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Gene expression reveals the pancreas of Aselli as a critical organ for plasma cell differentiation in the Eurasian common shrew

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Abstract

Background Almost all mammals rely on the thymus and bone marrow to generate and differentiate B and T cells essential for adaptive immunity. A few members of the family Soricidae, or true shrews within Eulipotyphla, have also evolved an enlarged pancreas of Aselli, a kidney-sized organ hypothesized to serve this primary immune role, and whose gene expression profile is unknown.

Results Here we introduce transcriptomes of juvenile Eurasian common shrews (*Sorex araneus*, family Soricidae) pancreas of Aselli. We compare these to those of the shrew spleen and chick bursa of Fabricius, an analogous, bird-specific organ, and explore differential expression overlaps with positively selected genes. Differential gene expression analyses revealed higher expression of genes that regulate the differentiation of B cells into long-term plasma cells (e.g., *IRF4*, *XBP1*, *PRDM1*) compared to the spleen and more convergent expression with the bursa of Fabricius than expected by chance (including *IRF4*). Overlaps with positive selection were as expected and included *PTPRCAP*, which regulates both T and B cell antigen responses and lymph node size.

Conclusions Our results support the specialized role of the pancreas of Aselli in adaptive immunity, and we propose this uniquely enlarged organ evolved at the intersection between extreme metabolic demands and high parasite burdens in tiny yet very active shrews.

Keywords Aselli's pancreas, *Sorex*, Transcriptomics, Plasma cells, Selection

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Background

Mammals rely on primary immune organs, such as the thymus and bone marrow, to generate and differentiate cells essential for adaptive immunity, including B and T cells [1, 2]. Yet, members of the family Soricidae (order Eulipotyphla), or true shrews, have evolved a uniquely large immune organ known as the pancreas of Aselli [3]. This enlarged mesenteric lymphoid organ has a dense aggregation of lymphocytes, with a significant proportion made up of plasma cells in varying degrees of maturation [4, 5]. In the Eurasian common shrew (*Sorex araneus*), it accounts for approximately 1% of the total body mass, comparable in size to a kidney [4]. This organ is especially notable in shrews for its unique size and structure. Unlike most mammals, which possess small, distributed mesenteric lymph nodes, shrews have a single, greatly enlarged node [3, 6]. Thus, based on cellular content, size, and location, it is hypothesized to serve as an additional primary immune organ to defend against their remarkably diverse array of parasites, including helminths (nematodes and cestodes), blood parasites (*Bartonella* sp.), and respiratory fungi (*Pneumocystis carinii*) [4, 7–10]. Despite its novelty among mammals [3, 6] and prominence in shrew physiology [4, 5, 7], the molecular mechanisms underlying the evolution and function of this immune organ remain largely uncharacterized.

While the pancreas of Aselli is an enlarged mesenteric lymph organ [6], other vertebrate clades have evolved other specializations to enhance sampling of gut-derived antigens (Fig. 1A), including gut-associated lymphatic tissue (GALT) extensions, like Peyer's patches [11]. For example, rabbits possess a more specialized GALT, called the *sacculus rotundus*, which is a distinct sac-like structure at the terminal portion of the ileum [12]. However, unlike a true primary immune organ [13], the *sacculus rotundus* does not generate immune cells independently and is instead seeded by the thymus. In contrast, the only comparable analogue to the pancreas of Aselli in evolutionary history (originally hypothesized by Tsiperson [4]) is the cloacal bursa [14], specifically, the bursa of Fabricius in birds [15, 16]. The bursa serves as the primary

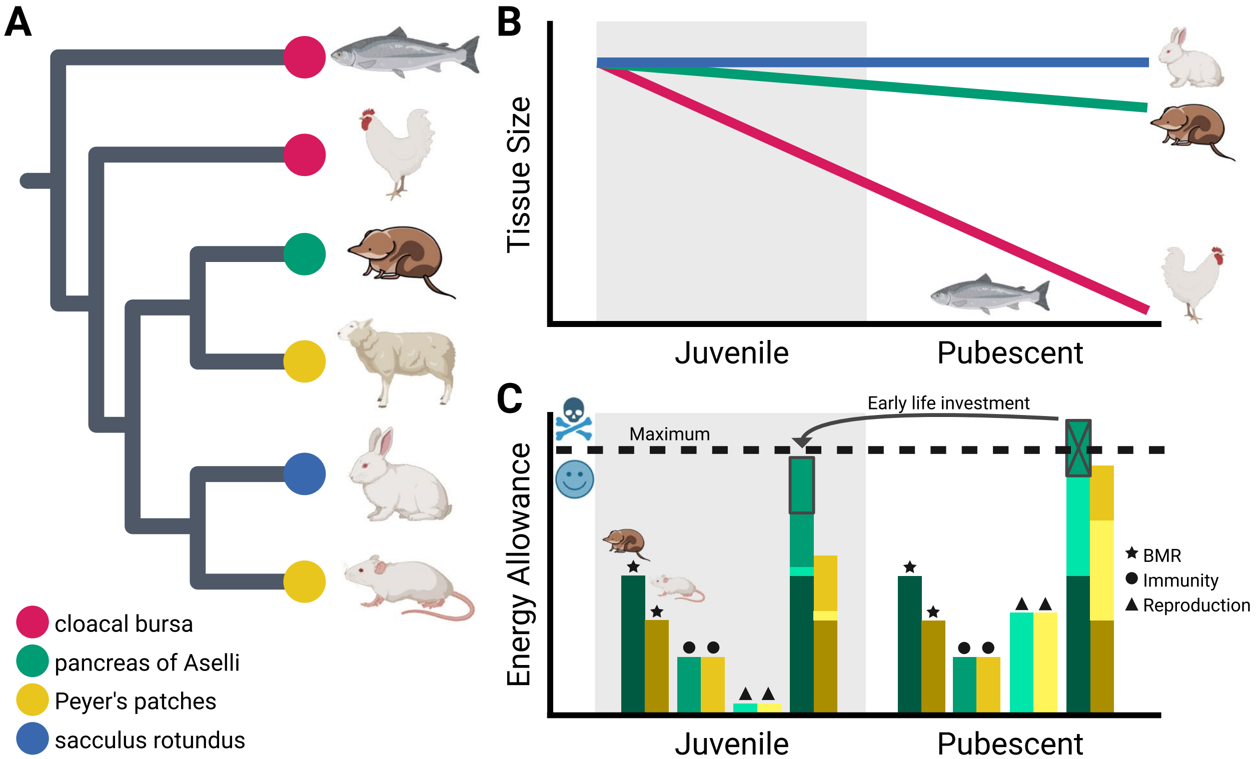

Fig. 1 **A** Independent evolution of gut-sampling lymphoid tissue across vertebrates, giving rise to unique organs such as the pancreas of Aselli in shrews and the sacculus rotundus in lagomorphs. **B** Both the pancreas of Aselli and cloacal bursa are prominent early in life, suggesting an important role in early immunity. However, unlike the cloacal bursa, the pancreas of Aselli persists into adulthood, emphasizing function beyond early-life immunity. **C** Shrews operate near their maximum energy allowance due to their exceptionally high basal metabolic rates. As they approach reproductive age, their energy demands may exceed sustainable levels, creating trade-offs between physiological functions. The pancreas of Aselli may represent an adaptive strategy that minimizes energetic cost of immune function during reproduction by serving as an early-life investment in immunity

