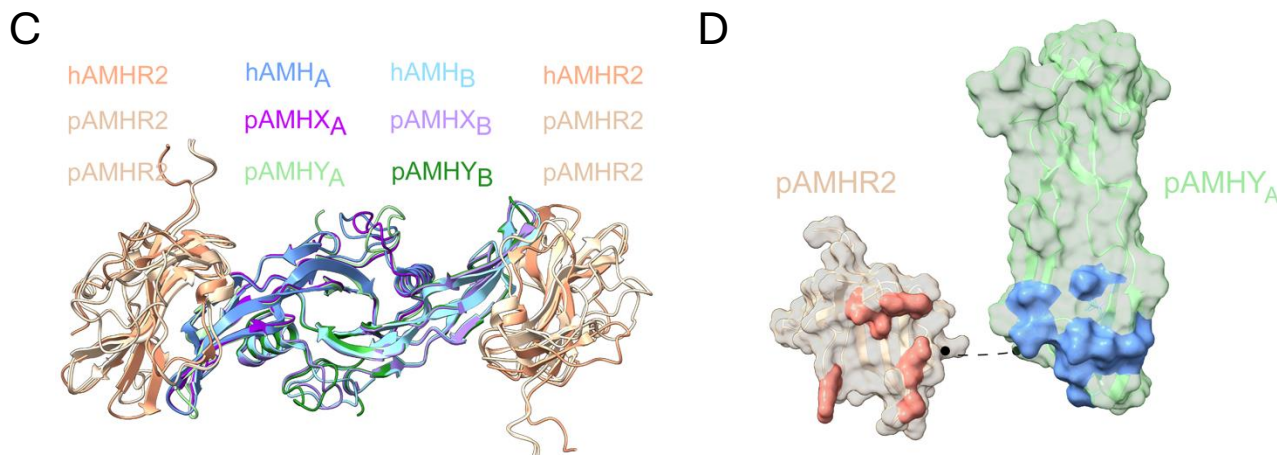
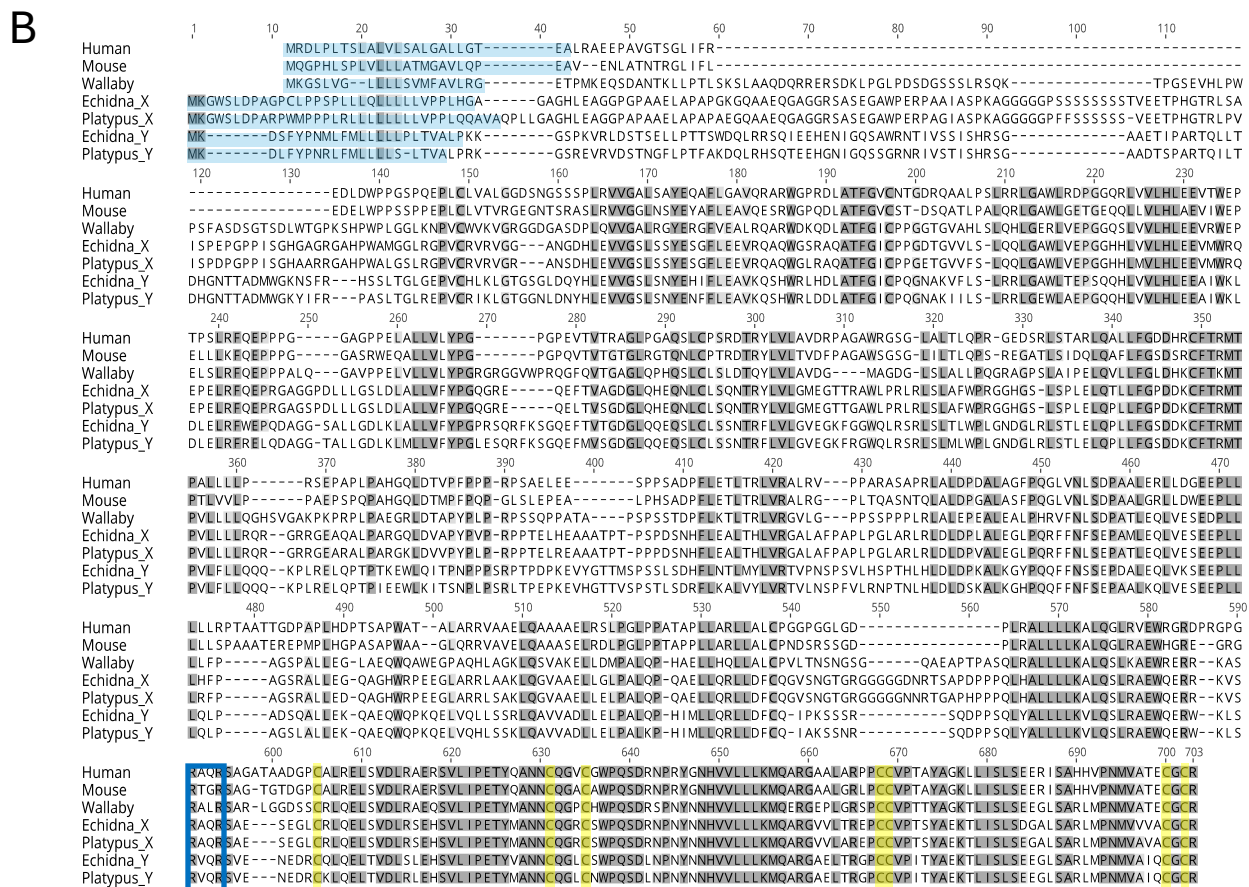
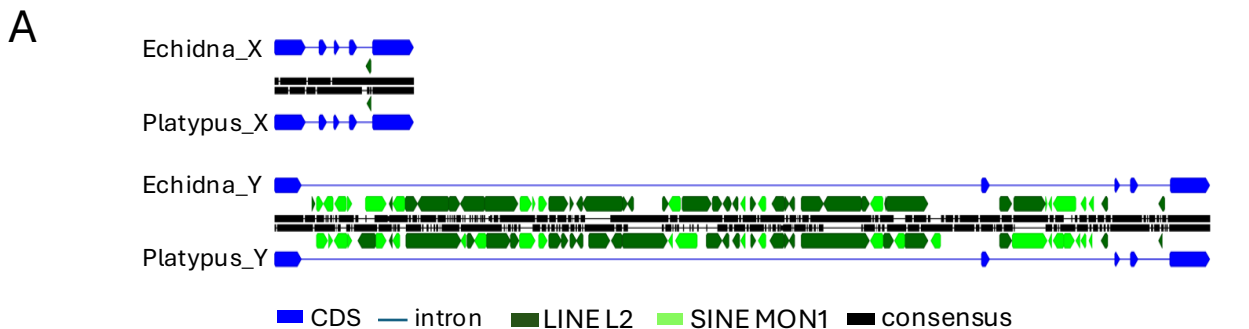




