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AMHY and sex determination in egg-laying mammals

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Abstract

Background: Egg-laying mammals (monotremes) evolved multiple sex chromosomes independently of therian mammals and lack the sex-determining gene *SRY*. The Y-localized anti-Müllerian hormone gene (*AMHY*) is the candidate sex-determination gene in monotremes. Here, we describe the evolution of monotreme *AMHX* and *AMHY* gametologues and for the first time, investigate their expression during gonad sexual differentiation in a monotreme.

Results: Monotreme *AMHX* and *AMHY* have significant sequence divergence at the promoter, gene, and protein level, likely following an original allele inversion in the early stages of monotreme sex chromosome differentiation but retaining the conserved features of TGF-β molecules. We show that the expression of sexual differentiation genes in the echidna fetal gonad, including *DMRT1* and *SOX9*, is significantly different from that of therian mammals. Importantly, *AMHY* is expressed exclusively in the male gonad during sexual differentiation consistent with a role as the primary sex-determination gene whereas *AMHX* is expressed in both sexes. Experimental ectopic expression of platypus AMHX or AMHY in the chicken embryo did not masculinize the female urogenital system, as does chicken AMH, a possible result of mammalian-specific changes to AMH proteins preventing function in the chicken.

Conclusions: Our results provide insight into the early steps of monotreme sex chromosome evolution and sex determination with developmental expression data strongly supporting *AMHY* as the primary male sex-determination gene of platypus and echidna.

Keywords: Anti-Müllerian hormone (AMH), Monotreme, Sex determination, Echidna, Platypus

Background

Monotremata, consisting of platypus and four echidna species, are the most basal, extant mammalian lineage, having diverged from therian mammals (marsupials and eutherians) around 188 million years ago (MYA) [1]. Monotremes are the only egg-laying mammals

and in the case of the short-beaked echidna, *Tachyglossus aculeatus*, eggs are laid into a transient pouch for incubation, after which the altricial young hatch and remain in the pouch for the first part of their development [2]. Monotremes are also distinguished by a multiple-sex chromosome system with five X chromosome pairs in females, five X and Y chromosome pairs in the male platypus, and five X and four Y chromosomes in the echidna [3, 4]. Monotreme sex chromosomes evolved independently from and share no homology with, therian sex chromosomes and lack the sex-determination gene, *SRY* [5–7].

The transition from autosome to sex chromosome is initiated by the acquisition of a sex-determining gene on an autosome [8, 9]. In therian mammals, changes to the *SOX3* gene and regulatory region gave rise to the sex-determining gene on the Y, *SRY* [10, 11]. During sex chromosome differentiation stepwise recombination suppression, surrounding the sex-determining gene, produces a punctuated pattern of sequence divergence, termed evolutionary strata [1, 12] with the sex-determining gene in the oldest stratum. A range of genes have become sex-determining throughout evolution and most commonly arise from genes with conserved roles in sexual development [13]. The search for the monotreme sex-determining gene has been hampered through the lack of Y chromosome sequence information and access to developmental stages. However, chromosome-level genome assemblies of male platypus and echidna have confirmed that a male-specific copy of the anti-Müllerian hormone (*AMH*) gene is the most likely sex-determining gene of monotremes [1, 12, 14]. This gene, and its X chromosome gametologue, are in the oldest stratum (S0) of the platypus and echidna sex chromosomes and is the only known gene in this stratum with a conserved role in vertebrate sexual development.

AMH is a secreted hormone of the TGF-β superfamily that functions as a sex-determining or differentiation gene in mammals, birds, reptiles, amphibians, and fish [15–18]. In therian mammals, *AMH* is expressed after the testis-determining *SRY* and *SOX9* genes and hence is a "downstream" component of the testis differentiation pathway. Sertoli cells secrete AMH, which causes regression of the Müllerian ducts that would otherwise develop into the female reproductive tract [16, 19, 20]. AMH binds to the AMH receptor type 2 (AMHR2) through the TGF-β domain to activate intra-cellular signaling via a type I receptor and the Smad proteins 1, 5, 8, and 4, leading to altered gene expression [18, 21]. Mutations in *AMH* or in *AMHR2,* cause persistent Müllerian duct syndrome in males and premature reduction of the follicle pool in females [22]. In species that lack Müllerian ducts, such as zebrafish, Amh promotes male development by regulating germ cell accumulation and inhibiting oocyte development or survival [23].

Amh has emerged as a master sex-determinant gene in a growing number of non-mammalian vertebrate species. The first discovery of a male-specific copy of *amh* on the Y chromosome (*amhy*) was in a fish, the Patagonian pejerrey, *Odontesthes hatcheri* where *amhy* knockdown caused male-to-female sex reversal [24]. Male-specific *amh* sex-determining genes have since been identified in several species of teleost fish [25–32]. In these instances, *Amhy* has arisen by independent, and lineage-specific, duplications rather than from a shared ancestral event [30]. Interestingly the region bearing the *AMH* gene has repeatedly and independently been recruited as sex chromosomes in vertebrate species [33, 34]. These include the ZZ/ZW sex chromosome systems of several

anguimorphs and geckos *Uroplatus* leaf-tail and *Coleonyx brevis* with *Amh* as the candidate sex-determining gene in these species [35, 36]. Independent emergence of *Amh* as the master sex-determinant gene in some teleost, anguimorph, and gecko species are examples of convergent evolution.

Identification of candidate sex-determining genes in monotremes has been extremely challenging due to limited access to early developmental stages, lack of Y chromosome data, and inability to perform genetic manipulation. However, the availability of new genome information and a significant improvement in captive breeding, have allowed us to access accurately timed and sexed echidna fetal and pouch young samples for the first time in over 150 years [37, 38]. In our study, comprehensive genetic analysis and structural modeling have shown that monotreme *AMHX* and *AMHY* differentiation in promoter, intronic, and coding sequence was an early step in monotreme sex chromosome evolution, with both proteins retaining the conserved features of TGF-β molecules. We highlight differences in gene expression during monotreme and therian sexual development and show that expression of *AMHY* occurs exclusively in the male echidna bipotential gonad throughout the period of gonadal sexual differentiation, strongly supporting the conclusion that *AMHY* is the primary sex-determining gene of monotremes. Over-expression of AMHX or AMHY in an avian model system (chicken embryo) suggests that AMH receptor binding and signaling have undergone specific changes in mammals, explaining the lack of a gonadal phenotype seen in the developing chicken embryo.

Results

An inversion involving *AMH* in the monotreme common ancestor

In platypus and echidna, which are separated by 55 MY of divergence, the *AMH* gene is located on the sex chromosomes, in contrast to all other mammals in which it is autosomal. We compared the local synteny and sequence similarity of monotreme *AMH* genes to other tetrapod species to identify changes that may be sex-specific in monotremes. *AMHX* is located in the X-specific region of the X1 chromosome in platypus and echidna in the oldest stratum [1] (S0, Fig. 1A). Genes in this stratum have an average Ka/Ks value of 0.126677 (range 0.008–0.328) and platypus *AMH* gametologues have a Ka/Ks of 0.1817. Synteny (collinearity) of genes flanking *AMH*, is conserved throughout tetrapod species including human chromosome 19 and chicken chromosome 28 with a chromosomal breakpoint flanking the *leucine-rich repeat and Ig domain containing 3* (*LING03*) gene (Fig. 1A, blue arrowhead). The relative orientation and gene order in this region is conserved in tetrapod species (aves, reptiles, amphibians, and mammals) and for the monotreme *AMHX* genes suggesting that sequences adjacent to *AMH* genes may be important for *AMH* expression. The *splicing factor 3a subunit 2* (*SF3A2*) gene lies immediately upstream of the *AMH* gene (within 0.6 kb of the start site) with the junctional sarcoplasmic reticulum protein 1 (*JSRP1*) gene downstream. In contrast, the collinearity of the region containing the monotreme *AMHY* genes has been lost on the monotreme Y chromosomes (non-recombining region of chromosome Y5, platypus and Y3, echidna), likely due to inversions and Y chromosome differentiation.

