}) decreases as oxygen saturation decreases for *G. caespitosa*. The rate of oxygen decline can also change based on: (1) the respirometer used – closed versus open; and (2) the sperm concentration measured – sperm at higher concentrations can deplete oxygen by 100% in a matter of minutes, whereas lower concentrations may take hours to deplete oxygen by just 20% (Table S4). Based on our data, we cannot rule out that sperm are oxy-conformers and reduce their oxygen uptake as the partial pressure of oxygen decreases (Marshall et al., 2013; Prosser, 1973), but this requires further explicit study.

We suspect that density-mediated changes in the pH and CO₂ concentration of the sperm environment drive the changes in metabolism that we observed, given the role of both in mediating sperm activity and function more generally. In high-density conditions, sperm tend to generate more CO₂ per volume, resulting in lower HCO₃⁻/pH_i and decreased activity. However, as sperm dilute into environments with elevated HCO₃⁻/pH_i, the spatial dispersion leads to reduced CO₂ levels per volume. As sperm transition from a state of low activity (higher densities) in the male reproductive organ to a state of higher activity (lower densities) in the female tract or aquatic environment, changes in the external environment, such as elevated HCO₃⁻ levels and pH, contribute to the initiation of sperm capacitation (Tresguerres et al., 2010). Sperm capacitation is a complex process that is crucial for fertilization and is initiated by various factors including CO₂, bicarbonate ions (HCO₃⁻), intracellular pH (pH_i) regulation and ATP dynamics (Chia and Bickell, 1983; Cohn, 1918; Foley and Williams, 1967; Grahn et al., 2023; Mohri and Yasumasu, 1963; Rothschild, 1956; Speer et al., 2021; Tresguerres et al., 2010; Zhou et al., 2015). HCO₃⁻:CO₂ plays a pivotal role in capacitation through intracellular alkalinization, resulting in an elevated pH_i, triggering hyperactivation (i.e. increase flagellar beating) (Grahn et al., 2023).

This process is likely conserved across mammals and free-spawning marine invertebrates, influencing flagellar beating and fertilization (Beltrán et al., 2007; Boulais et al., 2019; Christen et al., 1983; Grahm et al., 2023; Hess et al., 2005; Qi et al., 2007; Rothschild, 1956; Speer et al., 2021; Vacquier et al., 2014; Wang et al., 2007). Additionally, elevated intracellular HCO_3^- , similar to levels found in reproductive fluids, activates soluble adenylyl cyclase (sAC), which uses ATP to trigger cyclic adenosine 3',5'-monophosphate (cAMP) signalling pathways (Chen et al., 2000; Hess et al., 2005; Wandernoth et al., 2010), stimulating sperm motility (Wang et al., 2007) and respiration (Foley and Williams, 1967; Tajima et al., 1987). An important next step would be to disentangle CO_2 and pH from sperm density to determine exactly which covarying component drives sperm metabolism.

We found that sperm from endotherms have higher metabolism than sperm from ectotherms – metabolic rates are ~2-fold greater in endotherms. This is similar to patterns seen in whole organisms, though the effect was less strong: mass-specific metabolic rates can be ~6-fold higher for endotherms (Gillooly et al., 2017). Endotherms exhibit larger body sizes, higher internal temperatures and internal fertilization. Internal fertilizers produce much larger sperm (Kahrl et al., 2021) with a longer flagellum (i.e. tail+midpiece), and evidence suggests that larger sperm can swim faster (Fitzpatrick et al., 2009; Gomendio and Roldan, 1991, 2008; Simpson et al., 2014), implying a potential increase in sperm energy expenditure. Given that endotherms have sperm with higher metabolisms, and therefore higher rates of CO_2 production, they should show a steeper density–metabolism relationship relative to ectotherms. Instead, we found that endothermic sperm show similar density–metabolism relationships as ectotherms, implying that the way in which environmental changes alter density-dependent metabolism in sperm may be conserved across groups. Although we still favour the role of $\text{CO}_2/\text{HCO}_3^-/\text{pH}_i$ as an explanation, another plausible explanation for density–metabolism relationships in sperm is that sperm are activated by mechanical dilution whereby sperm become surrounded by free space once they are diluted, which provides room for movement and allows them to expend more energy than those in dense suspensions – termed allelostasis (Gray, 1928a).

