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Investigating the role of mitochondrial membrane potential in paternal inheritance of mitochondria

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ABSTRACT

The process of oxidative phosphorylation (OXPHOS) in mitochondria depends on an electrochemical gradient known as the mitochondrial membrane potential ($\Delta\psi$m). Reflecting high functionality, elevated $\Delta\psi$m usually depicts healthy mitochondria and contributes to organelle selection. This study investigates whether mitochondrial properties linked with bioenergetics, such as $\Delta\psi$m, play a role in paternal inheritance of mitochondria. More specifically, the study looks at how sperm $\Delta\psi$m responds to egg chemoattractants in bivalves characterized by distinct mitochondrial inheritance patterns: strict maternal inheritance (SMI) and doubly uniparental inheritance (DUI), the latter displaying sex-specific transmission of paternal mitochondrial DNA. Sperm $\Delta\psi$m was examined in four bivalve species: the blue mussel (*Mytilus edulis*) and the Manila clam (*Ruditapes philippinarum*) (DUI), plus the hard clam (*Mercenaria mercenaria*) and the soft-shell clam (*Mya arenaria*) (SMI). In the absence of egg chemoattractants, sperm $\Delta\psi$m did not vary between the two groups. However, there was a trend of increase in $\Delta\psi$m following egg detection only in sperm bearing paternally derived mitochondria (DUI). This suggests, along with bioenergetic changes, that $\Delta\psi$m modulation might be a specific property of at least some DUI species, possibly implicated in their unique ability to transmit their mitochondria in a sex-specific fashion.

Keywords: mitochondria; OXPHOS; mitochondrial membrane potential; doubly uniparental inheritance; sperm; egg chemoattractants

INTRODUCTION

Eukaryotic life depends on mitochondrial respiration to satisfy most of the cellular energy requirements. This is achieved through the process of oxidative phosphorylation (OXPHOS), which relies on an electrochemical gradient generated by proton pumping and substrate oxidation. This intermembrane electric potential (i.e. the mitochondrial membrane potential, $\Delta\psi$m) drives ATP production by the ATP synthase complex (Mitchell 1966). Given its fundamental role for energy production, loss of $\Delta\psi$m often depicts mitochondrial dysfunction and compromised bioenergetic efficiency, with downstream impact on cellular fitness (Zorova *et al.* 2018, Tworzydlo *et al.* 2020) (but see the potential benefits of mild depolarization; Brand 2000, Vyssokikh *et al.* 2020). Therefore, $\Delta\psi$m can be used to discriminate between healthy and dysfunctional mitochondria, and consequently contribute to the selective elimination of underperforming mitochondria by mitophagy to ensure viable organelle transmission (Twig *et al.* 2008, Jin *et al.* 2010, Westermann 2010, Youle and van der Bliek 2012, Zorova *et al.* 2018).

Strict maternal inheritance (SMI) of mitochondrial DNA (mtDNA), a near universal mitochondrial transmission system in animals, might have evolved as a way to prevent transmission of selfish mutations and preserve viable mitochondrial function in the offspring (Hoekstra 2000, Birky 2001). Discarding sperm mitochondria allows the removal of mitochondrial variants that have been potentially exposed to higher oxidative stress (Allen 1996, Xu *et al.* 2017). Moreover, it promotes homoplasmy (i.e. a single type of mtDNA in a cell or individual), therefore perpetuating efficient mitonuclear interactions (Lane 2011, 2012). Its opposing state, heteroplasmy, is recognized for its general negative effects on embryonic development, fitness, behaviour, cognition, age-related diseases and survival (Acton *et al.* 2007, Sharpley *et al.* 2012, Wallace and Chalkia 2013, Zhou *et al.* 2016, Hong *et al.* 2023). To prevent heteroplasmy, paternal mitochondria and mtDNA must systematically be eliminated by different

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pre- and postfertilization mechanisms, such as mtDNA removal during spermatogenesis in *Drosophila melanogaster*, degradation of ubiquitin-tagged paternal mitochondria in bovine and primate embryos, or mitophagy in embryos of *Caenorhabditis elegans*, to name just a few (Sutovsky *et al.* 1999, DeLuca and O'Farrell 2012, Mishra and Chan 2014, Zhou *et al.* 2016, Pickles *et al.* 2018).