The altered genomic environment for the monotreme *AMHY* genes compared to *AMHX* and *AMH* genes of other tetrapods, may introduce novel regulatory elements

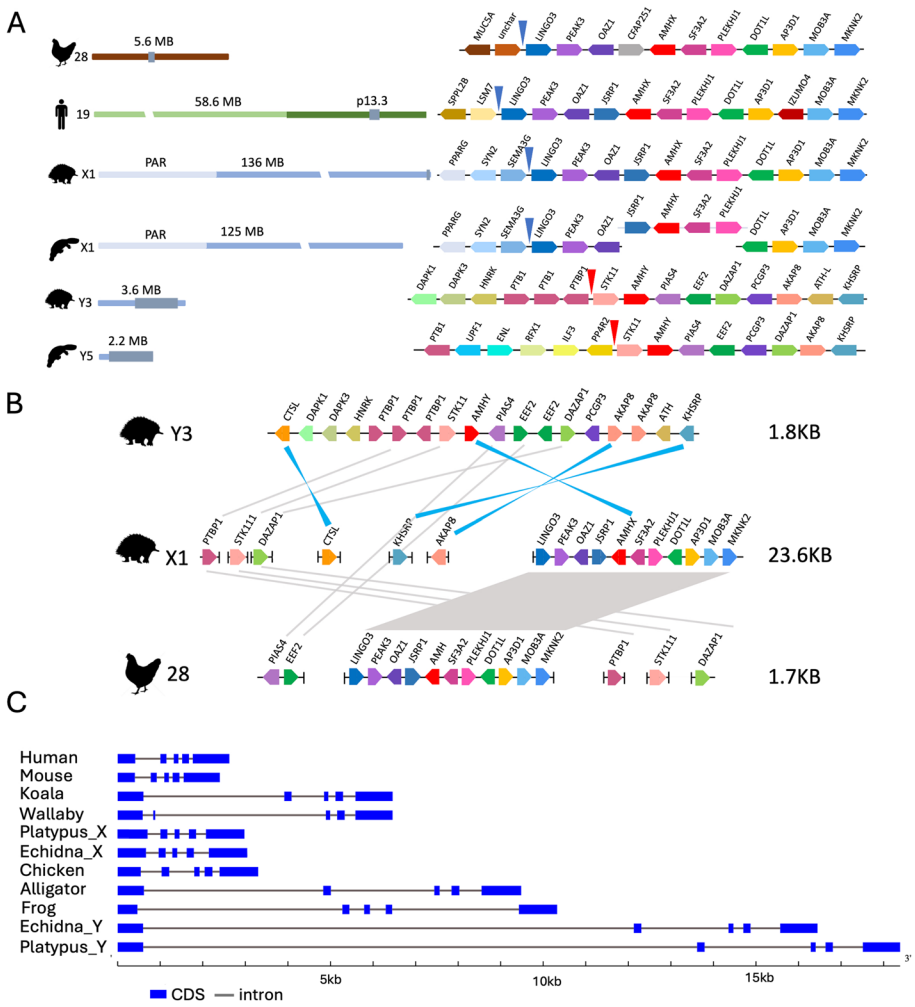


Fig. 1 Synteny and gene structure analysis of monotreme *AMHX* and *AMHY*. **A** Synteny between chicken human and monotreme *AMH* genes. Chicken chromosome 28, brown; human chromosome 19, green with q arm in light green; monotreme chromosomes blue, with pseudoautosomal region (PAR) in light blue; gray box syntenic region in expanded view showing genes as colored directional boxes. Blue arrow, break in synteny with monotreme X chromosomes; red arrow, break in synteny between monotreme Y chromosomes. **B** Comparison of echidna genes flanking AMHX/Y and chicken chromosome 28. Genes as colored directional boxes. Connecting lines gray, genes in the same orientation and blue, genes in opposite orientation; not to scale. **C** Gene organization of monotreme *AMHX* and *AMHY* compared to that of the human, mouse, koala, wallaby, chicken, alligator, and frog *AMH*. Each gene contains 5 translated exons. CDS, coding sequence

which impact *AMHY* expression. The *AMHY* genes are flanked by the *serine/threonine kinase 11* (*STK11*) and *protein inhibitor of activated STAT4* (*PIAS4*) genes in both platypus and echidna. In comparison, on the X chromosome, the *STK11* gene is 22 MB from *AMHX* and *PIAS4* is on chromosome X2 (Fig. 1A, B). Comparison of the orientation of echidna *AMHX* and *AMHY* genes to each other and flanking genes suggests that the *AMHY* gene has undergone an inversion relative to *AMHX* on the X1 chromosome. The genes immediately flanking *AMHY* within a 1.8 Kb region span over 23.6 Kb on the echidna X chromosome (the chicken is used as a comparison, Fig. 1B). The relative orientation of the *STK11* and *AMHY* genes is altered compared

to *AMHX* and *STK11* on platypus and echidna X1 (Fig. 1A, B). These comparisons suggest that an inversion event involving the *AMH* gene on the proto-Y occurred uniquely in the monotreme common ancestor.

The monotreme *AMHX* and *AMHY* genes consist of 5 exons with similar organization to that of other tetrapod *AMH* genes (Fig. 1C). The introns of the *AMHY* genes are considerably larger than those of the *AMHX* or autosomal *AMH* genes (Fig. 1C) largely as a result of repeat invasion (Additional file 1: Fig. S1A). Furthermore, repeat content is higher in the *AMHY* flanking regions compared to *AMHX*. In the 7 kb upstream of echidna *AMHX*, repeat coverage is 36.7% whereas the same region upstream of echidna *AMHY* has 71.4% repeat coverage (platypus *AMHY*, 61.6%). The *AMHY* genes also show specific examples of repeat invasion. For example, intron 1 has undergone large expansion due to LINE L2 and SINE intronic insertions in both platypus and echidna (Additonal file 1: Fig. S1A). One conserved LINE L2 insertion is present in intron 4 of the monotreme *AMHX* genes. Conservation of this L2 insertion in both platypus and echidna shows that this occurred before the divergence of platypus and echidna and its conservation for over 55 million years suggests functional importance.

AMHX and *AMHY* have diverged through sex chromosome differentiation

Comparative analysis of the monotreme *AMHX* and *AMHY* genes shows that the coding sequence of the gametologues in both platypus and echidna have substantially diverged from each other sharing only 62% DNA identity (Additional file 3: Supplementary Table S1). In contrast, the platypus and echidna *AMHX* genes are more similar to each other (93% identity) as are the platypus and echidna *AMHY* genes (90% identity). This is also reflected in the nucleotide substitution rate (Additional file 3: Supplementary Table S2). The Ka and Ks values, and total number of substitutions, are much lower between the platypus and echidna *AMHX* genes and between the monotreme *AMHY* genes (dark gray) compared to those of the *AMHX* and *AMHY* gametologues (light gray) within each species (Additional file 3: Supplementary Table S2). The Ka/Ks ratios of monotreme *AMHX* and *AMHY* compared to *AMH* of all other vertebrates analyzed is less than 1.

At the protein level, the monotreme AMHX sequences are 91% identical and the monotreme AMHY proteins are also highly similar with 86% identity. Importantly, the AMHX and AMHY gametologues within each monotreme species show greater divergence being only 52% identical (Additional file 3: Supplementary Table S3 and Fig. S2). The monotreme AMHX and AMHY proteins have the conserved features of AMH proteins, including a predicted signal peptide, conserved cleavage site between the N-terminal and C-terminal domains, and the 7 conserved cysteine residues of the TGF-β domain (Fig. 2A, Additional file 1: Fig. S1B). The TGF-β domain is the most highly conserved region of AMH proteins as it binds directly to the AMH receptor leading to signaling and this is also true of the monotreme AMHX and AMHY proteins (Additional file 3: Supplementary Table S4, Fig. 2A). Within the monotreme TGF-β domains there are only two amino acid differences, but the protease cleavage sites differ between AMHX (RAQR) and to AMHY (RVQR) which may impact on cleavage into the biologically active domains (Fig. 2A).