Evidence is mixed regarding the relationship between sperm metabolism and sperm activity (i.e. motility and velocity) across species. Some studies have found a link between metabolism and sperm activity (Boell and Burkus, 1984; Burness et al., 2004, 2005; Lahnsteiner et al., 1996, 1999) whereas others have found no relationship (Bernardini et al., 1988; Burness et al., 2005; Cardullo and Cone, 1986; Deutch et al., 1985; Lahnsteiner and Caberlotto, 2012; Murdoch et al., 1999). Additionally, swimming speed positively covaries with sperm density in some species (Reinhardt and Otti, 2012) but not others (Ginsburg and Armant, 1990; Levitan, 2000). Based on these studies and the negative density-dependent pattern we observed in our data, two things may be possible: either (1) the relationship is driven by reduced motility, where a significant proportion of sperm exhibit minimal movement at higher densities, resulting in lower oxygen consumption rates, or (2) it is driven by reduced velocity, where numerous sperm display sluggish movement, leading to lower oxygen consumption rates. We examined a subset of studies in our compilation and a few external studies to examine the relationship between percent motility and concentration across species. We found a strong positive relationship ($F_{1,69}=5.47$, $P=0.02$, coefficient \pm s.e.=3.10 \pm 1.30) – a higher percentage of sperm are moving in higher concentrations – the opposite of what would be expected if motility

was driving the sperm concentration–metabolism relationship. Therefore, based on the limited data available, it seems more likely that sperm velocity negatively covaries with concentration such that a higher percentage of sperm are moving at higher densities, but they are moving less quickly than in lower densities. We do not have enough data to test this inference as things stand, but believe it is an important next step for identifying the proximal drivers of the sperm metabolic plasticity.

Consequences of our findings

A practical consequence of our discovery that the RDE is widespread across the tree of life is that many of the standardized sperm metabolic rates reported in the literature are incorrect. A tradition in the field of sperm metabolic research is to measure sperm metabolism at whatever concentration is practical and then use a linear multiplier to convert that metabolic rate to the predicted metabolic rate (i.e. Z_{O_2}) at a common concentration based on a standardized sperm concentration (Redenz, 1933). For example, studies of mammals typically convert their estimates of metabolism to those expected at a concentration of 10^8 sperm ml^{-1} (see Table S1). Garrett et al. (2008) measured metabolism of freshly collected bull (*Bos taurus*) sperm at a concentration of 5×10^7 sperm ml^{-1} , but expressed metabolism in 10^8 sperm ml^{-1} . A nonlinear relationship between sperm metabolism and concentration means that using a linear conversion introduces systematic error. Simply put, when the test concentration is higher than the standardized reported concentration, such conversions underestimate the true metabolic rate; when the test concentration is lower than the standardized reported concentration, such conversions overestimate metabolic rate. As Fig. 5 shows, these misestimates can be substantial – across all the studies considered, metabolic rates can be misestimated by up to 5-fold. We suggest that future studies report the sperm metabolic rate at the concentration at which it was measured, and report the metabolic rate at that concentration without conversion to a standardized metabolic rate.

Although our analyses reveal general and consistent patterns of density-dependent sperm metabolism, some key uncertainties remain. First, sperm have finite energy reserves such that there should be a trade-off between sperm function (i.e. metabolic rate and velocity) and duration of energy use (longevity) – any increase in one will likely cause a decrease in the other (Ball and Parker, 1996; Pizzari and Parker, 2009). Sperm size has also been linked to swimming speed (and thus energy use), but the direction and magnitude of this relationship is variable (positive, negative or no relationship) and species-specific (Gomendio and Roldan, 1991, 2008; Lamunyon and Ward, 1998; Malo et al., 2006; Simpson et al., 2014). Determining how sperm size, swimming speed and longevity all interact with sperm concentration to affect metabolism is an important next step. Second, some internally fertilizing species can store diluted sperm in the female reproductive tract for extended periods of time (days to years) (Holt and Fazeli, 2016). Sperm that are densely packed into confined spaces within the female reproductive tract may be shielded from the effects of dilution (i.e. respiration and ageing) until they reach the site of fertilization (Reinhardt, 2007). A critical next step would be to explore how the RDE differs between internal fertilizers that store sperm versus those that do not. Finally, it would be interesting to determine whether there is similar density-dependent metabolism in externally shed female gametes (i.e. eggs). If eggs are less energy limited, which seems likely, we might expect that metabolism is unaffected by density. In contrast, the relatively small surface area to volume ratio of eggs means that oxygen availability can reduce egg

survival (Hendry and Day, 2003; Seymour and White, 2006). Thus, examining egg density–metabolism relationships seems like an interesting topic for further exploration.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.E.P., C.R.W., D.J.M.; Methodology: A.E.P., C.R.W., D.J.M.; Software: A.E.P.; Validation: A.E.P.; Formal analysis: A.E.P., C.R.W., D.J.M.; Investigation: A.E.P.; Data curation: A.E.P.; Writing - original draft: A.E.P.; Writing - review & editing: A.E.P., C.R.W., D.J.M.; Visualization: A.E.P., D.J.M.; Supervision: C.R.W., D.J.M.

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Data availability

The data and code are available from the Dryad digital repository (Potter et al., 2024): doi:10.5061/dryad.hmgqnm1.

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