The exception to SMI in animals resides in just over 100 bivalve species that possess a very different doubly uniparental inheritance system (DUI), where paternal mitochondria are typically transmitted to sons and populate male gametes, while maternal mitochondria populate all somatic tissues and female gametes (Breton *et al.* 2007, Passamonti and Ghiselli 2009, Gusman *et al.* 2016, Stewart *et al.* 2020). In other words, sperm mitochondria escape degradation in male bivalves (Zouros *et al.* 1994). The DUI system thus provides a unique opportunity to study the mechanisms involved in paternal mitochondria preservation and transmission to future generations, its possible adaptative value for male functions, and even its link with sex-determination (Breton *et al.* 2014, 2022).

Previous studies have suggested that the genetic divergence between female- and male-derived mtDNAs (F-mtDNA and M-mtDNA, respectively) result in the expression of different mitochondrial phenotypes, underpinning sex-specific bioenergetic adaptations with potential repercussions for fitness (Breton *et al.* 2009, Bettinazzi *et al.* 2019, 2020, 2021). The male-specific mitochondrial phenotype in DUI is characterized by a strong limitation exerted at the end of the electron transport chain, resulting in limited respiratory rates (Breton *et al.* 2009, Bettinazzi *et al.* 2019, 2021), a trait potentially in line with a high membrane potential in male mitochondria. Knowing that mechanisms of mitochondria quality control can exploit $\Delta\psi m$ to discriminate highly functional organelles (Tworzydlo *et al.* 2020), a putative role of $\Delta\psi m$ in driving paternal mitochondria persistence in DUI populations has been suggested (Milani 2015, Milani and Ghiselli 2015, Bettinazzi *et al.* 2020). Preliminary evidence supports this idea, suggesting that some DUI sperm might be able to modulate their $\Delta\psi m$, increasing it just before fertilization (Bettinazzi *et al.* 2020). However, whether $\Delta\psi m$ modulation could be a shared trait among DUI species and contribute to mitochondrial selection and inheritance remains to be demonstrated.

In this study, we took one step further in our understanding of the putative role played by sperm $\Delta\psi m$ in paternal inheritance of mitochondria. We examined mitochondrial properties in bivalve species with either a DUI [blue mussel (*Mytius edulis*) and Manila clam (*Ruditapes philippinarum*)] or an SMI [soft-shell clam (*Mya arenaria*) and hard clam (*Mercenaria mercenaria*)] system of mitochondrial transmission. We specifically tested: (i) whether DUI paternal mitochondria in sperm might have a constitutively higher membrane potential than SMI ones, and (ii) whether oocyte presence might induce a sudden increase in $\Delta\psi m$ just prior to fertilization in DUI sperm. Our results confirm an increased sperm $\Delta\psi m$ following egg detection in one DUI species compared to both SMI species, as well as an increasing trend in the second DUI species. These findings might represent a key step towards a better understanding of the mechanisms underpinning the unique ability of DUI paternal mitochondria to escape elimination and be transmitted to future generations.

MATERIALS AND METHODS

Adult bivalve specimens were obtained from a local fish market (Montreal, Quebec, Canada) during their spawning period, between June and August 2021. The DUI species examined were *Mytilus edulis* (Linnaeus, 1758) from Kensington (Canada) and *Ruditapes philippinarum* (Adam and Reeve, 1850) from Vancouver (Canada), representing independent origins of DUI (Plazzi and Passamonti 2019). The two SMI species were *Mercenaria mercenaria* (Linnaeus, 1758) and *Mya arenaria* (Linnaeus, 1758), both from Barnstable (USA).