Supplementary Fig. S1 Analysis of monotreme AMH genes and proteins. (A) Repeat content of the monotreme *AMH* and *AMHY* genes. Coding sequence, blue; introns, black or blue line, LINE L2, dark green and SINE MON1, light green. (B) Alignment of AMH proteins from mammalian species human and mouse (eutherian), wallaby (metatherian), echidna and platypus (prototherian). Shaded amino acids, identical amino acids; light blue shading, predicted signal peptide; yellow shading; conserved cysteine residues; blue outline, cleavage site between amino terminal and TGF- β domains and underline, TGF- β domain. (C) Overlay of platypus AMHR2-AMHX and AMHR2-AMHY multimeric complexes, predicted with AlphaFold multimer (Evans et al., 2021), using a stoichiometry of 2 AMHR2 domains and 2 AMHX (or Y) domains. The experimentally determined human hAMHR2-AMH structure (PDB 710J) is also shown in the overlay. (D) Pull apart view of the predicted platypus AMHR2 (peach) and AMHY (pale green) interface to highlight close contact residues conserved between the platypus and human complexes. Close contact residues from the interface in the hAMHR2-AMH complex (PDB 710J) are shown highlighted in the platypus cAMHR2-AMHY complex if the residue is identical (pink in AMHR2; pale blue in AMHY).