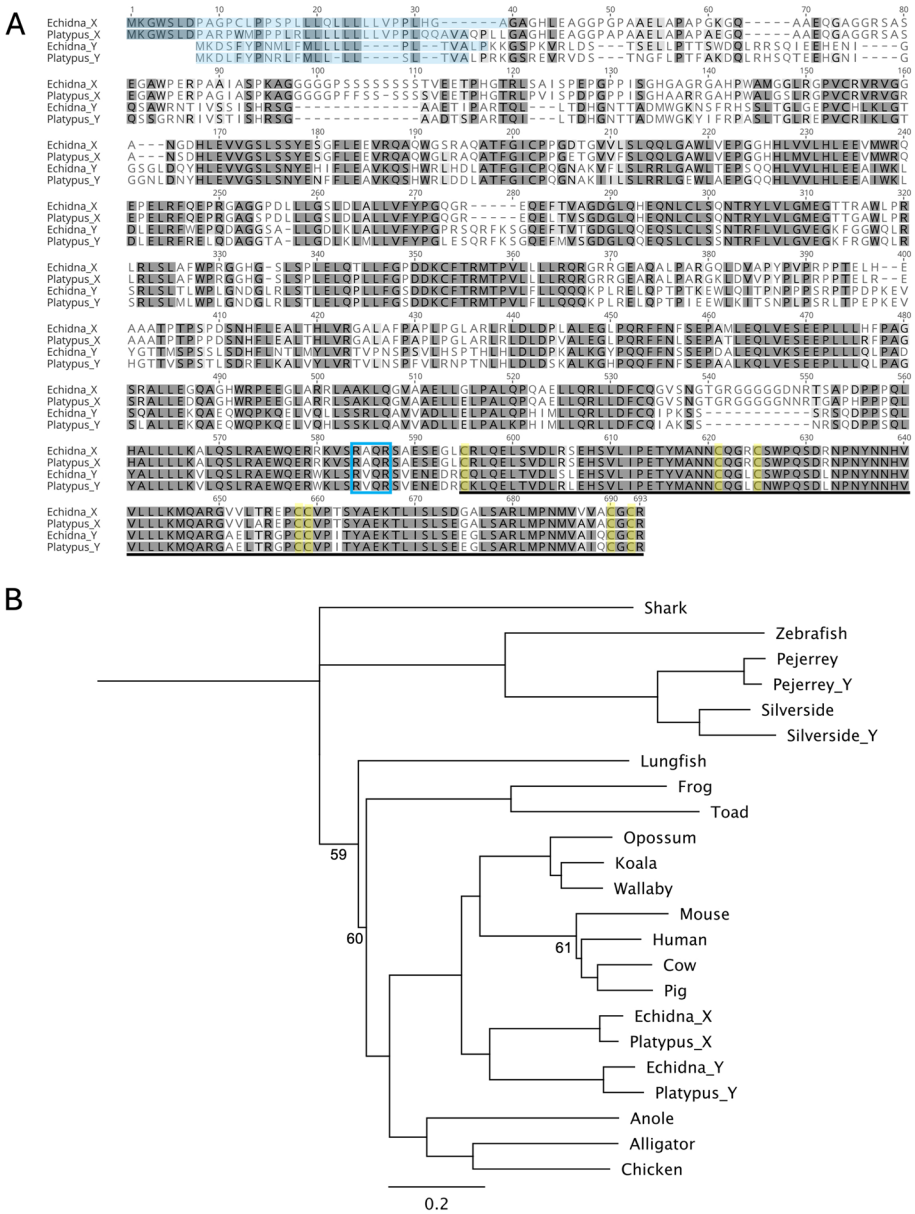


Fig. 2 Comparative analysis of monotreme AMH proteins. **A** Alignment of the monotreme AMHX and AMHY proteins. Gray shading, amino acid similarity with identical residues (dark gray); blue shading, signal peptides; blue box, protease cleavage site; underline, TGF-β domain with conserved cysteine residues highlighted in yellow. **B** Phylogenetic tree of vertebrate AMH proteins. Scale bar, branch length; branch labels, and bootstrap values shown if below 95%

The structure of the TGF-β domains of AMHX and AMHY from both platypus and echidna were assessed by in silico modeling [39, 40]. The best models obtained were all based on human AMH TGF-β domains with high confidence. As expected, based on the high degree of amino acid conservation, the predicted structures are very similar to each other with root mean square deviations of less than 0.2 Å across the full length (106 amino acids) of the TGF-β domain. The most variable regions were found in the loops at the distal tip of the structure, which interact with the AMH receptor AMHR2

[41]. All known TGF-β family ligands form dimers that can interact with two subunits of the extracellular domain of the cognate receptor. The monotreme *AMHR2* gene is located on platypus and echidna chromosome 10 in a region of conserved synteny. Prediction of platypus AMHR2-AMHX and AMHR2-AMHY multimeric complexes of the same stoichiometry was done with AlphaFold multimer [40], giving structures with topology very similar to the experimentally determined human AMHR2-AMH complex [41] (Additional file 1: Fig. S1C). The predicted platypus AMHR2 extracellular domain showed conservation of the "3 finger toxin" fold characteristic of the extracellular ligand binding domain of the TGF-β family. The majority of the key contact residues essential for AMH signaling in the human AMHR2 receptor [41] are conserved in the platypus receptor complexes (Additional file 1: Fig. S1D), suggesting that the monotreme AMHX and AMHY proteins both signal through the AMHR2 receptor.

AMHY has novel transcriptional control elements

As evolution of new sex-determining genes can be caused by alterations to regulatory regions that change the pattern of expression, we compared the proximal promoters of *AMHX* and *AMHY* to each other and to those of *AMH* from other mammalian species. Comparison of the human, mouse, tammar wallaby *AMH,* and the monotreme *AMHX* and *AMHY* promoters showed that the *AMHX* promoters are more similar to each other and to those of therian *AMH* genes, than they are to the monotreme *AMHY* promoters (Fig. 3A and Additional file 1: Fig. S2).

In humans, expression of AMH in Sertoli cells of the fetal testis is activated by direct binding of SOX9 to the proximal *AMH* promoter [42], following its activation by SRY. Binding of steroidogenic factor-1 (SF-1), GATA4 binding protein (GATA4), and Wilms' tumor protein (WT1) to the proximal promoter enhance transcription of *AMH* in the fetal gonad (Fig. 3B). Putative binding sites for SOX9, GATA4, SF-1, and HMG in the monotreme *AMHX* promoters are conserved in sequence and relative position with those of the mammalian *AMH* promoters. In contrast, binding sites for SOX9, GATA4, SF-1, and HMG are not found in monotreme *AMHY* promoters. Rather, putative binding sites for SF-1/Jun, GATA1, and DMRT1 are conserved in the promoter region in both platypus and echidna *AMHY*. The putative DMRT1 binding site, corresponding to the DMRT1 binding site identified by the SELEX method [43], is also present in the monotreme *AMHX* promoters (Fig. 3B and Supplementary Fig. S2). This shows that the *AMHY* promotor region has differentiated compared to that of *AMHX*, and the changes have been conserved between platypus and echidna *AMHY,* suggesting that they are functional.

AMHY is expressed in the male echidna gonad throughout sexual development

In situ hybridization was used to assess the cellular sites of expression of monotreme *AMHX* and *AMHY*, in adult gonads (Fig. 4A, B; expression purple stain). *AMHX* was expressed in the granulosa cells of the follicles in both echidna and platypus ovary (Fig. 4A) as is the case for therian AMH [44]. Both *AMHX* and *AMHY* were expressed in DMRT1/SOX9^{+} Sertoli cells of both platypus and echidna testis (Fig. 4B) as in therian mammals [45].