Individuals were dissected on ice, gonads were excised, and sex was determined by inspecting gonadial smears under a microscope. Gonads were then placed in a Petri dish containing artificial sea water (ASW, 3% Instant Ocean salt mix) and mature gametes were stripped by either letting them actively swim out for 5 min (sperm), or passively flow out of the gonad for 30 min (eggs) (Bettinazzi *et al.* 2019, 2020, 2021). Oocyte maturity and sperm motility were verified microscopically under 40× magnification (Vázquez *et al.* 2021). For each species, sperm were extracted from five different males and each sperm constituted a biological replicate (*Mytilus edulis* $N = 1678, 1959$; *R. philippinarum* $N = 895, 642$; *Mercenaria mercenaria* $N = 687, 559$; *Mya arenaria* $N = 1988, 1487$, control and treatment respectively) (Supporting Information Table S1). All sperm samples of a given species were exposed to the same species-specific oocyte sample, which consisted of a pool of homogenized mature eggs derived from three different females.

The solution of egg-derived compounds was achieved through homogenization of mature eggs with a Polytron PT1200E (Kinematica AG, Switzerland) in ASW (four rounds of 10 s homogenization interspersed with 30 s of cooling on ice). The cellular contents were then separated from larger debris by centrifugation (9391 g for 1 min) at 4°C. The supernatant was stored at −80°C prior to experiments. For each male, motile sperm were divided into two aliquots: a control group without egg-chemoattractants (350 µL of sperm-solution, 150 µL ASW) and a group infused with the species-specific egg-derived solution (350 µL of sperm-solution, 125 µL ASW, 25 µL egg-derived solution) (Bettinazzi *et al.* 2020). Sperm mitochondria were then stained with both MitoSpy™ Green FM and MitoSpy™ Red CMXRos probes (BioLegend, San Diego, CA, USA), which respectively localize to the mitochondria in a way that is either independent (MitoSpy Green FM) or dependent (MitoSpy Red CMXRos) from $\Delta\psi m$. The final concentration for each dye was 400 nM (Milani and Ghiselli 2015). Samples were then left to incubate at room temperature (RT) for 30 min in the dark and finally washed to remove the excess staining agent. The washing cycles consisted in removing the supernatant through centrifugation (9391 g, 1 min) followed by resuspension in 500 µL ASW, up to four times. Sperm motility was verified again after these steps.

Cellular imagery was obtained at 60× magnification on a dedicated EVOS™ M5000 Imaging System (Thermo Fischer Scientific, USA). FITC and TRITC filters were respectively

used to detect MitoSpy Green FM (excitation 490 nm, emission 516 nm) and MitoSpy Red CMXRos (excitation 577 nm, emission 598 nm). All parameters were kept constant across all captures. For each male, $N = 5$ separate pictures containing multiple sperm were taken for the control and another $N = 5$ pictures for the chemoattractant-treated sperm. Images were analysed through the Fiji distribution of Image J software (Schindelin *et al.* 2012). The fluorescence attributable to MitoSpy Green FM was used to visually inspect mitochondria position and verify the quality of each preparation. For all samples, the strict localization of the signal in the sperm midpiece (where mitochondria are located) was verified. The correct localization was also further confirmed with Mitotracker Red CMXRos probe, which localizes to the mitochondria, this time based on their membrane potential (i.e. the more signal, the higher the potential). The fluorescence associated with this $\Delta\psi$m-sensitive probe was quantified and used as a proxy of mitochondrial membrane potential (Milani and Ghiselli 2015). Fluorescence intensity was obtained as the mean grey value of the area corresponding to each sperm mitochondria, determined by automatic thresholding with Otsu's method (*Mytilus edulis*, *R. philippinarum*, *Mya arenaria*). In the case of *Mercenaria mercenaria*, threshold values were manually optimized and tested and did not differ statistically between treatments, therefore discarding any potential bias. Area was set at 50–500 pixels^2. Fluorescence intensities were corrected for the background intensity (Hemalatha *et al.* 2016).