site of B cell differentiation and proliferation, where gut-derived antigens that travel through the cloaca help shape antibody diversification through affinity maturation and gene conversions [17]. Mature B cells from the bursa then migrate to peripheral immune organs [18, 19], such as the spleen and lymph nodes, contributing immediately toward immune responses. The parallels between the bursa of Fabricius and the enlarged pancreas of Aselli are striking; both are unique within their respective clades (shrews and non-ratite birds), both house dense lymphoid populations, and both invest in early-life contributions to developing adaptive immunity.

Despite apparent similarities, development and cellular function indicate that the pancreas of Aselli and the bursa of Fabricius may not be direct evolutionary analogues [5]. One major distinction lies in their ontogenetic trajectories (Fig. 1B). The bursa of Fabricius reaches its largest size a few weeks after hatching, but rapidly involutes as birds reach sexual maturity [15, 20], much like the thymus in vertebrates [21]. In contrast, the pancreas of Aselli does not degenerate in size and function upon puberty. Instead, it increases plasma cell storage as the shrew reaches adulthood [5]. Plasma cells secrete an antibody against a specific antigen and are longer lived in comparison to mature B cells [22]. Thus, the bursa of Fabricius production of mature B cells is likely for a short-term increase in the number of adaptive immune cells as juveniles, while plasma cell stores in the shrew's pancreas of Aselli can be used for future antibody secretion as adults [5].

Life history traits and energy tradeoffs may help explain why the enlarged pancreas of Aselli is unique to shrews and absent or smaller in other mammals. Organisms must allocate limited energy between somatic growth, survival, and reproduction, creating evolutionary constraints. Shrews, however, face extreme challenges. With basal metabolic rates nearly twice what is expected for their size [23, 24], they have minimal energy reserves and low starvation endurance [25], with little flexibility. Mounting immune responses to their many parasites is especially taxing, particularly during reproduction, which is already energetically demanding for small mammals [26]. Unlike longer-lived species that can afford to delay reproduction to recover from disease, or short-lived rodents with biannual or continuous breeding shortly after birth, shrews must survive through one winter and then cannot miss their one reproductive opportunity [27]. Their exceptionally short lifespans compared to most mammals [28] restrict them to this single breeding season; their one chance to reproduce is critical. Evolving a larger mesenteric lymphoid organ may therefore be an adaptive strategy related to reproduction, allowing shrews to invest in long-lived immune cells early in life. Thus, immune investment would occur before reproduction drains their limited energy reserves, ensuring they can mount efficient immune responses through adulthood (Fig. 1C).

We investigated the regulatory functions of the pancreas of Aselli in *Sorex araneus* and its role in plasma cell development by conducting a transcriptomic comparison against a reference secondary immune organ, the shrew spleen. To explore the evolutionary significance of differentially expressed genes, we compared them to two independent analyses. First, we explored the effects of selection on the evolution of the enlarged pancreas of Aselli by comparing differentially expressed genes against those previously identified as evolving under positive selection in *S. araneus*. Then we assessed whether the pancreas of Aselli represents a functional analogue to the avian bursa of Fabricius by testing for differential expression between the avian bursa and spleen and comparing against within-shrew findings. These analyses revealed that adaptative immunity and genes involved in plasma cell differentiation (*XBP1*, *PRDM1*, *IRF4*) were expressed significantly higher in the pancreas of Aselli compared to the spleen, supporting its specialized role in long-term immune function. While more genes than expected by chance were significantly higher expressed in the pancreas of Aselli and the bursa of Fabricius compared to their corresponding spleens, key genes involved in B cell maintenance (*PAX5*, *BCL6*) did not overlap across the two organs, highlighting differences in adaptive immune function.

Results

RNA sequencing and alignment

RNA sequencing of *S. araneus* and *G. gallus* organs yielded high-quality data with comparable sequencing depths across organ type and species. *S. araneus* samples had a mean post-filtration sequencing depth of 41 million reads, with sequencing depths within an order of magnitude between the spleen (mean 34.8 million reads) and the pancreas of Aselli (48.1 million reads) (Additional file 1: Table S1). These results were consistent with publicly available transcriptomic datasets on the SRA. For *G. gallus*, the mean sequencing depth was 58.5 million reads, with specific BioProjects showing averages of 53.4 million reads for PRJNA494531 (bursa of Fabricius) [29, 30], 65.6 million reads for PRJNA511787 (bursa of Fabricius) [31], and 58.98 million reads for PRJNA623599 (spleen) [32, 33]. Mapping efficiency of sequencing data was similarly high across species. On average, 71.1% of *S. araneus* reads mapped to the transcriptome (GCF_027595985.1_mSorAra2.pri), with spleen samples achieving 77.5% mapping, slightly higher than the pancreas of Aselli mapping at 64.7%. *Gallus gallus*

(GCF_000002315.5_GRCg6a) mapping rates were comparable to those of the common shrew, averaging 77.3% for the spleen and 79.0% for the bursa of Fabricius.

