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SHORT COMMUNICATION

Disruption of thermogenic *UCP1* predated the divergence of pigs and peccaries

Thomas Jacob Fyda*, Connor Spencer*, Martin Jastroch and Michael J. Gaudry‡

ABSTRACT

Uncoupling protein 1 (UCP1) governs non-shivering thermogenesis in brown adipose tissue. It has been estimated that pigs lost *UCP1* ∼20 million years ago (MYA), dictating cold intolerance among piglets. Our current understanding of the root causes of *UCP1* loss are, however, incomplete. Thus, examination of additional species can shed light on these fundamental evolutionary questions. Here, we investigated *UCP1* in the Chacoan peccary (*Catagonus wagneri*), a member of the Tayassuid lineage that diverged from pigs during the late Eocene–mid Oligocene. Exons 1 and 2 have been deleted in peccary *UCP1* and the remaining exons display additional inactivating mutations. A common nonsense mutation in exon 6 revealed that *UCP1* was pseudogenized in a shared ancestor of pigs and peccaries. Our selection pressure analyses indicate that the inactivation occurred 36.2–44.3 MYA during the mid–late Eocene, which is much earlier than previously thought. Importantly, pseudogenized *UCP1* provides the molecular rationale for cold sensitivity and current tropical biogeography of extant peccaries.

KEY WORDS: Uncoupling protein 1, Endothermy, Evolution, Brown adipose tissue

INTRODUCTION

Brown adipose tissue (BAT) is a unique organ among eutherian mammals that is responsible for augmenting heat production during cold stress via adaptive non-shivering thermogenesis (NST). This mitochondria-rich tissue is especially important for rewarming from torpor bouts in hibernators and safeguarding high body temperatures in small-bodied species and newborns of larger species (Alexander et al., 1975; Cannon et al., 1977; Smith and Horwitz, 1969). The cold-inducible expression of uncoupling protein 1 (UCP1) governs the molecular mechanism of adaptive BAT-mediated thermogenesis. UCP1 resides in the mitochondrial inner membrane and, upon activation, promotes mitochondrial proton leak, dissipating oxidation energy as heat to support NST (Nicholls and Locke, 1984). While UCP1-mediated NST is key for the survival of many eutherian species, the first documented case of a *UCP1* gene inactivation within the mammalian lineage was discovered in pigs, occurring an estimated ∼20 million years ago (MYA) (Berg et al., 2006). In pigs, exons 3–5 have been deleted while the remaining exons of this six-exon gene each display an inactivating (frameshift insertion/deletion or nonsense) mutation. While the presence of UCP1 protein in pigs has been claimed by some (Mostyn et al., 2014), it has been refuted by others (Jastroch and Anderson, 2015) and experimentally verified to be not translated (Hou et al., 2017). The absence of UCP1 is thought to contribute to poor thermoregulatory abilities of piglets, dictating their well-described reliance on shivering thermogenesis (Herpin et al., 2002). Others have proposed a compensatory role of UCP3 in adipose tissue of some cold-adapted pig lineages (Lin et al., 2017), but many arguments have been put forward from phylogenetic inference, physiological and biochemical analyses of UCP3 in genetic mouse models, arguing against direct thermogenic function by uncoupling (Gaudry and Jastroch, 2019). While Berg et al. (2006) speculated that *UCP1* lost functionality owing to diminished selection pressures for NST during periods of evolution in warm tropical environments, the authors also raised the possibility that the wild boar (*Sus scrofa*), a species that inhabits temperate climates, evolved compensatory behavioural adaptations such as maternal nest building to insulate their young and overcome their lack of UCP1-mediated NST.

Although the described loss of UCP1 among pigs has been seminal to our understanding of the role of UCP1 for larger, cold-sensitive eutherians, Berg et al. (2006) pointed out limitations that should be resolved in future studies, such as increasing the genomic information by expanding species diversity with comparative studies that would enable more precise dating of inactivation events. While Berg and colleagues suggested including more suid species, molecular dating would also benefit from examining close suid relatives, determining whether the inactivation has occurred during or before suid evolution. Collectively, expanded genomic information would have important implications not only for the evolution of eutherian thermoregulation, but also for the understanding of speciation, migration and eventual extinction of mammals in our changing environment.