Data analysis was conducted on the software R (R Core Team 2021). Each sperm was considered a biological replicate and differences in sperm $\Delta\psi$m were analysed by means of two complementary linear mixed models (Supporting Information Table S2). The first model considered the factors species ('*species*'—four levels) and oocyte presence ('*treatment*'—2 levels, absence/presence of eggs) as categorical fixed effects, plus their interaction ('*species:treatment*'). This model also accounted for the variability among different males (factor '*subject*'—individual ID) and different pictures (factor '*picture*'—picture ID), which were fitted as random effects ('*picture*' nested into '*subject*'). The second model considered the different modes of mitochondrial inheritance (factor '*inheritance*'—two levels, DUI vs. SMI) and oocyte presence (factor '*treatment*'—two levels, absence/presence of eggs) as fixed effects, plus their interaction ('*inheritance:treatment*'). This model controlled for potential differences among species (random effect of factor '*species*'), males, and pictures (random effect of factor '*subject*' with factor '*picture*' nested in it). Model assumptions were verified graphically. Significance was determined through a type III ANOVA, followed by post-hoc multicomparison with multiple testing adjustment. Statistical significance was set at $P \leq .05$. The magnitude of the effect of each predictor was calculated as partial eta squared values (η_p^2). Detailed summaries are provided in Tables S1 and S2.

RESULTS

The effect of egg compound detection on sperm mitochondrial membrane potential ($\Delta\psi$m) was tested at the level of species and inheritance group (Fig. 1; Supporting Information Tables S1 and S2). Our analyses revealed an interaction effect between the presence/absence of egg compounds (factor '*treatment*') and the species tested, together with a large effect size [$F(3,173.65) = 9.6$, $P = 6.665\text{e-}06$; $\eta_p^2 = 0.14$] (Fig. 1A). A significant increase in sperm mitochondria fluorescence intensity after treatment was revealed for the DUI species *R. philippinarum* ($P < .0001$), whereas no significant changes were revealed for the other DUI species (*Mytilus edulis*, $P = .53$) and both SMI species (*Mercenaria mercenaria*, $P = .2952$; *Mya arenaria*, $P = .1974$) (Fig. 1A; Table S2). In line with these results, a significant relationship between the type of mitochondrial inheritance (DUI or SMI) and the response of mitochondria to the presence of egg compounds was also detected, together with a medium effect size [$F(1,175.49) = 16.4$, $P = 7.66\text{e-}05$; $\eta_p^2 = 0.09$] (Fig. 1B). Following a multicomparison by the factor '*inheritance*' (DUI vs. SMI), only the DUI group showed a significant fluorescence increase between control and treatment ($P = .0001$), whereas a nonsignificant but opposite trend was detected for the SMI group ($P = .1071$) (Fig. 1B; Table S2).

DISCUSSION

Sperm–egg interactions through chemical signalling have been revealed to be a complex communication system with direct consequences on sperm direction, speed, and even genetic selection (Riffell *et al.* 2004, Eisenbach and Giojalas 2006, Evans *et al.* 2012, Oliver and Evans 2014, Eads *et al.* 2016, Lymbery *et al.* 2017). However, little is known about the role played by mitochondrial and cellular bioenergetics in gamete interactions and external fertilization. Our results relate to this goal and provide evidence that egg detection can trigger a physiological response in sperm at the subcellular level. The ability to modulate sperm $\Delta\psi$m in the presence of egg chemical cues appears to be a specific property of paternal mitochondria in at least one DUI species where paternal mitochondria are selected and transmitted to male offspring. It was hypothesized that DUI sperm mitochondria might benefit from a constitutively high $\Delta\psi$m, but our results did not reveal any significant difference in $\Delta\psi$m in the absence of egg chemoattractants between DUI and SMI species (Supporting Information Table S2). The dissimilarity between the SMI group and at least one DUI species seems to reside in the ability to elevate $\Delta\psi$m according to an external triggering factor, in this case egg proximity (Fig. 1). This physiological property is in accordance with the previously acquired evidence that DUI sperm mitochondria express a male-specific phenotype able to sustain a high $\Delta\psi$m. This phenotype is characterized by low respiratory rates due to a limitation exerted by cytochrome *c* oxidase and ATP-synthase complexes (Breton *et al.* 2009, Bettinazzi *et al.* 2019, 2021). Together with specific mitochondrial properties, one way by which mitochondria can potentially preserve (and increase) $\Delta\psi$m is by reversing ATP-synthase activity at the expenses of ATP fuelled by glycolysis (Rieger *et al.* 2021, Valdebenito *et al.* 2023), or through proton release by lactate oxidation within the mitochondrial intermembrane space ('lactate shuttle') (Kane 2014). Although the involvement of these mechanisms in DUI $\Delta\psi$m modulation is purely speculative at this point, previous work on *Mytilus edulis* and *R. philippinarum* suggested that DUI sperm increasingly rely on fermentation following egg detection (Bettinazzi *et al.* 2020). This was also linked