Differential gene expression

Thousands of genes were differentially expressed between the *S. araneus* pancreas of Aselli and spleen, with several immune-related pathways, particularly those associated with adaptive immunity, increased in the pancreas of Aselli. To assess overall transcriptomic differences between these organs, we first performed a PCA (Fig. 2A). This analysis revealed a strong separation between the two organ types along PC1 (74.3% of variance), while intra-organ variation, particularly within the spleen, was captured on PC2 (6.9% of variance). Given this clear distinction in gene expression profiles, we proceeded with a differential gene expression analysis without a Log Fold Change (LFC) threshold to identify specific genes promoting these differences. Of the 24,205 genes tested (Additional file 2: Table S2), 5997 were significantly differentially expressed after multiple test correction ($p_{adj} < 0.05$), with 2500 increased in the pancreas of Aselli and 3497 decreased in comparison. Genes critical to plasma cell differentiation (Table 1) [34–36] were significantly higher, *XBP1* ($p_{adj} < 0.001$, LFC = 1.67), *PRDM1* ($p_{adj} < 0.001$, LFC = 1.76), and *IRF4* ($p_{adj} < 0.001$, LFC = 0.95), while most key genes in B cell maintenance [34, 37–40] were not, *PAX5* ($p_{adj} = 0.86$, LFC = −0.08), *BCL6* ($p_{adj} = 0.31$, LFC = 0.38), except *BACH2* ($p_{adj} < 0.001$, LFC = 1.70) (Fig. 2B). To further determine the functional significance of these differences, we conducted a ranked pathway enrichment analysis of KEGG pathways (Additional file 3: Table S3). This analysis identified 22 enriched pathways ($p_{adj} < 0.05$), with 11 showing increased expression and 11 with decreased expression in the pancreas of Aselli (Fig. 2C). Notable pathways with lower expression included porphyrin and chlorophyll metabolism ($p_{adj} < 0.001$, NES = −2.17), while key pathways with higher expression were predominantly immune-related. These included primary immunodeficiency ($p_{adj} < 0.001$, NES = 2.03), antigen processing and presentation ($p_{adj} < 0.001$, NES = 2.02), T cell receptor signaling ($p_{adj} < 0.001$, NES = 1.65), natural killer cell mediated cytotoxicity ($p_{adj} < 0.05$, NES = 1.46), and cytokine-cytokine receptor interaction ($p_{adj} < 0.01$, NES = 1.45).

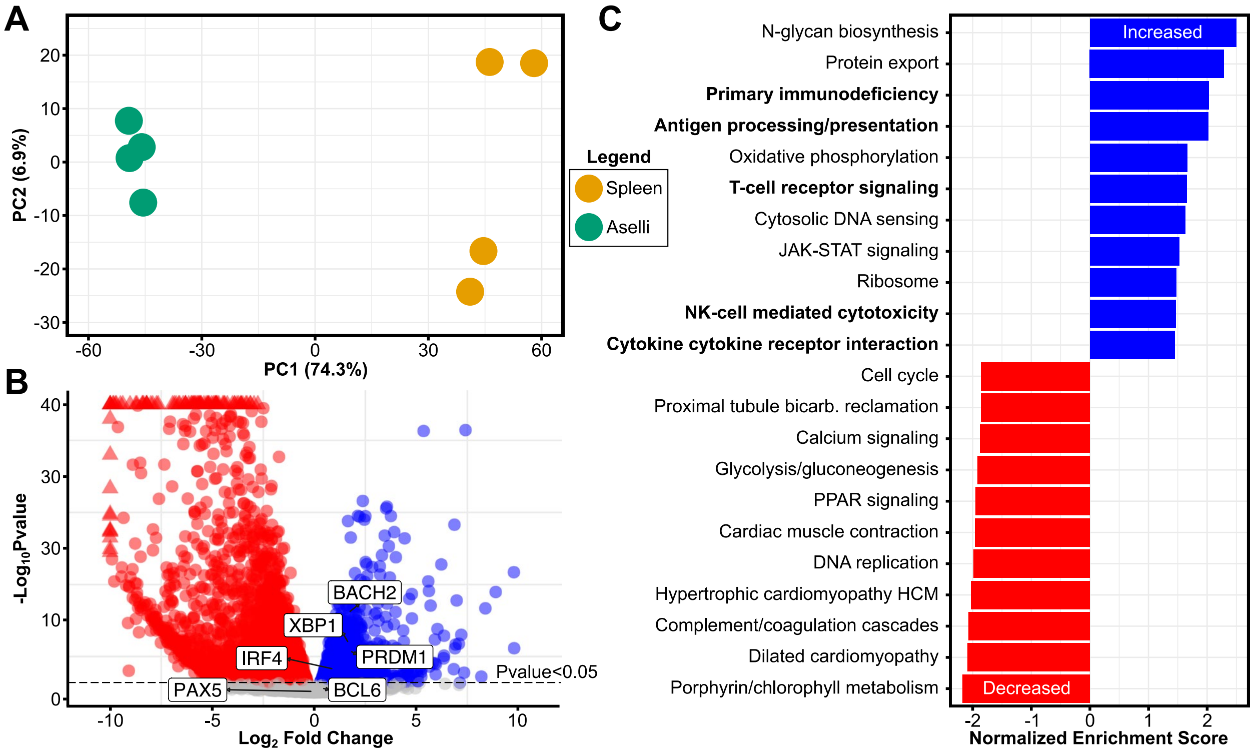

Fig. 2 **A** Principal component analysis (PCA) of the 5000 most variable genes shows distinct separation between the spleen and pancreas of Aselli along PC1. **B** Volcano plot quantifying differentially expressed genes ($p_{adj} < 0.05$), with 2500 significantly expressed genes with increased expression (blue) and 3497 genes with decreased expression (red) in the pancreas of Aselli. Noted genes include those associated with B cell maintenance (*PAX5*, *BCL6*, *BACH2*) and plasma cell differentiation (*XBP1*, *PRDM1*, *IRF4*). **C** Pathway enrichment analysis revealed 11 pathways with increased expression (blue) and 11 pathways with decreased expression (red) in the pancreas of Aselli, suggesting adaptive immune-related function

Table 1 List of key genes involved in immune cell development of the pancreas of Aselli

Gene	Function
PAX5	Master regulator of B cell program needed for initial commitment of lymphoid progenitors to B cells. Represses plasma cell pathway. [30–35]
BCL6	Required for B cell proliferation and somatic hypermutation in the germinal center. Represses *PRDM1* and antibody secreting cell differentiation. [30–32, 36]
BACH2	Regulated by PAX5 and expressed during B cell differentiation. Represses *PRDM1*, therefore controlling the timing of plasma cell differentiation. [30–32, 35]
XBP1	Transcription factor upregulated in protein secreting cells to regulate genes involved in antibody secreting cell differentiation (e.g., plasma cells). Repressed by *PAX5*. [30, 32]
PRDM1	Encodes BLIMP1, a transcriptional repressor (*PAX5*, BCL6) required for terminal differentiation of B cells into antibody secreting cells. Upregulated in long-lived plasma cells. [30–32]
IRF4	Transcriptional regulator important for immunoglobin recombination, B cell formation, and plasma cell differentiation. Represses *BCL6* and activates *PRDM1*. [30, 32]

We compared differentially expressed genes to those inferred to be evolving under positive selection in *S. araneus* from previous work [41, 42] and identified some overlap in significant genes. Of the 2500 genes increased for expression in the pancreas of Aselli and the 231 inferred to be under common shrew specific positive selection from previous work [41], 21 were found to overlap between both data sets. These genes notably include the plasma cell differentiation gene *PRDM1* (Additional file 4: Fig. S1), as well as the protein tyrosine phosphatase receptor type C- associated protein, *PTPRCAP* (Fig. 3). However, this overlap was not greater than expected by chance ($\chi^2_1 = 1.1$, pval = 0.29).