Peccaries (members of the family Tayassuidae) are the closest living relatives of pigs (family Suidae) and have both been grouped into the suborder Suina (Fig. 1). The peccary lineage originated in Southeast Asia, with members later colonizing the 'New World', the Americas (Prothero, 2015). Pigs, on the other hand, remained in the 'Old World' until human intervention precipitated their invasion of the Americas ∼500 years ago (Burgos-Paz et al., 2013). The fossil record shows that peccaries were once diverse; however, only three extant species remain: the collared peccary (*Pecari tajacu*), the white-lipped peccary (*Tayassu pecari*) and the Chacoan peccary (*Catagonus wagneri*). Pigs and peccaries diverged in the late Eocene–mid Oligocene according to fossil and molecular data (Orliac et al., 2010; Parisi Dutra et al., 2017; Prothero, 2009, 2015), predating the estimated pig *UCP1* inactivation event (Berg et al., 2006). We show that this locus is also pseudogenized in peccaries, indicating either an independent inactivation event or pushing back the loss of *UCP1* in pigs from the Miocene to the Eocene, in a common ancestor of modern suinans. Given identical inactivating

Department of Molecular Biosciences, The Wenner-Gren Institute, The Arrhenius Laboratories F3, Stockholm University, SE-106 91 Stockholm, Sweden.
*These authors contributed equally to this work

‡Author for correspondence (michael.gaudry@su.se)

M.J., 0000-0003-0358-3865; M.J.G., 0000-0001-8411-0415

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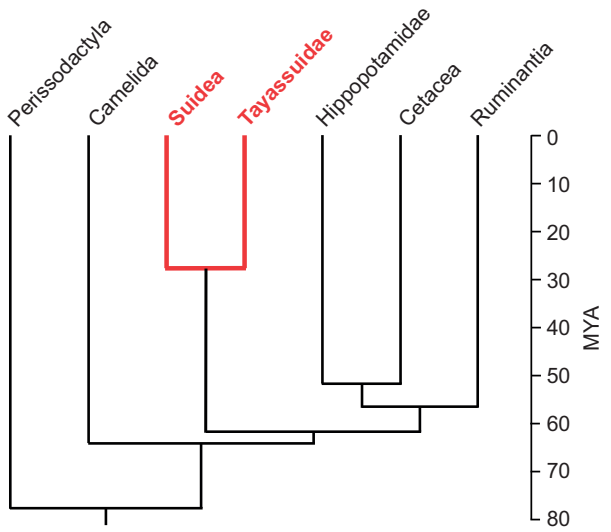

Fig. 1. Phylogenetic relationships among ungulates based on Meredith et al. (2011). The Suina lineage is highlighted with red branches. MYA, millions of years ago.

mutation events in exon 6, we provide evidence for the latter scenario that *UCP1* pseudogenization occurred in a common suinan 36.2–44.3 MYA according to our molecular dating analyses. Our examination of this lineage provides a more complete picture of *UCP1* inactivations among eutherian mammals and may even explain current biogeographical distributions and cold sensitivity of the remaining extant peccaries.

MATERIALS AND METHODS

We utilized human *UCP1* mRNA (accession number: NM_021833.4) as a query to perform nucleotide discontinuous megablasts against whole genome shotgun projects of the Chacoan peccary (*Catagonus wagneri* Rusconi 1930), domestic pig (*Sus scrofa domesticus* Erxleben 1777) and 57 other ungulate species on the NCBI webserver. Top hit contigs (see Table S1 – for accession numbers) were annotated in Geneious Prime 2019.2.1 using human *UCP1* exons as references. Contigs were manually inspected to ensure proper reading frame and correct exon/intron boundary splice sites according to the AG-GT rule. We also included two other Suid (*Sus verrucosus* and *Sus cebifrons*) *UCP1* pseudogene sequences in our dataset previously acquired (Gaudry et al., 2017) through the NCBI SRA database. As both the pig and peccary *UCP1* loci display major deletions, dot plots were generated using the EMBOSS 6.5.7 dotmatcher tool. We also annotated the flanking *TBCD19* and *ELMOD2*, respectively located upstream and downstream of *UCP1* to confirm gene orthology and used Easyfig 2.2.2 to evaluate the conserved synteny of the gene clusters with sequences that span 2 kbp upstream of the termination codons of *TBC1D9* and *ELMOD2*.