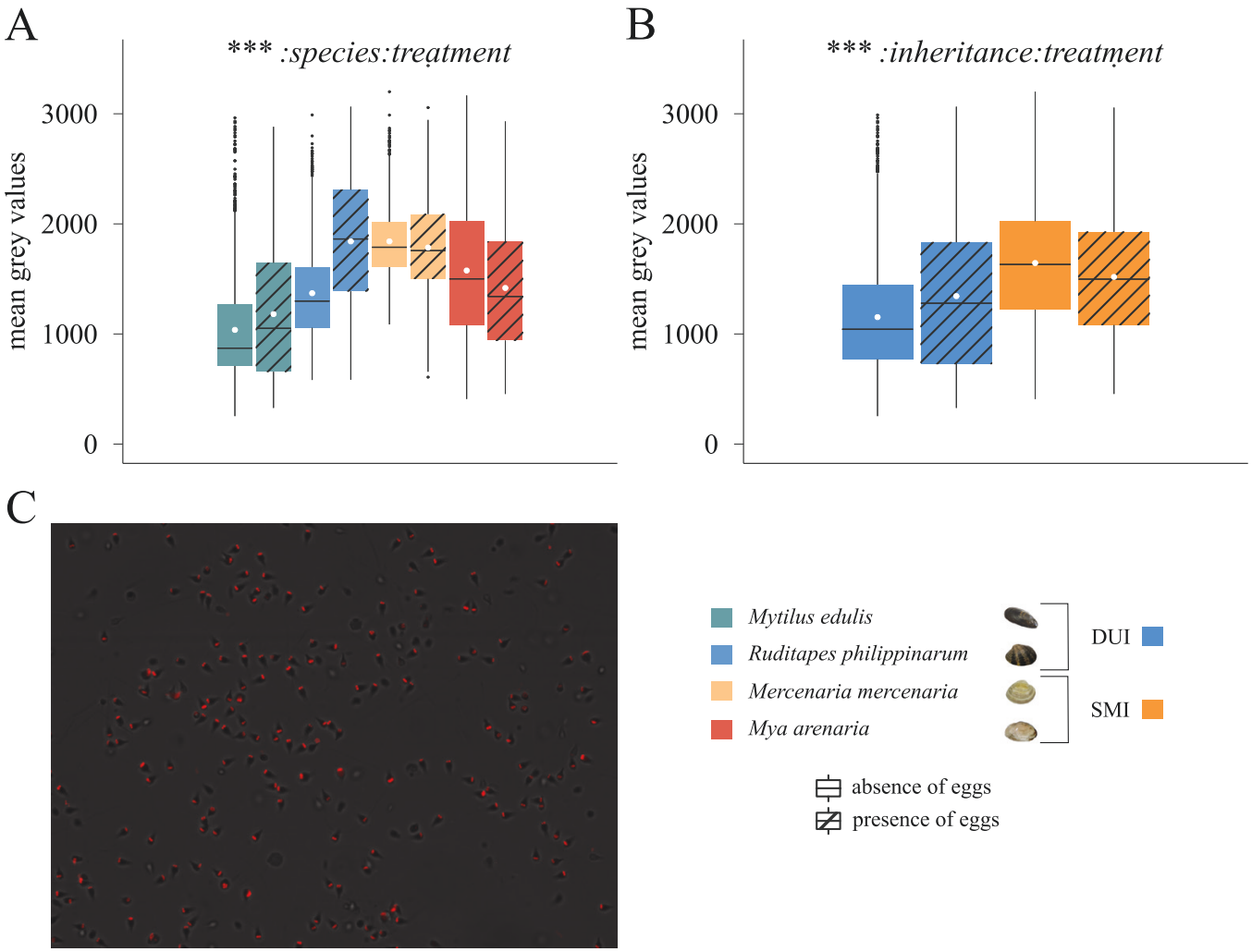

Figure 1. Mitochondrial membrane potential of bivalve sperm. Mitochondrial membrane potential was detected fluorometrically through use of MitoSpy™ Red CMXRos probe and its intensity is reported as mean grey values. A, mitochondrial membrane potential across the four species analysed, *Mytilus edulis*, *Ruditapes philippinarum*, *Mercenaria mercenaria*, and *Mya arenaria*, in the absence and presence of egg chemoattractants. B, mitochondrial membrane potential across DUI and SMI species, in the absence and presence of egg chemoattractants. C, representative image of sperm mitochondria fluorescence associated with membrane potential. Statistical analyses: A, linear mixed model with fixed effects of '*species*' and '*treatment*', plus their interaction; B, linear mixed model with fixed effects of '*inheritance*' and '*treatment*', plus their interaction. Significance was determined by means of a type III ANOVA. $^{*}P \leq .05$; $^{**}P \leq .01$; $^{***}P \leq .001$. A detailed summary is reported in Supporting Information Tables S1 and S2.

to no detectable change in swimming performance following egg detection in both species (Stewart *et al.* 2012, Bettinazzi *et al.* 2020), although changes in sperm speed have been observed in the closed DUI species *Mytilus galloprovincialis* (Oliver and Evans 2014, Hadlow *et al.* 2023). Moreover, the low respiration rates of DUI paternal mitochondria together with the generally slow speed of sperm carrying them (Jha *et al.* 2008, Bettinazzi *et al.* 2020) suggest a mitochondrial phenotype tailored for functions that go beyond intense energy production. It is therefore possible (although yet to be proven) that this metabolic switch might provide the supply of glycolytic end products (ATP and/or lactate) needed by sperm mitochondria to modulate $\Delta\psi$m just before fertilization.

Our results provide new evidence that the sex-specific divergence of DUI mitogenomes underpins variation in bioenergetic properties and support the possible link between mitochondrial physiology and organelle transmission in DUI species. It is possible, yet unproven, that a sudden increase in sperm mitochondria $\Delta\psi$m when in proximity with oocytes might underpin their ability to be transmitted to the offspring in a sex-specific fashion. This occurs through both preferential selection (Zhou *et al.