P_Amhy	ATTATCCATTTTTAAAGGCTGCAGAC-ATACTACTTGAAATCACATAAA-----G	49
E_Amhy	-----AT-ACACTGCTTGAAAACACATAAAGTAGCCTTG-G	34
W_Amh	-----CAAGGACAGTATTTTGACAAATCTGGTCAAC-----AGCTTGAA	39
P_Amhx	-----TCTCCTGCTCTG-----GCTCAAA	19
E_Amhx	-----AAGGACGGCGTTTCTCCTGCTCTG-----GCTCAAA	31
H_Amh	-----CGCTGTCTAGTTTGGTTGCTTGGCCGTCCTCC-CAGCCTGGTTCCCACTCCTG	53
M_Amh	-----AGTGGGGAGGGTGGATACT-GCTTGTCTCTGTTGAAGCTGTGGTGACCTGGGG	52

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P_Amhy	TAAAATCTTATCTTTATCATTAC---AGACCCAAAATCACCAAGTGCATTTTTTCGGC-	105
E_Amhy	AAAAATTTCACATTTTACCATTAC---ATACTCAGATTACACA--GTACGATTTTCGCC-	88
W_Amh	ATTTCATATCACTTGTAAAG---AAGCAAGATACCCAGCCAAAAATAGAACAAGAAACC-	95
P_Amhx	TTTATATCATTGTCCGAAGCAGATATACCCAGCCCGAAAAATAGAACATA-AGGTTT-	77
E_Amhx	TTTATATCATTGTACAAAGCAGATATACCCAGCCCTGAAAAATAGAACAAGAAAGGTTT-	90
H_Amh	TGTCTTCTGGGGATGGCCCTCAAGGACGGCATGTTGACACATCAGGCCAGCTCTATCA-	112
M_Amh	CGTCTTCAGGTGGGCTCCCCAG---GGAGATGGGAGCTACTCAAGGACAGCTCAGGCCT	109

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Sox9-151

P_Amhy	ATATCAACTACGTCACTTCCGTTTGAAAGAATATCCGCTTTTCAAGAGC-----	155
E_Amhy	ATATCTACTAGGTCACTTCCGTTTCGAAAGATTATCCGCTTTTAAAGGC-----	138
W_Amh	-CTTTGAAAGGGCCTCCCCTC-ACTTTACACTGTTGGTCTCCAGGGTCCCAAAGGATGT	153
P_Amhx	-TTTTGAAAGAGGTTTCGCTCA-GTTTCAGGCCATTGGCCTTTCAAGTTGCCAAACGAGCT	135
E_Amhx	-TTTTGAAAGAGGTTTCCTCA-GTGTGAGGCCATTGGCCTTTCAAGTTGCCAAAGGATCT	148
H_Amh	CTGGGGAGGGAGATAGGCTGCCAGGGACAGAAAGGGCTCTTTGAGAAAGGCCACTCTG---	169
M_Amh	CTGCAGTTATGGGCCAGCTCTGAGGACAGAAAGCCCTTTGAGACAGTCGCTCCACCT	169

SF-1-102

GATA4-84

P_Amhy	--GGGACTTCGACCATT---T-----AGGTAAGGAAGGGGTGGAAG	192
E_Amhy	--GGGGCTCGGCCTCGC---GATAGTGTGCGATTTTACATAATGAAAGGGCCGGTG	191
W_Amh	GCTCTGCATTGTCAATTC---AAGGTACCTCCGTTGG---GAAGAGATAACCATC	203
P_Amhx	GTTGGGCATTGTGACTTC---AAGGTACCTCCGTTCA---CAAGAGATAGCTGCC	185
E_Amhx	GTTGGGCATTGTGACTTC---AAGGTACCTCCGTTCA---CAAGAGATAGCTGCC	198
H_Amh	--CCTGGAGTGGGGGCGCCGGGCACTGTCCCAAGGTTCGGCAGAGGAGATAGGGGTC	227
M_Amh	GCTGGGCATGAAAAGTGCAGGCACTGTCCCAAGGTACCTTTGGTGTGATAGGGGC	229

HMG-66

TATA

P_Amhy	CGTAGCCGCTGGCTTTTATCCT-GCCGGTGACTTACTTCCACACGGCGGAAAG---ATT	248
E_Amhy	TTTAGCTGCTGGCTGTTTGTCTGCCGGGGGACTTGCTTCCACGCGGAAAGAAG---GTT	248
W_Amh	CTTCCCTCCTCATAAACAGTCTGGAGAAGGCATTTCTGCCACCTTAGTCTCATAAAGC	263
P_Amhx	TTTGCCTCCTTCGCAAAAGGTTTGGGAA-AGCATCGCTGCCACCCTGGCCTCATATAAGG	244
E_Amhx	CTTGCCTCCTTCGCAAAAGGTTTGGGAA-AGCATCGCTGCCACCCTGGCCTCATATAAGG	257
H_Amh	TGTCCTGCACAAACACCCACCTTCCACTCGGCTCACTTA-----AGG	270
M_Amh	GTCCC-TCCCAAGCAAGCAATCTGGCTCAGCCATACATAT-----AAG	271