Selection pressure analyses were performed using CODEML in the PAML 4.8 software package (Yang, 2007) following the description outlined by Gaudry et al. (2017). Briefly, we first constructed a species tree to reflect the evolutionary relationships between *C. wagneri*, *Sus* spp. and other ungulate species (Table S1) based on the phylogenies of previous literature (Agnarsson and May-Collado, 2008; Bibi, 2013; McGowen et al., 2009; Meredith et al., 2011; Steeman et al., 2009). *UCP1* coding sequences were aligned using the alignment algorithm in Geneious Prime 2019.2.1 and manually adjusted to accommodate insertion/deletion pseudogenization sites. The free ratio model in CODEML was used to assess selection pressures along each branch of the tree, providing an initial dN/dS ratio (ω) prior to targeting individual branches for more robust analyses using the M2 model.

To estimate the inactivation date of the *UCP1* gene in the shared ancestor of both *C. wagneri* and *Sus* spp., we first categorized each branch in the tree as 'functional', 'pseudogenic' or 'transitional' according to Meredith et al. (2009) and Gaudry et al. (2017), where 'transitional' refers to the branch upon which the transition from a functional to non-functional gene arose. Using the M2 model with parameters identical to those described in Gaudry et al. (2017), we estimated selection pressures for all functional branches as a single category, all pseudogenic branches as a single category, and each transitional branch as its own category. The selection pressure corresponding to the 'transitional' Suina branch was then used to estimate the inactivation date of the *UCP1* gene using the fossil constrained molecular time tree 28.8 MYA global mean divergence date of Meredith et al. (2011) and the 37 MYA split considered by Prothero (2009, 2015) and following the calculations outlined by Meredith et al. (2009) and Gaudry et al. (2017).

RESULTS

Conserved synteny of the *UCP1* locus in a peccary

A single contig (GenBank accession: PVHT010004494.1) of the Chacoan peccary was retrieved that encompasses the 5′-*TBC1D9*-*UCP1*-*ELMOD2*-3′ gene cluster. As expected, the synteny of *UCP1* is conserved for both the pig and peccary (Fig. 2A). A distinct contig (GenBank accession: PVHT010002981.1) containing the *UCP3*-*UCP2* gene cluster was also retrieved from the peccary genome, both of which have intact open reading frames (see Fig. S1 for amino acid translations of *UCP3* and *UCP2* genes).

UCP1 pseudogenization

The Chacoan peccary *UCP1* locus displays numerous inactivating mutations. A dot plot comparing sequence identity of the pig versus peccary *UCP1* genes (Fig. 2B) reveals a ∼5.3 kbp deletion in the peccary that eliminates exons 1 and 2. At least part of the *UCP1* enhancer is also deleted, though a small (∼85 bp) upstream section displays ∼83% similarity to the ∼220 bp enhancer of the pig. Pig *UCP1* has been inactivated in part by an alternative ∼2.3 kbp deletion that eliminates exons 3, 4 and 5 (Fig. 2B). While *UCP1* exons 3, 4, and 5 are present in the peccary, they exhibit several points of disruption (Fig. S2). In addition to a 57 bp in-frame deletion in exon 3, both exons 3 and 4 contain single nucleotide frameshift deletions. Exon 5 displays a single bp insertion followed by a 4 bp deletion, but all GT–AG splice sites remain intact. Exon 6 is the only shared exon that has been retained in both *UCP1* pseudogenes of the pig and peccary and contains a premature nonsense mutation in both Suina species (Fig. S2) that, excluding all other inactivating mutations, would truncate the C-terminus of the protein by 30 amino acids. This shared inactivating mutation among both pigs and peccaries suggests *UCP1* was inactivated in a common suinan ancestor prior to their divergence.