To test whether the bursa of Fabricius is a functional analogue of the pancreas of Aselli, at least at the transcriptomic level, we performed a differential expression analysis of the chicken bursa of Fabricius to the chicken spleen and compared these results to the same analysis

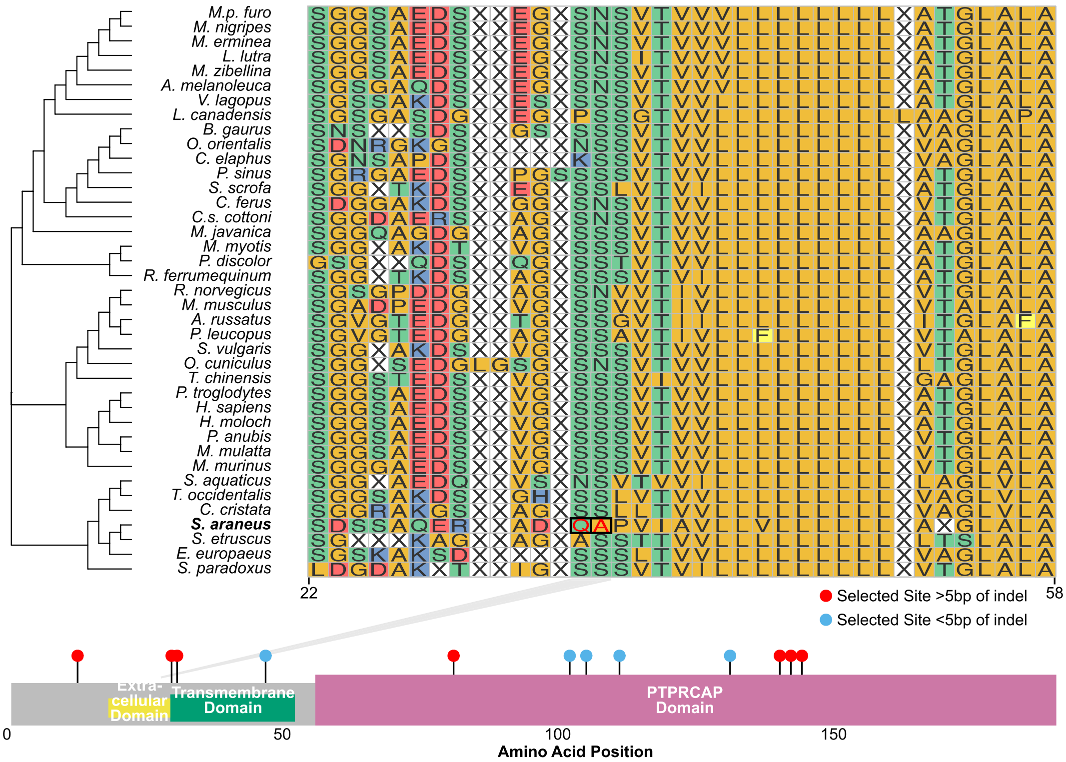

Fig. 3 Multiple sequence alignment of the extracellular and transmembrane domains of the positively selected gene, *PTPRCAP* (amino acids 22–58), across 39 mammalian species from a previous analysis [41]. Two of the 12 sites identified to be under positive selection (indicated by pins on the protein domains) by MEME (Mixed Effect Models of Evolution) are highlighted, which may have functional consequences for *PTPRCAP* binding capabilities to CD45

in the common shrew. Of the 23,747 genes tested (Additional file 5: Table S4), 11,184 were significantly differentially expressed, with 5744 increased and 5440 decreased in the bursa of Fabricius. Among these, 964 genes were significantly lower in both the pancreas of Aselli and the bursa of Fabricius and 543 higher in both. This overlap is significantly more than expected by chance (hypergeometric test, $p < 0.001$). Next, we examined the genes critical to plasma cell differentiation (*XBP1*, *PRDM1*, *IRF4*) and B cell maintenance (*PAX5*, *BCL6*, *BACH2*). All three of the B cell maintenance genes were increased in the *G. gallus* bursa of Fabricius: *PAX5* ($p_{adj} < 0.001$, LFC = 3.08), *BCL6* ($p_{adj} < 0.001$, LFC = 1.01), and *BACH2* ($p_{adj} < 0.001$, LFC = 1.61). However, only two plasma cell differentiation genes, *XBP1* ($p_{adj} < 0.001$, LFC = −0.48), *PRDM1* ($p_{adj} < 0.001$, LFC = 0.38), and *IRF4* ($p_{adj} < 0.001$, LFC = 1.14), were significantly higher in the bursa of Fabricius, all of which were increased in the common shrew pancreas of Aselli (Fig. 4).