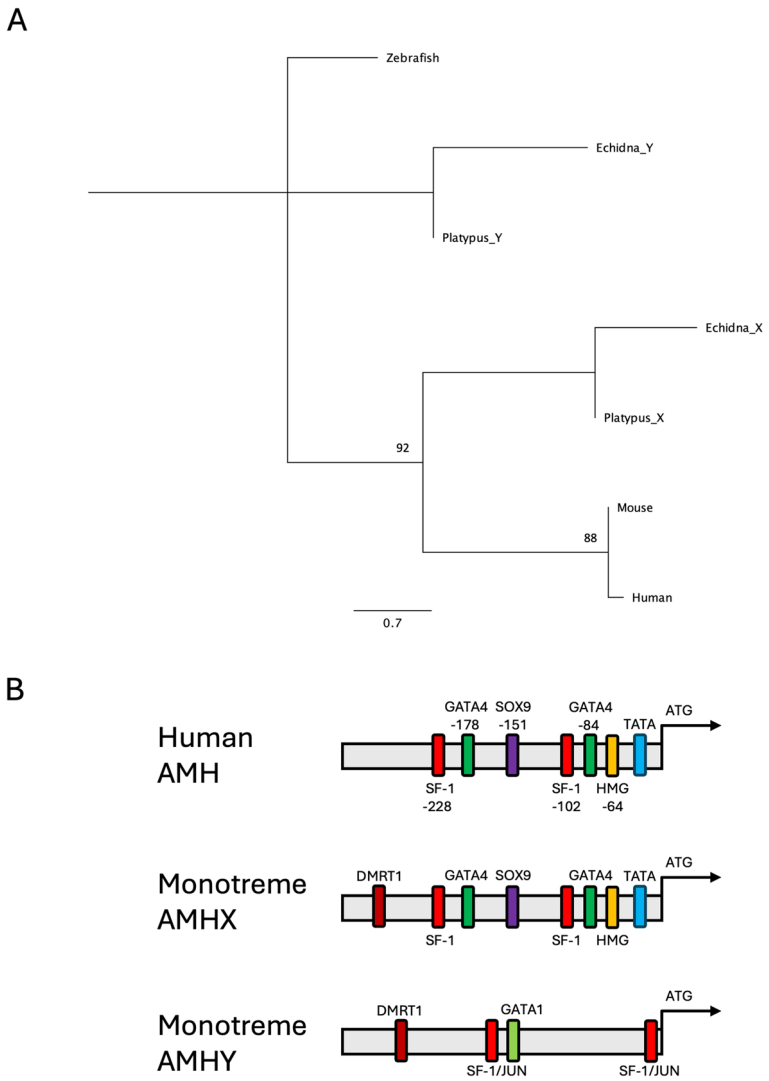


Fig. 3 Comparative analysis of *AMH* proximal promoters. **A** Phylogenetic tree of mammalian AMH promoters 300 bp with Zebrafish as the outgroup. Scale bar, branch length; branch labels, and bootstrap values shown if below 95%. **B** Schematic representation of the human *AMH*, monotreme *AMHX*, and *AMHY* promoters showing transcription factor binding sites (not to scale). Binding sites for GATA1, GATA1 transcription factor; GATA4, GATA4 transcription factor; SF-1, steroidogenic factor-1; JUN, Jun; DMRT1, doublesex and mab-3 related transcription factor 1; SOX9, *Sry*-type box 9; HMG, high mobility group protein; TATA, TATA binding factor; arrow, + 1 translation start site

For a gene product to be male sex determining, it must be present in the male fetal gonad during the period of gonad specification. In echidna, the ovary and testis first become morphologically differentiated after hatching between day 2 to day 4 of the pouch young (py) stage (JC Fenelon and MB Renfree unpublished results, Fig. 4C). We assessed gene expression in developing echidna gonads from a day 6 fetus (in egg, where the day of egg laying is designated d0) through to day 11 post-hatching pouch young stage. The sex of all echidna fetuses and pouch young was determined by PCR using the published male markers *CRISPY* [46] and *RADseq116* [47] (Additional file 1: Fig. S3A and B). In situ hybridization shows *AMHX*, *AMHY*, *SOX9*, and *DMRT1* expression in the echidna fetal gonad throughout the period of sexual development (Fig. 4C) and