Selection pressure analyses and *UCP1* inactivation date

The free ratio CODEML model provided initial individual selection pressures for each branch. Notably, under this model, the Chacoan peccary *UCP1* pseudogene displayed a near-neutral dN/dS ratio (ω=0.9933; Fig. S3). Further analyses using the M2 model revealed an elevated ω value of 0.4095 for the stem Suina transitional branch (Fig. 3A), while ω values for functional (ω=0.1755) and pseudogenic branch (ω=1.0025) categories are indicative of

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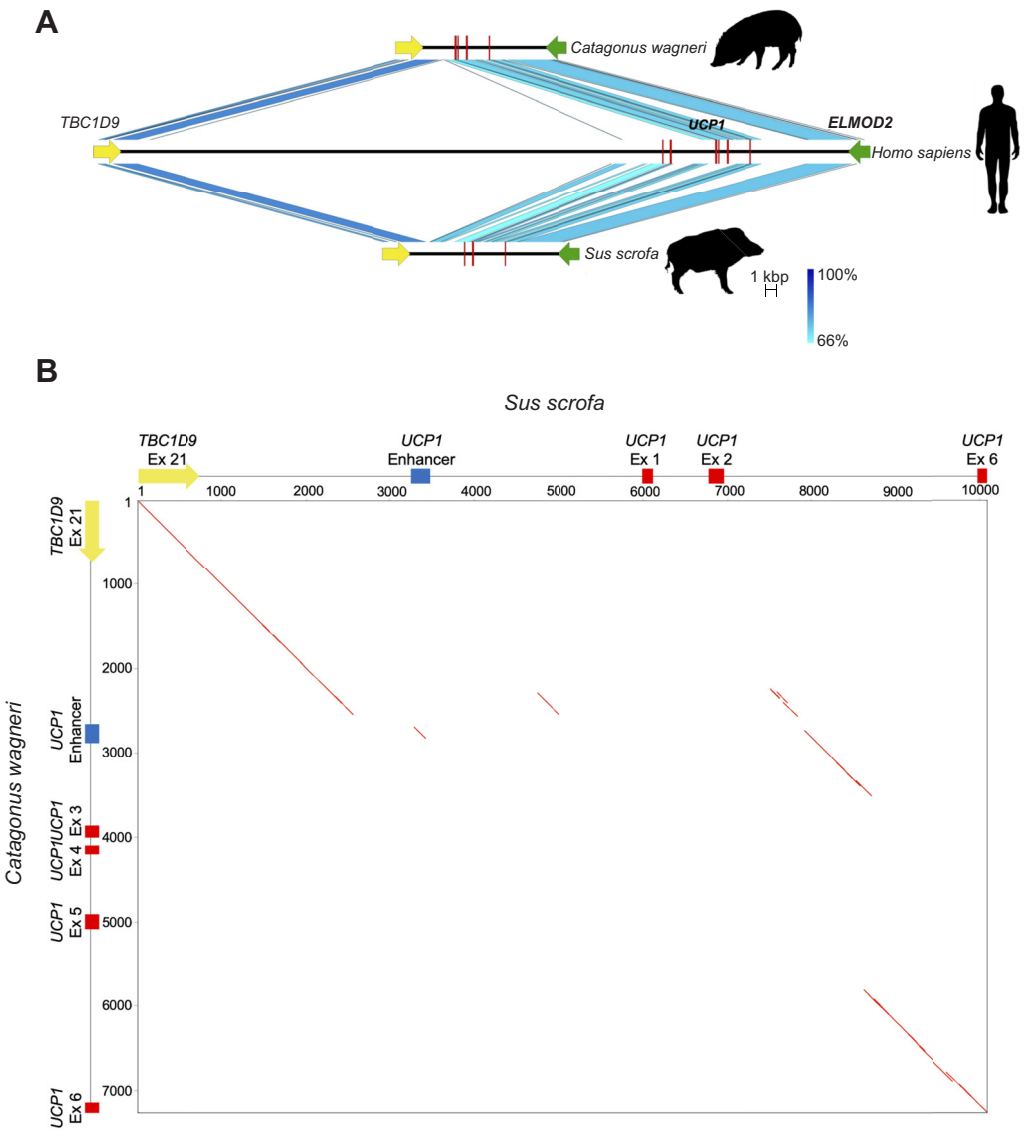