Immunoglobulin repertoire diversity

To assess adaptive immune diversity, we used ImReP to assemble immunoglobulin receptor sequences from transcriptomic data across four pancreas of Aselli (Table 2; sequences available in Additional file 6: Table S5). A total of 6077 unique receptor sequences were recovered across all samples. The number of identified immunoglobulin heavy chain (IGH) transcripts ranged from 751 to 1730 per individual, with kappa and lambda light chain (IGK and IGL) transcripts detected at lower frequencies (8

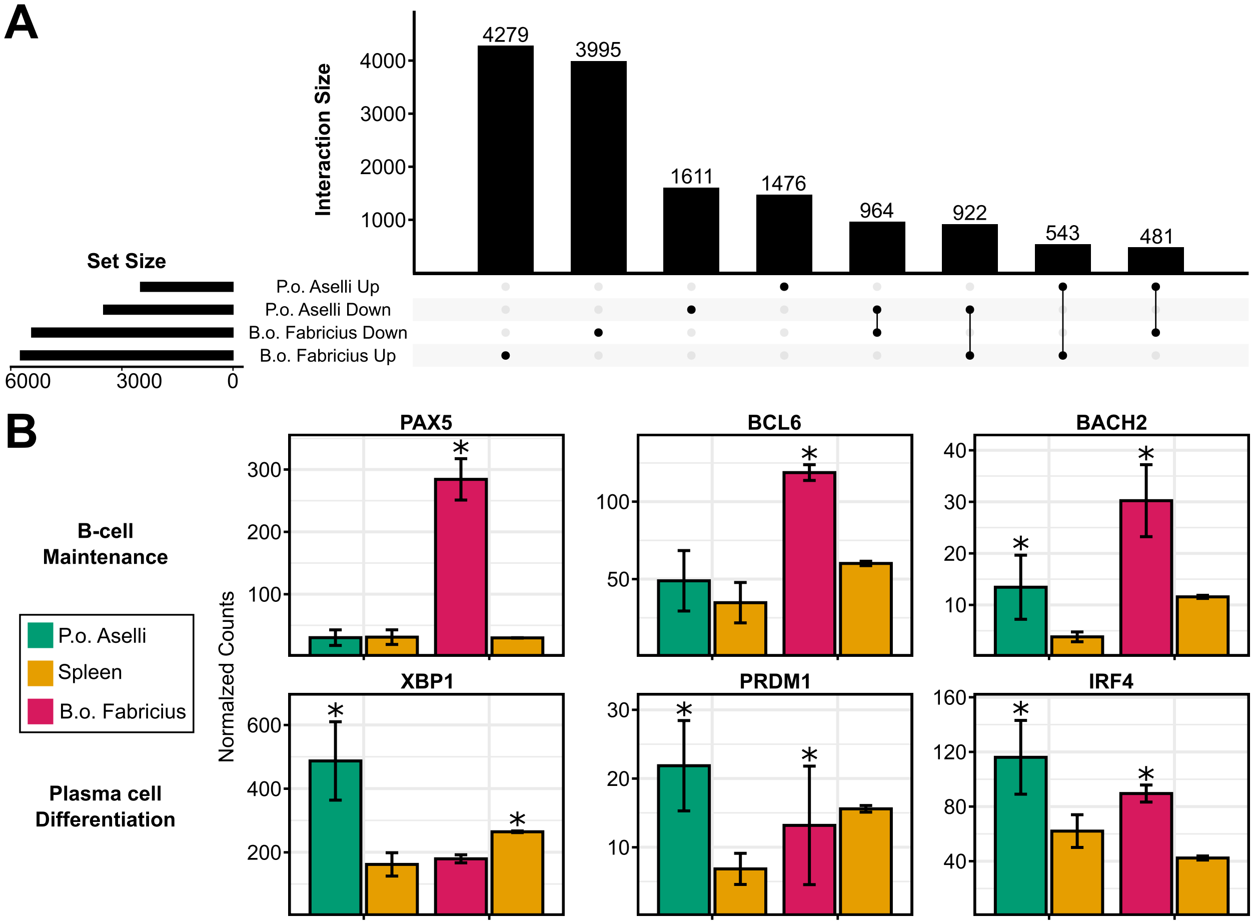

Fig. 4 **A** Upset plot showing overlap in significantly differentially expressed genes between the pancreas of Aselli ($n = 4$) vs. spleen ($n = 4$) in *Sorex araneus* and the bursa of Fabricius ($n = 5$) vs. spleen ($n = 3$) in *Gallus gallus*. The total significant genes of each set is found to the left of each result (Set Size), with overlapping significant genes between analyses indicated by connected interactions (Interaction Size). Here, 1507 (543 up, 964 down) genes exhibited concordant regulation between the pancreas of Aselli and bursa of Fabricius. **B** Comparative analysis of important B cell differentiation genes (*PAX5*, *BACH2*, *BCL6*) and plasma cell differentiation genes (*XBP1*, *PRDM1*, *IRF4*) reveals differences in regulatory patterns between the shrew's pancreas of Aselli and the chicken's bursa of Fabricius, suggesting divergent adaptive immune function. Significant comparisons are indicated by an asterisk ($p_{adj} < 0.05$). Error bars represent one standard deviation from the mean of the data

Table 2 Diversity of immunoglobulin receptors in each pancreas of Aselli

Individual	ID	IGH	IGK	IGL	TRA	TRB	TRD	TRG	partial-V CDR3	partial-J CDR3
1	ML6	1666	12	20	101	11	0	0	819,361	174,417
2	ML7	751	8	12	146	21	0	0	923,810	120,245
3	ML8	1562	13	20	91	14	0	0	711,354	170,959
4	ML9	1730	13	18	107	16	0	0	878,294	183,076

to 20). T cell receptor alpha (TRA) and beta (TRB) were also recovered, while delta (TRD) and gamma (TRG) were not detected. However, the partial-V and partial-J CDR3 sequences identified by ImReP numbered in the hundreds of thousands for each individual. This suggests that there is a high repertoire complexity that may not be fully assembled due to our short-read sequencing (100 bp/read).

Discussion

To gain insight into its functional and evolutionarily adaptive role in Eurasian common shew immunity, we characterized gene expression of the pancreas of Aselli. Our analysis revealed significant differences in gene expression between the pancreas of Aselli and the spleen (Fig. 2A), with 2500 genes with increased expression and 3497 genes with decreased expression (Fig. 2B). Pathway enrichment analysis indicated that genes with higher expression in comparison to the spleen were functionally linked to immunity, such as primary immunodeficiency, antigen processing and presentation, T cell receptor signaling, natural killer cell-mediated cytotoxicity, and cytokine-cytokine receptor interactions (Fig. 2C). These pathways are predominantly associated with adaptive instead of innate immunity [22, 43–45], suggesting the pancreas of Aselli facilitates antigen-dependent immune responses instead of rapid, non-specific immunity. Supporting this, we observed high immunoglobulin receptor diversity in the pancreas of Aselli (Table 2; Additional file 6: Table S5), with thousands of unique IGH, IGK, and IGL transcripts identified, in addition to many partial CDR3 sequences. Conversely, genes involved in porphyrin metabolism, or the production and degradation of heme for oxygen transport, were lower in the pancreas of Aselli. This evidence aligns well with a previous morphological study [5] showing that the pancreas of Aselli shares a cellular composition similar to that of a lymph node, containing adaptive immune cells such as T cells, B cells, neutrophils, and macrophages, and plays a little role in filtering oxygen-carrying red blood cells, a key function of the spleen.