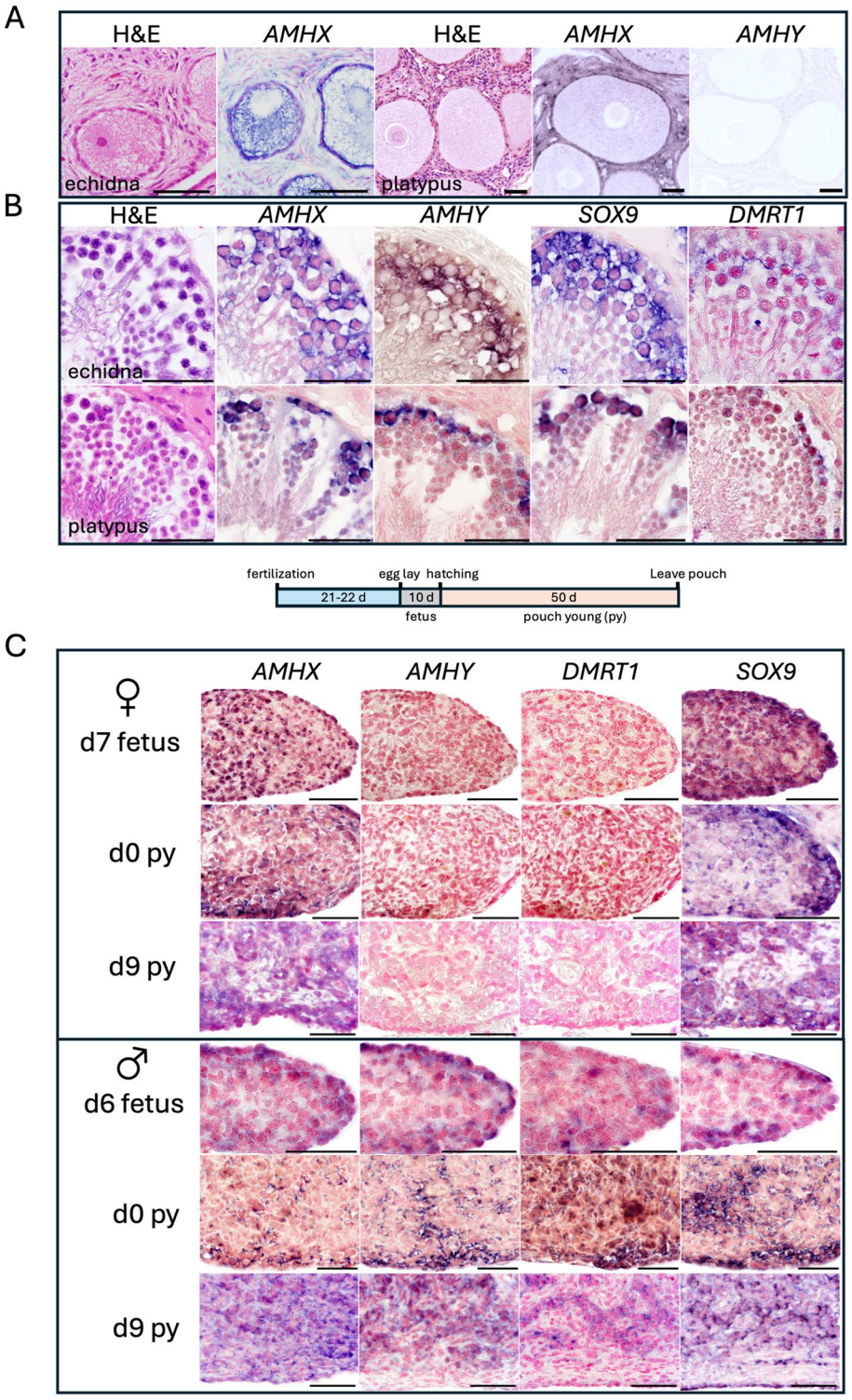


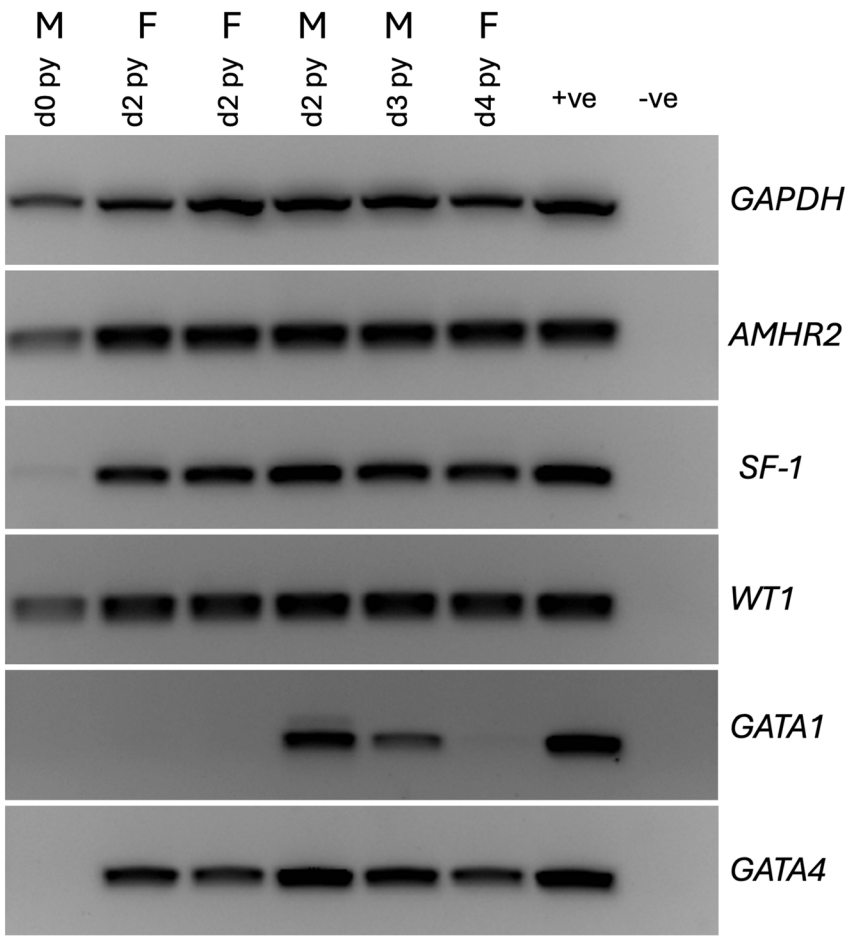
Fig. 4 Expression analysis in monotreme gonads by in situ hybridization. **A** Hematoxylin and eosin stained adult ovary sections showing *AMHX* expression in the echidna and platypus and *AMHY* in the platypus. **B** Hematoxylin and eosin stained adult testis sections showing *AMHX*, *AMHY*, *SOX9*, and *DMRT1* expression in echidna and platypus. **C** Timeline of early echidna developmental stages (upper). Expression of *AMHX*, *AMHY*, *DMRT1*, and *Sox9* in echidna female (middle) and male (lower) gonads during gonadal sexual differentiation. Scale bars: 50 μm

specifically in the bipotential male gonad from the d6 in egg fetus (earliest time point when the gonad had developed) through to d0 py. By the d9 py, *AMHY* was clearly expressed in the Sertoli cells of the testis cords, similar to *DMRT1* and *SOX9*. *AMHX* and *SOX9* were also expressed in the developing female gonad at all stages examined but expression of *AMHY* and *DMRT1* were not detected (Fig. 4C). These results were confirmed by RT-PCR (Additional file 1: Fig. S3C). RT-PCR showed expression of *DMRT1* in female d2 samples but not in d4 samples. Genes important for therian AMH expression (*SF-1*, *WT1*, and *GATA*) or function (*AMHR2*) were expressed from d0-d4 py in both the male and female gonads whilst *GATA4* was expressed in all except the male d0 py gonad (Fig. 5A). In contrast, *GATA1* was only present in the d2 and d3 py male gonads (Fig. 5A). Expression of the *AMHR2* in the bipotential gonads of both sexes suggests that *AMHX* and *AMHY* signaling is required throughout this period of development. Importantly, expression of *AMHY* in the male, but not female, gonad throughout the period of gonadal specification supports its role as the primary male sex-determining gene. These data also indicate that *GATA1* and *DMRT1* may be involved in monotreme male sex determination (summarized in Fig. 5B).

Ectopic expression of *AMH* proteins during sexual development in a non-mammalian model system

In order to investigate whether AMHX and AMHY proteins can affect sexual development we had to identify a suitable in vivo model, as genetic manipulation is not possible in monotremes. Expression of the AMH receptor in the gonad prior to and during gonad specification is vital for assessing the effects of monotreme AMH proteins on gonad development. In the mouse, expression of gonadal AMHR2 occurs after gonad specification, ruling it out as a suitable model system for these experiments. In fish, the low similarity of the AMHR2 to that of mammalian species is likely to hamper the binding and signaling of monotreme AMHX and AMHY. Chicken AMHR2 has higher similarity to monotreme AMHR2 and the receptor is expressed in the gonad of both sexes from early embryonic stages, prior to sexual differentiation [48, 49]. Viral delivery systems for ectopic expression in the chicken embryo, deliver high-level protein expression which may be sufficient to overcome the potential reduced affinity of the monotreme AMH proteins for the chicken receptor. Importantly, forced over-expression of AMH in chicken embryos gives a discernible phenotype (masculinized gonads in females) and knockdown of AMH blocks proper gonad formation in both sexes [50, 51]. This early timing of receptor expression, together with the known masculinizing effects of exogenous chicken AMH on avian gonads, makes the chicken embryo the most suitable model for assessing monotreme AMH function during gonad specification. Chicken embryos were infected with viral vectors over-expressing either chicken AMH or platypus AMHX or AMHY (each GFP-tagged) at the blastoderm stages. Embryonic chicken urogenital systems were examined at embryonic day 9.5 (Hamburger-Hamilton stage 35). Over-expression of chicken AMH in the chicken embryo resulted in the expected phenotype, characterized by small elongated (male-like) gonads and regression of Müllerian ducts in both sexes (Additional file 1: Fig. S4) proving successful over-expression as previously described [51]. Over-expression of platypus AMHX or AMHY proteins did not result in a discernible phenotype in the developing gonads of either males or females (Additional

A



B

	Fetus		d0 PY		d2 PY		d3-d4 PY		d9 PY		Adult Gonad	
	F	M	F	M	F	M	F	M	F	M	F	M
AMHX	+	+	+	+	-	+	-	+	+	+	+	+
AMHY	-	+	-	+	-	+	-	+	-	+	-	+
DMRT1	-	+	-	+	+	+	-	+	-	+	na	+
SOX9	+	+	+	+	+	+	+	+	+	+	+	+
AMHR2	na	na	na	+	+	+	+	+	na	na	na	na
SF-1	na	na	na	+	+	+	+	+	na	na	na	na
WT1	na	na	na	+	+	+	+	+	na	na	na	na
GATA4	na	na	na	-	+	+	+	+	na	na	na	na
GATA1	na	na	na	-	-	+	-	+	na	na	na	na

Fig. 5 RT-PCR Echidna pouch young gene expression analysis. (**A**) Expression of *AMHR2*, *SF-1*, *WT1*, *GATA1*, and *GATA4* with *GAPDH* as loading control in pouch young gonads from d0-d4 py with either adult testis or ovary as a positive control. *GAPDH*, *AMHR2*, *SF-1*, and *GATA4* were expressed in both sexes at all stages examined whereas GATA1 was only expressed in the d2 and d3 py males. py, pouch young (**B**) Summary table of echidna gene expression. +, expression; −, no expression; PY, pouch young; F, female; M, male; py, pouch young; na, data not available

file 1: Fig. S4) even with the chicken-specific cleavage site required for processing into the functional AMH domains (Additional file 1: Fig. S4, panels labeled + CC). To explain this result, we analyzed the potential interaction surface between the platypus AMH proteins and chicken AMHR2 based on that of the recently defined human AMH/AMHR2 binding interface [41] (Additional file 1: Fig S5). This revealed differences in key residues between the chicken and monotreme AMH proteins that are required for receptor binding in human (Additional file 1: Fig. S5A). Comparison of the TGF-β domains of AMH across different taxa shows that the residues implicated in binding and/or activity of human AMH and AMHR2 are completely conserved in the eutherian mammal species examined and largely conserved within all three mammalian groups but not in non-mammalian vertebrates (Additional file 1: Fig. S5B).