* 2010, Tworzydlo *et al.* 2020) and active transport into the primordial germ cells by $\Delta\psi$m-dependent trafficking mechanisms (Milani 2015). The increase in $\Delta\psi$m upon egg detection was, however, only statistically significant for *R. philippinarum* sperm. Although our current results do not facilitate generalization to all DUI species, it is nonetheless important to highlight that both DUI species share a consistent pattern of $\Delta\psi$m change, distinguishing them from the SMI species analysed. Additionally, species-specific variation in the magnitude of the effect could be expected if we consider that both DUI species are phylogenetically distant and might even represent separate origins of the DUI system (Plazzi and Passamonti 2019). Further research involving a greater number of species is certainly needed.

The results presented here suggest that sperm $\Delta\psi$m modulation might be an important trait in at least some DUI species, with potential implications for paternal mitochondria transmission. To further explore this hypothesis, we might be able to impair sperm $\Delta\psi$m prior to fertilization and explore the possibility that the usual DUI aggregation pattern, which involves paternal mitochondria segregating in the blastomere giving rise to the germline, could be disrupted in male embryos (Cao *et al.* 2004, Cogswell *et al.* 2006, Milani *et al.* 2012). We may also be able to observe a bias towards females in the offspring sex ratio if mitochondrial selection is indeed a trait involved in sex determination in DUI species.

SUPPLEMENTARY DATA

Supplementary data are available at *Biological Journal of the Linnean Society* online.

CREDIT STATEMENT

Ariane Pouliot-Drouin (data analysis, writing-original draft), Thierry Niaison (data collection and analysis), Sophie Breton (conceptualization and supervision), and Stefano Bettinazzi (conceptualization, methodology, data analysis, and supervision). All authors contributed substantially to revisions.

CONFLICT OF INTEREST

We declare no conflict of interest.

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DATA AVAILABILITY

Supporting information is provided in Tables S1 and S2. All data and scripts used in this study are available on the figshare online repository (https://doi.org/10.6084/m9.figshare.24459604.v1).

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