Functionally, the pancreas of Aselli differs from a typical lymph node in that its medulla is almost entirely composed of plasma cells [5], or terminally differentiated B cells reflecting prior immune experience or antigen exposure. This cellular composition is strongly supported by gene expression data, which indicated a regulatory shift toward plasma cell production. We observed minimal changes in the expression of genes associated with B cell maintenance (*PAX5*, *BCL6*, *BACH2*) [34], with only *BACH2* showing increased expression (Fig. 2B, 4B). These transcriptional repressors play a crucial role in maintaining B cell identity [34, 37–40] and preventing premature differentiation into plasma cells [35, 36] by inhibiting key genes such as *PRDM1* and *XBP1*. In contrast, three key genes involved in plasma cell differentiation (*XBP1*, *PRDM1*, and *IRF4*) were all significantly increased in the pancreas of Aselli. This reinforces this organ's role as a site for plasma cell rather than naïve B cell storage, as *PRDM1*, *IRF4*, and *XBP1* drive the transition to antibody-secreting plasma cells [34], with *PRDM1* repressing B cell associated genes [46, 47], which can be activated by *IRF4* to promote plasma cell fate [48]. This pattern in the pancreas of Aselli aligns with single-cell sequencing of mouse immune cells, where *PAX5* and *BACH2* are highly expressed in naïve follicular B cells but absent in long-lived plasma cells, which instead express *PRDM1*, *IRF4*, and *XBP1* [49]. The significant increased expression of plasma cell differentiation genes in the pancreas of Aselli, alongside minimal changes in B cell maintenance genes, highlights its specialized role in long-term plasma cell production instead of the generalized B cell development typical of lymph nodes, and may explain why more plasma cells are found in the lymph nodes of the shrew compared to other species [5].

Gene expression evidence suggests the pancreas of Aselli is not a direct analogue of the bursa of Fabricius, supporting earlier morphological studies [5] (Fig. 4A). When comparing the chicken bursa of Fabricius to the spleen, we found that genes associated with naïve B cells (*PAX5*, *BACH2*, *BCL6*) were increased in the bursa, a pattern that contrasts with the minimal change generally observed in the pancreas of Aselli, except in *BACH2* (Fig. 2B, 4B). Despite these differences, a comparison of differentially expressed genes across both analyses revealed a greater overlap than expected by chance (hypergeometric test, $p < 0.001$) of concordant changes. These similarities can be observed in the plasma cell differentiation genes. Some genes upregulated in the shrew pancreas of Aselli, including *IRF4* and *PRDM1*, were also increased in the bursa of Fabricius (Fig. 4B). These findings indicate that the pancreas of Aselli and the bursa of Fabricius are not precise functional equivalents but have evolved independently to enhance adaptive immunity using some similar regulatory mechanisms.

Natural selection may shape some of the key regulatory genes of the pancreas of Aselli compared to other mammals. We compared genes increased in expression in the pancreas of Aselli to those we previously inferred to be under positive selection specific to the common shrew [41] in comparison to 39 other mammals. In this comparison, continuous evolution, refinement, and innovations of complex immune systems [50, 51], or the pancreas of Aselli having increased selection to refine its immune function, would be indicated by greater than expected overlap. However, we did not find more overlap than expected by chance ($\chi_1^2 = 1.1$, $p = 0.29$). Instead, we find few overlapping genomic changes, indicating that many genes under positive selection are unrelated to those differentially expressed in the pancreas of Aselli.

As *PRDM1* appears critical to the adaptive immune functions of the pancreas of Aselli, we note that *PRDM1* was inferred to be under positive selection (Additional file 4: Fig. S1) in our previous work [41]. However, 70% of the sites inferred to be under positive selection were found in the first 100 amino acids, which could represent an alternative start site [52]. Importantly though, in previous work we also found *PTPRCAP* was under positive selection (Fig. 3) [41], including at the first two amino acids of its transmembrane domain. *PTPRCAP* is typically expressed in lymphocytes [53], where it interacts with *CD45*, a key regulator of antigen receptor–mediated signal transduction and lymphocyte development [54]. In mice, deletions in the transmembrane, rather than the extracellular domain, disrupt *PTPRCAP* binding with *CD45* [55], with deficient or knockout mice showing decreased *CD45* expression [56] that can, in turn, impair the responses of T and B cells to antigen receptor stimulation [57]. Yet, *PTPRCAP*-deficient mice have increased cellularity and size of lymph nodes [56, 58], suggesting a role in regulating lymphocyte expansion. Evidence of adaptation in the common shrew, particularly in the organ where it has higher expression, may alter antigen presentation while simultaneously supporting lymphocyte development in this enlarged mesenteric lymphoid organ.

Together, our gene expression analyses support a model in which the pancreas of Aselli and the bursa of Fabricius serve differing roles in immune development and maintenance, corresponding to the evolved life history of their organisms. The bursa degenerates at sexual maturity (Fig. 1B), emphasizing its early-life role in B cell maturation, consistent with the elevated gene expression that sustains mature B cells (*PAX5*, *BCL6*, *BACH2*) in comparison to the avian spleen (Fig. 4B). Meanwhile, the pancreas of Aselli endures past puberty and throughout the brief lifespan of the shrew. Its gene expression profile, which shows increased expression of plasma cell differentiation genes (*PRDM1*, *XBP1*, *IRF4*) in comparison to the spleen, validates its role as a store of plasma cells. These overexpressed genes likely facilitate the long-term production and secretion of diverse antibodies in response to heavy parasite loads, which continue throughout the shrew's lifespan, tend to be higher than in other mammals, yet appear to cause no obvious clinical illness [7, 59]. This proposed function may be reflected in the diversity of immunoglobulin receptor sequences identified in the pancreas of Aselli. Overall, while the persistence and gene expression of the pancreas of Aselli suggest it functions as a long-term source of adaptive immunity into adulthood, both it and the bursa contribute to immune function along a developmental continuum to enhance survival toward adulthood and, ultimately, reproductive success.