Discussion

The divergence of monotremes from therians predates the evolution of *SRY* as the sex-determination gene in therian mammals. Monotremes evolved a complex XY sex chromosome system that shares no homology with therian sex chromosomes. Instead, these chromosomes have homology to the avian Z sex chromosome which includes the conserved sex-determining gene, *DMRT1* on an X chromosome [3, 52, 53]. The absence of a *DMRT1* gametologue on one of the Y chromosomes makes it an unlikely primary monotreme sex-determination gene [1].

Identification of Y-specific *AMH* gametologues in the oldest stratum of the monotreme sex chromosomes (platypus Y5, echidna Y3 [1, 12]) makes *AMH* the frontrunner as the monotreme sex-determining gene. The most recent estimate of the age of the oldest sex chromosome stratum suggests that monotreme sex chromosomes evolved at least 100 MYA [54]. *AMHX* and *AMHY* gametologues of platypus and echidna, within this stratum began differentiation at around this time, long before the divergence of the two monotreme lineages at 55MYA. Analysis of the substitutions in the monotreme *AMHX* and *AMHY* genes shows that the genes are under purifying selection with *AMHX* under stronger purifying selection than *AMHY*. The greater difference between the *AMHX* and *AMHY* gametologues compared to the monotreme *AMHX* (or monotreme *AMHY*) may reflect the neofunctionalization of the monotreme AMHY proteins which largely occurred prior to the divergence of the echidna and platypus and has been maintained since then. This is fundamentally different to all but one example in fish where *Amh* has become a sex-determining gene by duplication and translocation onto the future or existing Y chromosome [55] and can maintain a high level of sequence identity with the autosomal *Amh* gene [26]. By contrast, monotreme AMHX/AMHY proteins are highly differentiated and the evolutionarily conserved syntenic block that contains the *AMH* gene is maintained for monotreme *AMHX* but is significantly different for *AMHY*. This includes loss of the Follicle-Stimulating Hormone-responsive enhancer upstream of the human *AMH* gene, which overlaps with *SF3A2* and is involved in upregulation of *AMH* expression in Sertoli cells of the prepubertal testis [56]. Loss of *SF3A2* upstream of monotreme *AMHY* may remove important regulatory elements, thus leading to altered expression of this gene compared to *AMHX*. Comparison of the platypus and echidna *AMH* alleles to each other and to *AMH* genes of representative tetrapod species supports the idea that inversion of one *AMH* allele was an early step in the

differentiation of the proto-Y chromosome in the monotreme ancestor. Recombination suppression caused by inversion, could be a first step in sex chromosome differentiation in monotremes. Conserved changes in transcription factor binding sites in the proximal regulatory region of *AMHY* and loss of the upstream *SF3A2* gene, which impacts *AMH* expression in therian mammals [35], further support a male-specific function. The accumulation of intronic transposable element insertions in *AMHY*, common in Y chromosome gametologues [57, 58] may also affect the expression of *AMHY* compared to *AMHX*. This can be particularly important for developmental control genes where precise temporal control is required and large introns can cause transcriptional delay [59]. Interestingly most of the LINE L2 and SINE MON1 insertions are conserved between platypus and echidna suggesting that repeat invasion occurred in the common ancestor more than 55 MYA.

Phylogenetic analysis shows the separation of the AMHX and AMHY proteins, signifying evolutionarily conserved differences in the gametologues. Both proteins retain the features of functional TGF-β family members with subtle substitutions that may affect signaling and proteolytic cleavage. Together these results provide evidence that the monotreme X- and Y-localized *AMH* genes encode functional signaling molecules with significant differences in both coding and regulatory sequence which may have conferred a sex-determining function on *AMHY*.

For the first time, access to echidna embryonic and pouch young stages allowed us to investigate the expression of key genes potentially involved in monotreme sexual development, although extremely limited access to these stages, restricted further investigation of comparative temporal gene expression. We show that *AMHX* is expressed in the granulosa cells of the ovary and Sertoli cells of the testis, similar to other mammals, while *AMHY* expression is restricted to Sertoli cells of the testis. Importantly, in the developing echidna fetus *AMHX* is expressed at least from the day 6 fetal stage in males and females, about 8 days before the gonads are morphologically different. *AMHR2* was also present from at least the d0 py stage (unfortunately sufficient earlier stages were not available). Regardless, this is notably earlier than the onset of *AMH* expression in therian mammals. In the developing echidna male gonad, *AMHY* shows expression in all stages examined, from the earliest day 6 fetal stage to the day 9 pouch young stages. Expression throughout these stages is consistent with a role in monotreme gonad specification. In therians, *AMH* expression is upregulated in response to increased *SOX9* expression after gonad specification in the developing mouse testis by E12.5 when it results in loss of the Müllerian ducts in males [44]. Echidna *AMHY* expression is more similar to non-mammalian vertebrates (chicken, turtle, American alligator, and bearded dragon, *P. vitticeps*) in which *Amh* expression also precedes that of *Sox9* [60–63]. Expression of autosomal *Amh* and sex-determining *Amhy* in the gonad during sexual differentiation is also seen in the Northern Pike (*Esox lucius*) where expression of *Amhy* precedes that of *Amh* and both genes are expressed from stage 75 in males before morphological differentiation of the gonad [30]. Our data show that both *AMHX* and *AMHY* are expressed in the male gonad in the day 6 fetus. Expression of genes before that time could not be assessed as gonads have not developed at earlier time points, thus whether *AMHY* expression precedes that of other male expressed genes could not be determined. We suggest that expression of AMHX and AMHY in the monotreme male gonad may be required to

trigger male development, whereas female development would occur in the absence of AMHY. In males this could produce a dosage effect, absent in females. Alternatively AMHX/Y heterodimers may result in altered signaling through AMHR2 resulting in testis formation. In other examples where *amhy* is sex determining (e.g., *O. hatcheri*) knockdown experiments suggest that *amhy* represses the female pathway genes *foxl2* and *cyp19a1a* required for ovary development. *AMHY* could play a similar role in the monotremes, repressing expression of genes required for ovarian development.

In therian mammals, *SOX9* expression is low in the bipotential gonad of both sexes but is the first gene upregulated in the male gonad by SRY and causes upregulation of *AMH* expression [42]. It was therefore unexpected to see *SOX9* expression in the echidna throughout the sexual development period in both the female and male gonads. In therians SOX9, SF-1, GATA4, and WT1 directly activate *AMH* expression later during male gonad development [64]. Here we show that *SOX9*, *SF-1*, *GATA4*, and *WT1* are expressed during the echidna gonadal differentiation period in both sexes. We also show that *DMRT1* has male-biased expression in the bipotential echidna gonad from the earliest stage examined up until at least the day 9 pouch young, the latest stage examined, and *GATA1* is exclusively expressed in the male gonad together with *AMHY*. Conserved potential binding sites for GATA1 in the monotreme *AMHY* promoters and for DMRT1 in the promoters of monotreme *AMHX* and *AMHY* suggest that these factors may be involved in regulating *AMHY* expression during gonad specification in monotremes. These data highlight significant differences between mammalian sexual development pathways with *SRY* (therian) or without (monotremes).