Fig. 2. *UCP1* is pseudogenized in the Chacoan peccary (*Catagonus wagneri*). (A) Sequence comparison of the peccary 5′-*TBC1D9-UCP1-ELOMD2*-3′ gene cluster versus that of humans and pigs, displaying conserved synteny in peccaries. Exons are highlighted in red, and *TBC1D9* and *ELMOD2* denoted by yellow and green arrows, respectively. Sequences spanned 2 kbp upstream of *TBC1D9* and *ELMOD2* termination codons. Varying shades of blue denote sequence identity across species and scale bar indicates the length of 1 kbp. (B) Dot plot spanning *TBC1D9* exon 21 (yellow arrow) to *UCP1* exon 6 of *S. scrofa* (*x*-axis) versus *C. wagneri* (*y*-axis). The *UCP1* enhancer is represented with the blue rectangle, while red rectangles indicate the remaining exons of *UCP1* pseudogenes.

purifying selection and neutral evolution, respectively. Given these dN/dS ratios and the 28.8 MYA Tayassuidae-Suidae divergence date from molecular data (Meredith et al., 2011), we determined that *UCP1* was pseudogenized in a common suinan ancestor 36.2–38.5 MYA. On the other hand, if this divergence date is considered to be 37 MYA based on fossil data (Prothero, 2009, 2015), our calculations place the *UCP1* inactivation 42.6–44.3 MYA.

DISCUSSION

The shared inactivating nonsense mutation in exon 6 among pigs and peccaries reveals *UCP1* inactivation in a common suinan ancestor. These findings provide the molecular basis for early seminal anatomical investigations that examined four newborn collard peccaries, all of which visually lacked BAT (Rowlatt et al., 1971). Given the genomic information of peccaries, our inactivation date estimates based on selection pressure analyses provide a better estimate of the *UCP1* pseudogenization event during the late Eocene (36.2–44.3 MYA), pushing back the previous Miocene (~20 MYA) inactivation date, which was only based on a single pig genus within the Suid lineage (Berg et al., 2006). Given the lack of exons 1 and 2, and partial deletion of the enhancer box, we expect that *UCP1* is not transcribed in peccaries, whereas some vestigial transcription of these exons occurs in pigs (Hou et al., 2017), which have retained the *UCP1* enhancer (Gaudry and Campbell, 2017).

The fairly wide range of our inactivation date estimate stems from the ambiguous phylogenetic relationships of late Eocene suinans. Prothero (2009, 2015) regards *Perchoerus* spp. as early members of the Tayassuidae lineage based on fossil data, placing the suinan radiation ~37 MYA. By contrast, others characterize *Perchoerus* spp. as a sister lineage to Suids and Tayassuids based on molecular and fossil data, placing the suinan radiation at ~30 MYA (Parisi Dutra et al., 2017). Using a fossil-constrained ~36 kbp molecular time tree, Meredith et al. (2011) place the divergence of Suids and Tayassuids at 28.8 MYA. We calculated our inactivation date estimates based on both these most recent and most ancient divergence dates. Nevertheless, our data indicate *UCP1* functionality was lost in an ‘Old World’ stem suinan, preceding the Tayassuidea split and dispersal into the ‘New World’.

Among peccaries, cold intolerance is a broadly known characteristic. For instance, collared peccaries (*Pecari tajacu*) halt summer nocturnal activity and instead huddle to reduce heat loss during the cooler winter nights, seek shelter and visibly shiver (Bissonette, 1982; Zervanos and Hadley, 1973). This species also