Limitations

There are methodological limitations in this study. A key challenge in assessing whether the pancreas of Aselli is a direct analogue of the bursa of Fabricius lies in the availability of appropriate comparative sequencing data. To make a robust comparison within *G. gallus*, the bursa and secondary immune organ (the spleen) should ideally be sampled at the same developmental stage with a sufficiently large sample size (at least 4 individuals), as low sample sizes increase false positives from high variation in species outside the laboratory (i.e., *PRDM1* in bursa) [60]. However, after scanning the SRA, the only available high-quality data sets for the spleen and bursa came from 2-week-old chicks. This age discrepancy introduces confounders when comparing to the pancreas of Aselli, as adaptive immune function can be influenced by developmental stage. Experimental constraints prevent us from fully resolving the mechanisms underlying the formation and evolutionary emergence of this organ. One possibility is that shrews experience a distinct developmental fitness landscape due to their exceptionally short lifespans [28], which may result in a novel immune solution. However, our current approach does not allow us to distinguish between genes involved in the organ's development versus those contributing toward its immune function. Future research integrating embryonic time-series data could provide regulatory insights into the developmental and evolutionary origins of this immune innovation.

Conclusions

This study provides a robust characterization of gene expression for the pancreas of Aselli, offering new insights into mammalian immune system evolution.

Our findings reveal that this organ is functionally distinct from the spleen, with gene expression patterns supporting a specialized role in adaptive immunity, particularly in long-term plasma cell storage. While not a direct analogue of the bursa of Fabricius, as evident by differences in B cell maintenance genes (*PAX5*, *BACH2*, *BCL6*) and plasma cell differentiation genes (*IRF4*, *XBP1*, *PRDM1*), similarities in regulatory mechanisms indicated by greater than expected differentially expressed gene overlap suggest independent evolutionary solutions for enhancing adaptive immunity. Additionally, evidence of positive selection in key immune genes, such as *PTPRCAP*, suggests a dynamic interplay between adaptive immunity and natural selection, potentially fine-tuning the function of the pancreas of Aselli to meet the immunological demands of the common shrew. Characterizing the regulatory mechanisms of the pancreas of Aselli alongside selective processes, we highlight how evolution and immune function can act synergistically to generate adaptation, expanding our understanding of mammalian immune system diversity.

Methods

Animals and tissue collection

Sorex araneus were collected ($n = 4$) in November of 2020 from a single German (47.9684 N, 8.9761 E) population (protocols authorized by Regierungspräsidium Freiburg, Baden-Württemberg; 35–9185.81/G-19/131). Shrews were captured with insulated wooden traps baited with mealworms, which were monitored every two hours in hope of reducing trap-related stress, food insufficiency, or captivity on gene expression [61]. Once trapped, shrews were aged and sexed (two male and two female) based on tooth wear, fur condition, and gonad development. Notably, Eurasian common shrews have a single breeding season in late spring or early summer and typically do not survive in the wild beyond their second fall. Thus, all shrews captured were prepubescent individuals approximately 6 months of age. Within the transport chamber, shrews are first anesthetized by filling the container with isoflurane in a cotton ball. The anesthetized animal is then injected intraperitoneally with 2 mg/kg of Butorphanol to achieve adequate analgesia. Shrews were then perfused transcardially with PAXgene Tissue Fixative. Then the pancreas of Aselli and spleen were removed and placed in PAXgene Tissue Stabilizer for 2–24 h. These organs were then frozen and stored in liquid nitrogen (− 180 °C).

RNA extraction and sequencing

RNA was extracted from each organ using a modified version of the Micro RNeasy Micro kit (Qiagen). Modifications included [1] tissues were ground using glass mortar and pestles on dry ice for 1–2 min to reduce heat-associated RNA degradation, [2] column binding efficiency and lysing were improved by adding 7μL of 2 M dithiothreitol and 5 μL of 4 ng/μL carrier RNA to the lysate, [3] lysate was ground for an additional minute and homogenized with QIAshredder columns, and [4] DNAse incubation was reduced from 15 to 2 min. Azenta Life Sciences (New Jersey) performed quality control analyses (nanodrop and RNA ScreenTape), library preparation (poly-A selection), and sequencing (approx. 15–25 million PE reads/sample, 150 bp PE) of the spleen on an Illumina HiSeq platform. For the pancreas of Aselli, BGI (Beijing Genomics Institute, California) conducted quality control (RNA ScreenTape), library preparation (DNBseq Eukaryotic Strand-specific mRNA library), and sequencing (~ 25 million PE reads/sample, 100 bp PE) on a DNBseq platform.

Differential gene expression

Raw reads for each sequencing run were analyzed with fastp (v0.20.1) [62] to trim adapters and remove low quality sequences. Sequences were then aligned to the *S. araneus* transcriptome (GCF_027595985.1_mSorAra2.pri) using Kallisto (v0.46.2) [63]. Read counts were normalized using the median of ratios from DESeq2 (v1.36) [64] which accounts for library content and size. This step was critical to our analysis as organs were sequenced at different sequencing companies. We then ran a principal component analysis (PCA) using the 5000 genes with the most variation in the data set. Next, we tested for differential expression between the spleen and the pancreas of Aselli samples using DESeq2 [64], where gene ($n = 24{,}205$) counts are fitted with a negative binomial generalized linear model and then tested for differential expression using a Wald test, with sex as an additional covariate (~ sex + organ). The spleen was selected as a reference because, although it supports immune cell responses by developing germinal centers for B cell proliferation, it is considered a secondary immune organ rather than a primary site of immune cell development. This allowed us to identify differential gene expression related to this primary immune role that is specifically increased in the pancreas of Aselli. Significant results are identified following a multiple test correction (Benjamini Hochberg; $p_{\text{adj}} < 0.05$) [65]. We did not include an arbitrary log fold change (LFC) threshold, as comparison effect size appeared high based on our preliminary PCA, and our ranked Gene Ontology (GO) enrichment incorporates LFC into gene ranking. DESeq2 results were then run through a ranked GO enrichment using Kyoto Encyclopedia of Gene and Genomes (KEGG) pathways with the fsgea (v1.22) package [66] to explore the molecular functions of differentially expressed genes. We then examined which genes had higher expression in the pancreas of Aselli and whether they overlapped with genes identified as evolving under positive selection specific to the common shrew from previous comparative analyses [41]. A chi-squared test was used to test if observed overlap with positively selected genes were greater than expected by chance ($p < 0.05$).