In order to prove that a gene is sex-determining, ectopic expression or gene ablation experiments in the developing gonad are required. As such experiments are not possible in native species, such as monotremes, we carried out experiments in an animal model to probe the function of monotreme AMH proteins during gonad specification. In chicken AMH and AMHR2 are expressed before gonadal differentiation and overexpression of chicken AMH causes masculinization of female gonads [51] making it the most suitable animal model for these experiments. Over-expression of monotreme AMHX and AMHY in the chicken embryo did not recapitulate the chicken AMH over-expression phenotype. To explain this result, we investigated the AMH AMHR2 interface recently defined from human proteins [40]. This suggested that changes in the AMH/AMHR2 system in early mammalian evolution may hamper mammalian AMH proteins binding to/signaling through the chicken receptor. In the case of the monotreme AMHX and AMHY proteins it appears that even at high over-expression levels, these proteins are unable to signal through the chicken receptor. Interestingly, chicken AMH can induce regression of mammalian (rat) Müllerian ducts, while rat AMH has no effect in chicken [65]. Analysis of AMH proteins of vertebrate species suggests that the residues involved in AMHR2 binding by the human TGF-β domain of AMH [41] are only conserved in mammals, suggesting that mammalian AMH proteins more broadly may not function in bird models.

Conclusions

Monotreme sex determination has been a mystery since the first unsuccessful attempts to identify the mammalian sex-determination gene *SRY* over thirty years ago. Here we provide a comprehensive analysis of the monotreme *AMHX* and *AMHY* gametologues and the first insight into the sexual development pathway of this mammalian lineage which lacks *SRY*. We show that the *AMHX* and *AMHY* gametologues are highly differentiated in genomic context and sequence, likely caused by early events in the evolution of monotreme sex chromosomes. Importantly in the developing echidna gonad, *AMHX* is expressed earlier than *AMH* in therian mammals and *AMHY* is exclusively expressed in the developing male fetal gonad throughout the period of gonad specification, supporting a role for *AMHY* as the primary male sex-determination gene. This would be the only mammalian example of convergent evolution in which AMH was co-opted as the male sex-determination gene and the first example of a soluble growth factor acting as a sex-determination gene in mammals.

Methods

Gene and protein analysis

Platypus AMHX (NC_041749.1). Platypus AMHY Y5 (NC_053179.1): 1,478,061–1508837, geneID 114808765. Echidna AMHX X1(NC_052101.1):135,469,955–135,473,524 geneID 119919435; AMHY Y3 (NC_052095.1):2,513,435–2,535,704 geneID11946726. Gene maps were generated using the Gene Structure Display Server 2.0 [66]. To identify transposable elements present in AMHX, AMHY, and the flanking regions of these genes, we used BLASTN [67] (-task dc-megablast) to search for previously described monotreme TEs from the Repbase database [68]. Sequence alignments and the Phylogenetic trees (Geneious Tree Builder; Genetic distance model: Jukes-Cantor; Tree build method: Neighbor-joining; Resampling method: Bootstrap; 500 replicates) were generated using the Geneious Prime 2022.0.1 and FigTree v1.4.4. The non-synonymous (Ka) to synonymous (Ks) rate ratio (Ka/Ks) between platypus AMHX and AMHY, or between AMHX or AMHY and AMH(s) in other 10 species, including Alligator, Chicken, Echidna, Human, Koala, Mouse, Opossum, Pig, Wallaby and Zebrafish, were calculated using two different tools, including KaKS_calculator (v2.0) [69] and PAML (v4.10.6) [70], independently. Only coding sequences of AMHs were included for the Ka/Ks calculation. "Model Averaged" method was used for KaKS_calculator and "Nei-Gojobori method" was used for PAML [71]. Proteins were modeled at the Phyre2 server (sbg.bio.ic.uk/~phyre2 [39]) Protein structures were predicted using the Phyre2 server [39] and AlphaFold multimer [40] and images produced with ChimeraX (cgl.ucsf.edu/chimerax). Promoter alignments were produced with Clustal Omega (ebi.ac.uk/Tools/msa/clustalo/ [72] and transcription factor binding sites identified with MatInspector (genomatix.de [73]) and CIS-BP Database (cisbp.ccbr.utoronto.ca).

Animals

Adult male and female short-beaked echidna (*Tachyglossus aculeatus*) testis and ovaries were collected opportunistically from injured animals brought into the Currumbin Wildlife Hospital, Queensland, Australia, that required euthanasia for animal welfare reasons. Fetal and pouch young echidna samples were collected from a research

breeding colony at Currumbin Wildlife Sanctuary (CWS), Queensland, Australia. Fetal samples were dated based on the date of egg laying (oviposition, designated d0 E), with samples varying in age from day 6 to day 9 after oviposition (hatching occurs on day 10) (d6 $n=1$, d7 $n=2$, d8 $n=3$, d9 $n=1$). Pouch young (py) samples were dated based on the day of hatching (where day of hatching is designated d0 py) (d0 $n=3$, d2 $n=2$, d3 $n=1$, d4 $n=1$, d9 $n=3$, d11 $n=2$). Tissues were collected from wild-caught adult male platypuses under permits from NSW Parks and Wildlife and ethically approved by the University of Melbourne Animal Ethics committees. Tissues collected were either stored in RNAlater or fixed in 4% paraformaldehyde and washed in graded changes of phosphate-buffered saline (PBS):methanol before storing in methanol at 5 °C.

Gonad histology

Briefly, tissues were fixed in 4% paraformaldehyde overnight at 4 °C, rinsed several times with 1X PBS and transferred to ethanol, embedded in paraffin, and sectioned at 5 μm. After dewaxing and rehydration, slides were stained with hematoxylin and eosin according to standard procedures. Sections were examined using an Olympus BX51 microscope and photographed using the attached Olympus DP70 camera (Olympus Corporation).

Fetal and pouch young gene expression

Total RNA was extracted from pouch young liver, kidney, and gonads where available and from adult testis and ovaries ($n=1$ of each as positive controls) with the GenElute Mammalian Total RNA Miniprep Kit (Sigma) and DNase treated using DNA-free (Ambion) according to the manufacturer's instructions. RNA was then reversed and transcribed using the Superscript IV kit (Invitrogen) with oligo(dT) priming according to the manufacturer's instructions. All other fetuses and pouch young, used for the in situ hybridization studies ($n=9$) were fixed whole in paraformaldehyde (PFA) and processed, in order to sex these young, gDNA was extracted from the formalin-fixed tissues with the ReliaPrep formalin-fixed paraffin-embedded (FFPE) gDNA Miniprep System (Promega) according to the manufacturer's instructions. For positive gDNA controls, adult testis and ovary were extracted with the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. To identify the sex of PFA-fixed fetuses and pouch young in which sex could not be determined morphologically (younger than d4 py, $n=9$), RT-PCR was first carried out as previously described [47] using the male-specific markers *CRSPY* and the RADSeq marker, *RADseq116* on the gDNA samples with *GAPDH* used to confirm the presence of echidna gDNA. To identify the sex of the pouch young where RNA was available ($n=7$), RT-PCR was first carried out using the male-specific marker *CRSPY* on the pouch young liver and kidney with adult testis and ovary as positive controls and *GAPDH* as the loading control. RT-PCR was then used to examine the expression profiles of *AMHR2*, *SF-1*, *GATA1*, *GATA4*, and *WT1* across the pouch young gonads from d0-d4 py ($n=7$) with *GAPDH* as the reference gene and adult testis as the positive control. PCR reactions were amplified with an initial denaturation 95 °C for 5 min, followed by 39 cycles of denaturation at 95 °C for

30 s, annealing at either 56 °C (*CRSPY*) or 60 °C (all remaining genes) for 30 s, and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 5 min.