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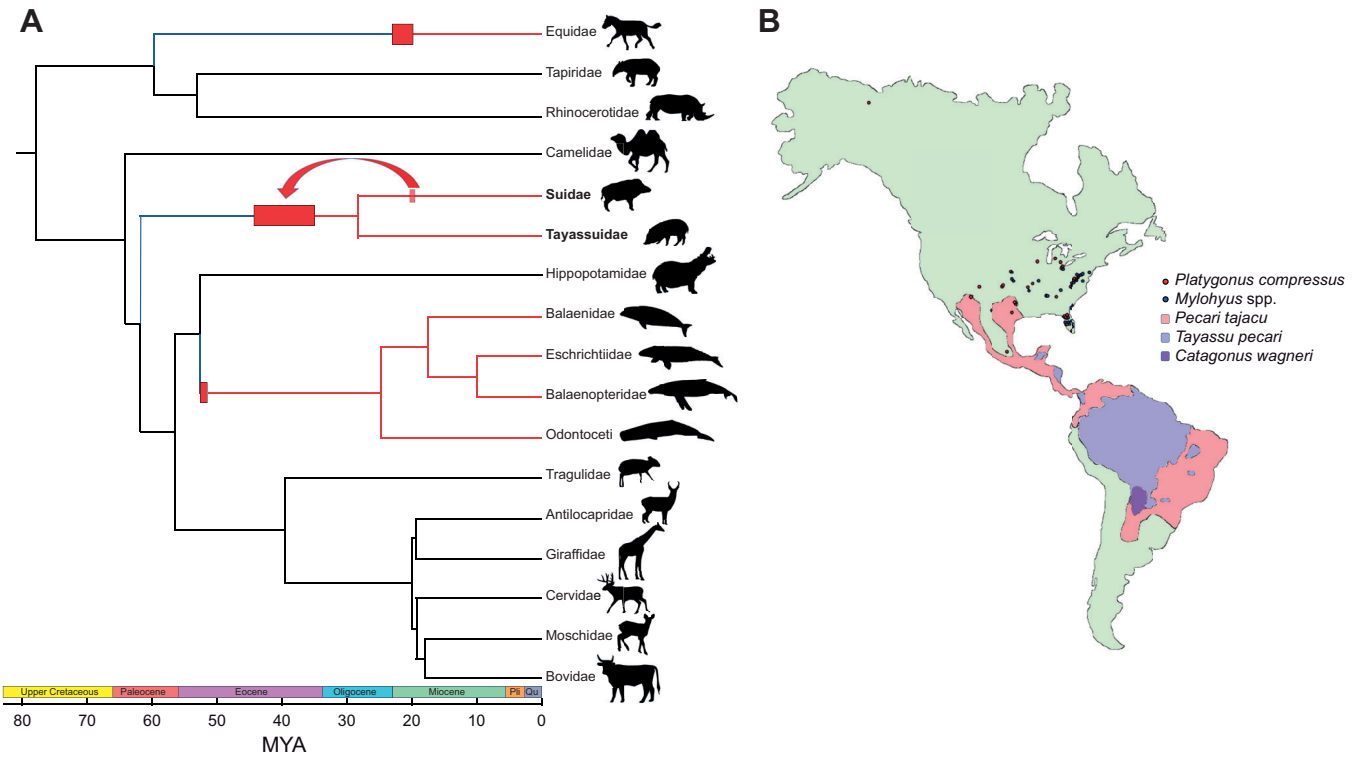

Fig. 3. *UCP1* was inactivated in a common ancestor of pigs and may contribute to the current biogeography of modern peccaries. (A) *UCP1* inactivations mapped to the ungulate phylogeny. Branch lengths are adapted from Meredith et al. (2011). Branches where *UCP1* remains intact are shown in black, while branches that display a *UCP1* pseudogene are in red. The transitional branches, along which *UCP1* switched from functional to pseudogenic, are a mixture of blue and red, while red rectangles denote our estimated date ranges for these inactivation events. The red arrow signifies the pushing back of the previous 20 million years ago (MYA) inactivation estimate by Berg et al. (2006) to a common Suina ancestor. *UCP1* has also been inactivated in stem ancestors of equids and cetaceans (Gaudry et al., 2017). Note that as in Gaudry et al. (2017), ω for the transitional cetacean branch (ω=1.1089) is slightly higher than that of the pseudogenic branch category (ω=1.0025), thus the inactivation date is assumed to be at the base of the transitional branch. (B) Tropical and subtropical geographic distribution of the three extant peccary species (*Pecari tajacu*, *Tayassu pecari* and *Catagonus wagneri*). Locations of fossil recoveries of extinct Tayassuids (*Platygonus compressus* and *Mylohyus* spp.) indicate peccaries were selected out of northern latitudes. Fossil localities of extinct Tayassuids were predominantly data-mined from the Fossilworks database (see Table S2 for references of fossil localities), while the current distributions of extant peccaries were adapted from the IUCN red list database.