To test if the bursa of Fabricius is a functional analogue of the pancreas of Aselli, we required age-matched transcriptomic data from the bursa of Fabricius and spleen in a bird species. RNA sequencing data for these tissues were obtained from the Sequencing Read Archive (SRA) on NCBI (NIH National Center for Biotechnology Information). We searched the SRA using the keyword “bursa” and restricted results to RNA-seq data from chickens (*Gallus gallus*). Combining three experiments (BioProject PRJNA494531, PRJNA623599, PRJNA511787) provided age-matched control spleen ($n = 3$) and bursa of Fabricius (n = 5) samples. Although these chickens were prepubescent, akin to the shrews, they were only 2 to 3 weeks old, whereas the shrews were 6 months old. Older bursa of Fabricius samples exists, however with a sample size that cannot measure variation (PRJNA901530, $n = 2$). This age difference introduced a potential confounding variable in our comparative analyses. Additionally, all bursa of Fabricius samples were from male chicks. To process the sequencing data, we trimmed adapter sequences and removed low-quality reads using fastp (v0.20.1) [62]. The cleaned reads were then mapped to the chicken transcriptome (GCF_000002315.5_GRCg6a) using Kallisto (v0.46.2) [63]. Read counts were normalized using the same approach as for *S. araneus*, and differential expression between the spleen and bursa of Fabricius was assessed using DESeq2 (v1.36) (~ breed + organ), with p-values corrected via the Benjamini–Hochberg method [65]. A hypergeometric test ($n = 100{,}000$) was used to test if overlap in significantly higher gene expression with the bursa of Fabricius was higher than expected by chance.

Immunoglobulin repertoire analysis

To explore immunoglobulin diversity in the pancreas of Aselli, we applied the ImRep pipeline (v1.0) [67] to bulk RNA sequencing reads for the pancreas of Aselli. ImReP reconstructs complementarity-determining region 3 (CDR3) sequences from transcriptomic data by scanning reads and anchoring them to the variable (V) and joining (J) segments amino acids, without a need for genome alignment. For each sample, we ran ImReP on the forward read, generating a set of CDR3 amino acid sequences and read counts. Only full, in-frame CDR3s were retained for comparison between samples.

This approach enables a rapid, transcriptome-based assessment of B cell receptor (BCR) diversity without requiring specialized library preparations. However, it is important to note the ImReP has its limitations, only recovering 69% of the immune repertoire obtained by specialized sequencing like BCR-Seq [67]. ImReP cannot distinguish between isotype or fully resolve light and heavy chain pairing. Additionally, because the data is derived from bulk tissue, we also cannot resolve clonality or somatic hypermutation patterns at the single-cell level. Nevertheless, the number and diversity of CDR3s recovered provide a useful proxy for B and T cell activity within the pancreas of Aselli.

Abbreviations

BCR	B cell receptor
BGI	Beijing Genomics Institute
bp	Base pairs
CDR3	Complementarity-determining region 3
GALT	Gut associated lymphatic tissue
GO	Gene Ontology
HPC	High performance computing
IGH	Immunoglobulin heavy chain
IGK	Immunoglobulin kappa light chain
IGL	Immunoglobulin lambda light chain
KEGG	Kyoto Encyclopedia of Gene and Genomes
LFC	Log Fold Change
MEME	Mixed Effect Models of Evolution
mRNA	Messenger ribonucleic acid
NCBI	NIH National Center for Biotechnology Information
NES	Normalized Enrichment Score
PC1	Principal component 1
PC2	Principal component 2
PCA	Principal component analysis
PE	Paired end
RNA	Ribonucleic acid
SRA	Sequencing Read Archive
TRA	T cell receptor alpha
TRB	T cell receptor beta
TRD	T cell receptor delta
TRG	T cell receptor gamma

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12915-025-02420-7.

Additional file 1: Table S1—Sample information and sequencing quality. Information for *Sorex araneus* includes the individual number, the organ, sex, read count before and after filtration, and the mapping percentage for each sample. *Gallus gallus* information contains the same information, as well as the SRA ID, breed, age, and Bioproject number for each individual.

Additional file 2: Table S2 – Differential expression analysis for the pancreas of Aselli. This table consists of the DESeq2 differential gene expression results for comparing the *Sorex araneus* pancreas of Aselli to the *S. araneus* spleen. Results include the gene, mean expression, log fold change and standard error, the Wald test statistic, *p*-value, and the adjusted *p*-value.

Additional file 3: Table S3 – Gene set enrichment statistics. This table is the pathway enrichment for genes in the pancreas of Aselli that are differentially expressed. This table includes statistics for the *p*-value, adjusted *p*-value, error, enrichment score, normalized enrichment score, leading edge size, and the list of genes on the leading edge.

Additional file 4: Fig. S1—Multiple sequence alignment *PRDM1*. While this gene is indicated to be under positive selection from previous work [41], many (7 of 10) positively selected sites (indicated by pins on the protein domains) were found within the first 100 amino acids and near indels, reducing confidence in this result.

Additional file 5: Table S4—Differential expression analysis for the burs of Fabricius. This table consists of the DESeq2 differential gene expression results for comparing the *Gallus gallus* bursa of Fabricius to the *G. gallus* spleen. Results include the gene, mean expression, log fold change and standard error, the Wald test statistic, *p*-value, and the adjusted *p*-value.

Additional file 6: Table S5 – Immunoglobulin receptor diversity. This table includes the immunoglobulin receptor sequences from transcriptomic data across each pancreas of Aselli tissue generated from ImReP. A total of 6,077 unique receptor sequences were identified, including immunoglobulin heavy chain (IGH), kappa and lambda light chain (IGK and IGL) transcripts, and T cell receptor alpha (TRA) and beta (TRB). However, T cell receptor delta (TRD) and gamma (TRG) were not found.

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Authors' contributions
Conceptualization: WRT; Methodology: WRT; Validation: WRT; Formal analysis: WRT; Investigation: WRT; Resources: LMD, TL; Data Curation: WRT, CB; Writing – Original Draft: WRT; Writing – Review and Editing: LMD, CB, DE, TL, YZ, AC, JN; Visualization: WRT; Supervision: LMD; Project administration: LMD; Funding acquisition: LMD, DD, JN, DE, APC.

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Data availability
The datasets (raw sequencing) generated in the current study are available in the NCBI Sequencing Read Archive, under BioProject PRJNA941271. The datasets analyzed in the current study are available on Github https://github.com/wrthomas315/project_aselli (analysis, supplementary tables, data, results, and code) and with in the article (and its additional files).

Declarations

Ethics approval and consent to participate
Ethical approval for the use of animals in this study was approved by Regierungspräsidium Freiburg, Baden-Württemberg (reference number 35–9185.81/G-19/131).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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