Gene cloning and adult expression

Genes were cloned by RT-PCR from platypus or echidna testis cDNA *AMHY* and platypus ovary cDNA *AMH*. RNA was prepared using TRIzol Reagent (Ambion) and reverse transcription was carried out with Superscript III First-Strand Synthesis System (Invitrogen) (for cloning) or iScript cDNA Synthesis Kit (Bio-Rad) (for expression analysis). PCR was carried out with One Taq DNA polymerase (NEB, cloning) using primers listed in Additional file 3: Supplementary Table 5 and products cloned using the pGEM-T Easy Vector System I (Promega) or Phusion HF DNA polymerase (NEB) expression analysis.

In situ hybridization

Regions of low sequence identity between the *AMH* and *AMHY* cDNAs of approximately 500 bp in length were identified, amplified by PCR, and subcloned using the pGEM-T Easy Vector System I for use in in situ hybridization analysis. The cDNA of platypus *SOX9* in pUCIDT-Amp was obtained from IDT and subcloned into pGEM-T Easy following removal with *EcoRI* (PCR product 499 bp) for use in in situ hybridization analysis. Clones were linearized and in vitro transcribed using SP6/T7 Transcription Kit with Digoxigenin-11-UTP, and detection was carried out using NBT/BCIP stock solution (Roche). The *SOX9* clone was linearized with SacII or PstI and transcribed with SP6 or T7 RNA polymerase respectively to generate sense and anti-sense in situ probes. The *AMHX* and *AMHY* clones were linearized with NcoI or PstI and transcribed with SP6 or T7 RNA polymerase respectively to generate sense or anti-sense in situ probes. In situ hybridizations were carried out using a standard protocol on a total of 14 echidna fetuses and pouch young gonads and adult echidna and platypus testis and adult echidna ovaries as positive controls. Briefly, tissues were fixed in 4% paraformaldehyde overnight at 4 °C, rinsed several times with 1X phosphate-buffered saline (PBS), embedded in paraffin, and sectioned onto polylysine slides (Menzel-Glaser, Braunschweig, Germany). After dewaxing and rehydration, the sections were washed several times with 1X PBS, glycine, Triton X-100, and triethanolamine buffer and were then immediately hybridized with probes for 16–18 h at 42 °C. Hybridization signals were detected using anti-Dig alkaline phosphatase-conjugated antibody and visualized with NBT/BCIP chromogen, according to the manufacturer's instructions (Roche GmbH). The sections were counterstained with 0.1% Fast Red (Aldrich Chemical Corp., Milwaukee, WI, USA).

Chicken methods

To use the developing chicken embryo as an AMH-responsive sex-differentiation model system, the integrating retroviral vector RCASBP was used to overexpress platypus AMH. RCASBP(A) constructs were prepared that express GFP and used a T2A-linker to also express AMH (such that GFP acts as a marker of AMH expression). The "empty" negative control construct encodes just a downstream FLAG-tag (GFP-T2A-FLAG).

The chicken AMH ORF was used as a positive control, with known effects [51] on the chicken urogenital system (GFP-T2A-cAMH). The platypus AMHX and AMHY ORFs were cloned with (+CC) and without the chicken-specific cleavage sites (GFP-T2A-pAMHX, GFP-T2A-pAMHX+CC, GFP-T2A-pAMHY, GFP-T2A-pAMHY+CC). Constructs were assembled using homology-based cloning methods (HiFi DNA Assembly, NEB Australia) with a combination of synthesized DNA and PCR products with primers to introduce overhangs or sequence modifications. Sanger sequencing was used to confirm correct construct assemblies (Micromon Genomics, Monash University, Clayton Australia).

High titer ($>1\times10^7$ infectious units per mL) purified virus preparations were made by transfecting each RCASBP construct into DF-1 cells (chicken fibroblast cell line), collecting conditioned media, and concentrating using ultracentrifugation as previously described [52]. Purified virus particles were injected into the blastoderms of embryonic day 0 (E0) fertile SPF chicken eggs (Australian SPF Services) and incubated in a humidified environment at 37 °C with hourly rocking until E9.5. Eggs were then opened and embryos dissected to expose the urogenital systems for imaging on a Zeiss SteREO Discovery.V12 microscope with Axiocam506Colour camera.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13059-025-03546-1.

Photographic composite and uncropped images are available at 10.6084/m9.figshare.28545305 [75].Additional file 1: Fig. S1 Analysis of monotreme *AMH* genes and proteins showing repeat content in the monotreme *AMH* genes, AMH protein alignment and predicted structures of platypus AMHX and AMHY proteins bound to AMHR2. Fig S2 Mammalian *AMH* proximal promoter alignment showing 300 bp upstream of the translation start sites. Fig S3 Echidna sexing and expression analysis showing sexing of fetuses or pouch young by PCR from gDNA or RT-PCR and expression of *AMHX*, *AMHY*, *DMRT1* and *SOX9* in gonads of pouch young by RT-PCR. Fig S4 Overexpression of platypus AMHX and AMHY in the chicken gonad at E9.5. Fig S5 Comparative analysis of residues involved in AMH binding to AMHR2. Supplementary Figures S1-S5 and figure legend.

Additional file 2: Extra data for Figures 1-3 and Supplementary Figures S1, S5 and Tables S1-S4. Supplementary Tables S1-S5. Supplementary Table S1 Percentage identities of *AMH* coding sequences (base pairs). Supplementary Table S2 Amino acid substitution rates of AMH protein. Supplementary Table S3 Percentage identities of AMH proteins (amino acids). Supplementary Table S4 Percentage identities of AMH TGF-b domains (amino acids). Supplementary Table S5 Primers used for PCR screening and generation of in situ hybridization probes.

Additional file 3: Supplementary Table S1 Percentage identities of AMH coding sequences (base pairs). Supplementary Table S2 Amino acid substitution rates of AMH protein. Supplementary Table S3 Percentage identities of AMH proteins (amino acids). Supplementary Table S4 Percentage identities of AMH TGF-β domains (amino acids). Supplementary Table S5 Primers used for PCR screening and generation of in situ hybridization probes.

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Peer review
Tim Sands was the primary editor of this article and managed its editorial process and peer review in collaboration with the rest of the editorial team. The peer-review history is available in the online version of this article.

Authors' contributions
LS-W and FG designed the experiments to characterise the monotreme *AMH* genes and proteins. LS-W cloned and sequenced the monotreme *AMH* genes, carried out synteny analysis, gene analysis, protein promoter analysis and prepared the in situ hybridisations of platypus samples. JF, SJ, MP and MR designed the echidna experiments. MR and JF collected the fetal and pouch young echidna samples and MP supervised the echidna colony. MR, JF and SJ dissected the gonad samples. JF prepared the fetal and pouch young gonad RT-PCRs. HY and JF prepared the in situ hybridisations of echidna samples. YZ and GZ provided the monotreme genomic sequences of *AMHX* and *AMHY*. LS-W, FG, AM and CS designed the experiments in the chicken model system, with AM and CS performing the in ovo work and data acquisition. ZQ carried out the Ka/Ks analysis. KS carried out the AMH and AMHR2 protein modelling and analysis. JG, AS and DA carried out the repeat analysis. LS-W, JF, CS, MR and FG wrote the manuscript. All authors revised the manuscript and approved the paper.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Wild short-beaked echidnas were obtained and maintained under the Queensland Government EPA scientific purposes permit (WISP153546614). The University of Queensland Animal Experimentation Ethics Committee approved all sampling for echidnas and the University of Melbourne Animal Experimentation Ethics Committee approved all sampling of platypuses, in accordance with the National Health and Medical Research Council of Australia Guidelines (2013) [74]. Ethics approval was not required for the chicken experiments.

Competing interests
The authors declare no competing interests.

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