increases the dark coloration and density of their pelt over the winter months, facilitating heat absorption during extended periods of sun basking (Zervanos and Hadley, 1973). The lower critical temperature of the collared peccary thermal neutral zone ranges between 25 and 28°C (Zervanos, 1975) and their cold limit is much higher (–12°C ambient temperature without air movement) as compared to domestic swine (Porter and Gates 1969; Zervanos and Hadley, 1973). On the other hand, the thermoregulatory strategies of peccaries is shifted towards higher heat dissipation, likely facilitating life in the tropics (Zervanos and Hadley, 1973). Thus, it is conceivable that the lack of UCP1-mediated NST contributes to physiological cold sensitivity of both pigs and peccaries, as well as interesting behavioral adaptations such as huddling and basking in peccaries. A strikingly high mortality rate (50–100%) among collared peccary young has not been attributed to a single factor (Bissonette, 1982), but may be exacerbated by the lack of UCP1, making offspring more vulnerable towards cold temperatures. While sarcolipin-mediated muscle NST has been claimed to overcome the lack of functional UCP1 in wild boars (Nowack et al., 2019), the potential thermogenic contributions of sarcolipin have been questioned by others (Campbell and Dicke, 2018) and have yet to be experimentally demonstrated. It has been proposed that UCP3 in adipose tissue may be thermogenic in cold-tolerant pig breeds Lin et al., (2017), yet no direct evidence has so far shown that UCP3 uncouples respiration (Jastroch et al., 2018), contributing to NST. However, these potential compensatory mechanisms would still be worth investigating in peccaries and pigs.

Overall, *UCP1* pseudogenization in peccaries further exemplifies that UCP1-mediated NST is a ‘use it or lose it’ phenomenon, likely resulting from its specific function and expression as a thermogenic protein. We previously demonstrated that *UCP1* is also lost in whales and dolphins, horses, elephants, hyraxes, sea cows, xenarthrans and pangolins (Gaudry et al., 2017; and McGaugh and Schwartz, 2017). Inactivation dates calculated for other ungulate *UCP1* pseudogenes, among equids and cetaceans, were highly congruent with Gaudry et al. (2017) (Fig. 3A). While the majority of *UCP1* pseudogenizations appear to be temporally correlated to evolutionary increases in body size, we currently have only a rudimentary picture of the root causes of these inactivations. Thus, the examination of additional eutherian species is key to enhancing the dating precision of these genetic events in order to reconstruct a complete image of environmental and physiological constraints that led to the repeated pseudogenization of *UCP1*.

Hypothetically, if BAT is unnecessary because of warm ambient temperatures and/or large body size, for example, selection pressures on *UCP1* are presumably minimal or non-existent; thus, the locus is free to accumulate random mutations that may lead to its inactivation without physiological consequences. However, the detriments of such reductions to the genetic repertoire for future lineages are perhaps evident in the biogeographical confinement to subtropical/

tropical habitats, increased cold sensitivity, low species diversity and high extinction rates. Indeed, xenarthrans and pangolins also are confined to tropical habitats and lack functional *UCP1* (Gaudry et al., 2017). Extinct peccary species (e.g. *Platygonus compressus* and *Mylohyus* spp.) once ranged over more northern parts of the current USA, with one fossil even being recovered from Yukon, Canada (Beebe, 1980). However, all extant species are currently restricted to tropical/subtropical latitudes (Fig. 3B).

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.G.; Methodology: T.J.F., C.S., M.G.; Formal analysis: T.J.F., C.S., M.G.; Investigation: M.G.; Resources: M.J.; Data curation: T.J.F., C.S.; Writing - original draft: T.J.F., C.S., M.G.; Writing - review & editing: M.J., M.G.; Visualization: M.G.; Supervision: M.J., M.G.; Funding acquisition: M.J.

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Supplementary information

Supplementary information available online at
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