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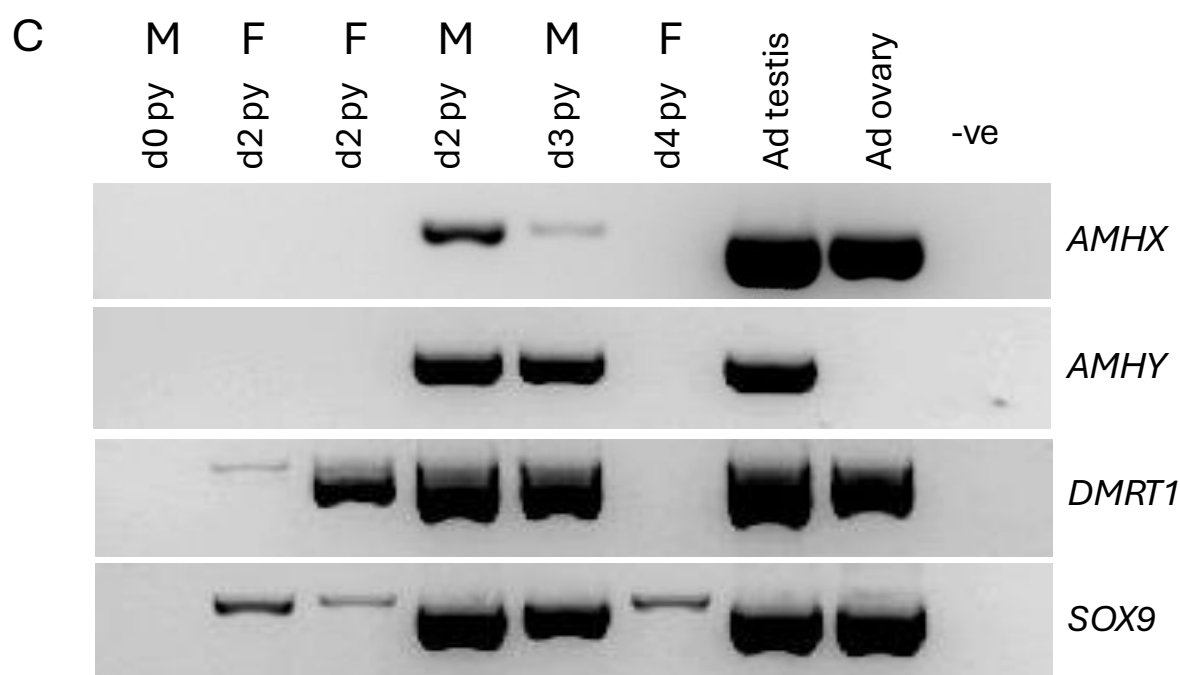
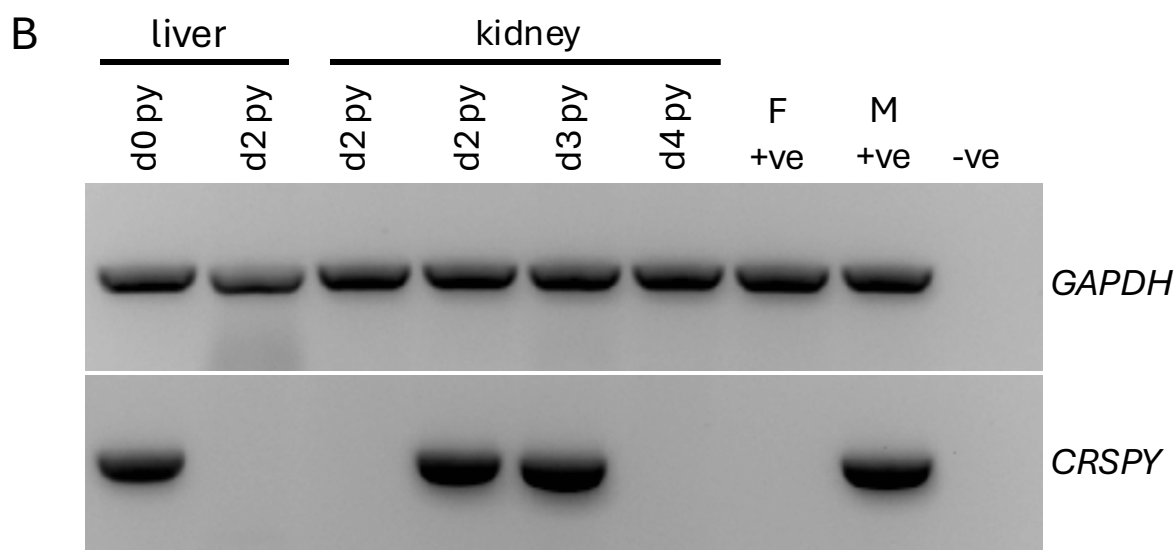
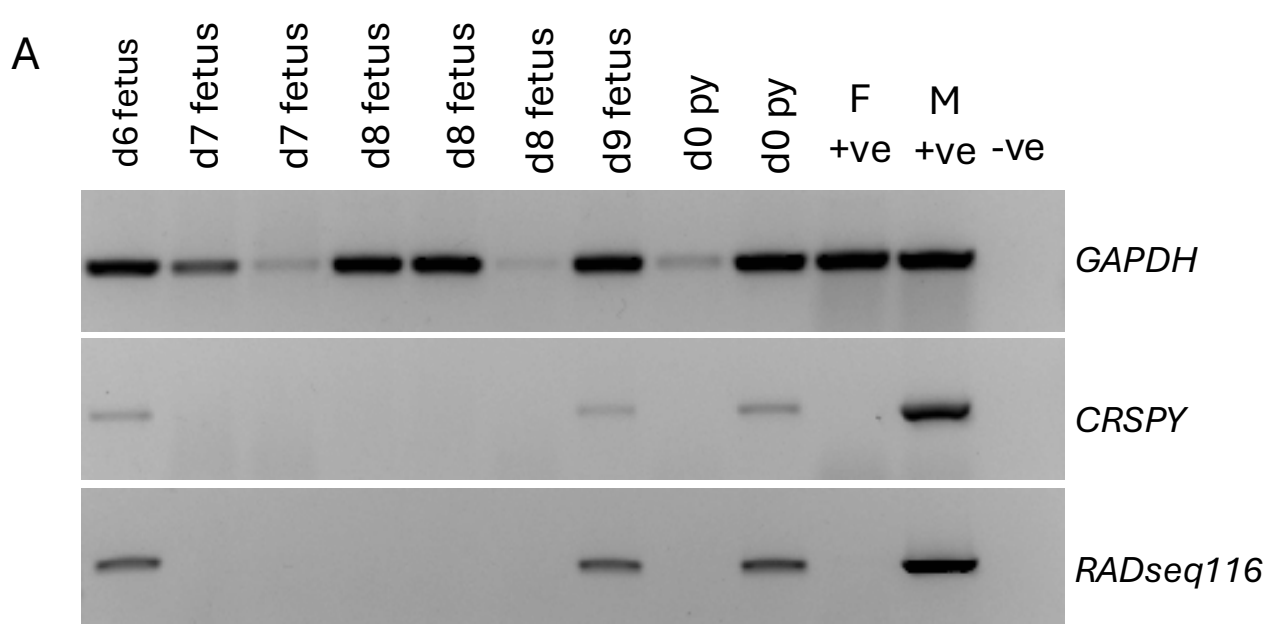
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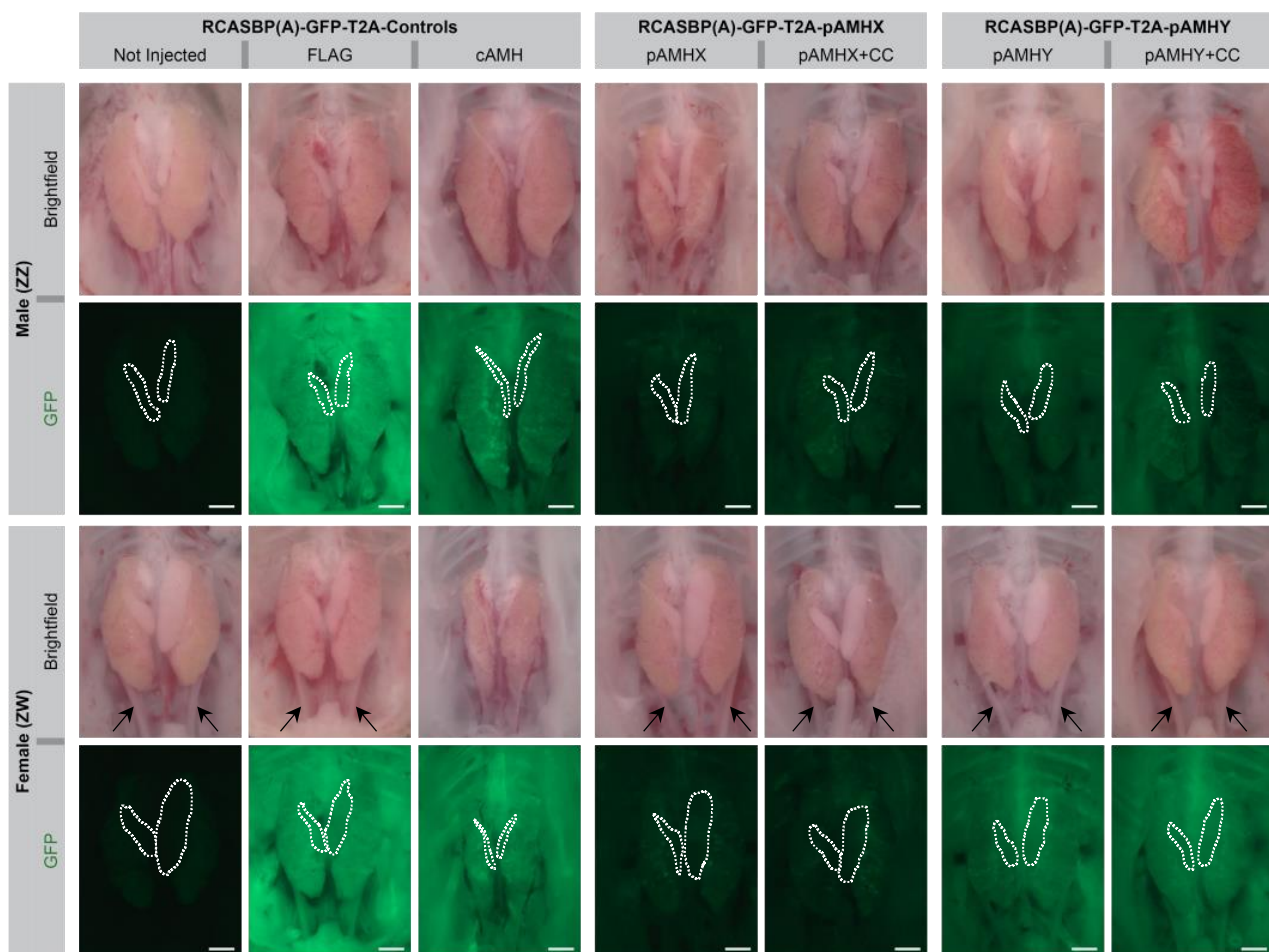
P_Amhy	CATAG---TCTTCAAGCTGTTAGATTCCAAAAAGTTTCTGAGTCATTAAAGCCAAAATG	303
E_Amhy	CATAA---TCTTCAAGTCGTTGATTCCAAGCGTTTCTGAGTCATTAAAGCCAAAATG	303
W_Amh	CCCAGTTAGCCAAAGGCTCGGTACAGTC-----AGCCCAAGATG	303
P_Amhx	CGGAGGCAGCCGGGAACTCGGGTGCACACATACTCATGCAACATACCCTGCTCAAAATG	303
E_Amhx	CGGAGGCAGCCGGGAACTCGGATGCGCACACA-----CCCGCTCAAAATG	303
H_Amh	CAGGCAG---CCCAGCCCTGGCAGCAC-----CCACGATG	303
M_Amh	CAGGGCC---ACCCGGACCTTGCTGTAC-----CACC-ATG	303

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Supplementary Fig. S3 Echidna sexing and expression analysis by RT-PCR. Expression of *CRSPY* and *RADSeq 116* with *GAPDH* as loading control detected by RT-PCR from either FFPE gDNA (A) or somatic pouch young tissues (B) to identify the sex of all fetuses and pouch young with adult testis and ovary used as male and female positive controls. (C) Expression of *AMHX*, *AMHY*, *DMRT1* and *SOX9* in pouch young gonads from d0-d4 py with adult testis and adult ovary as a positive control. py, pouch young; Ad, adult.

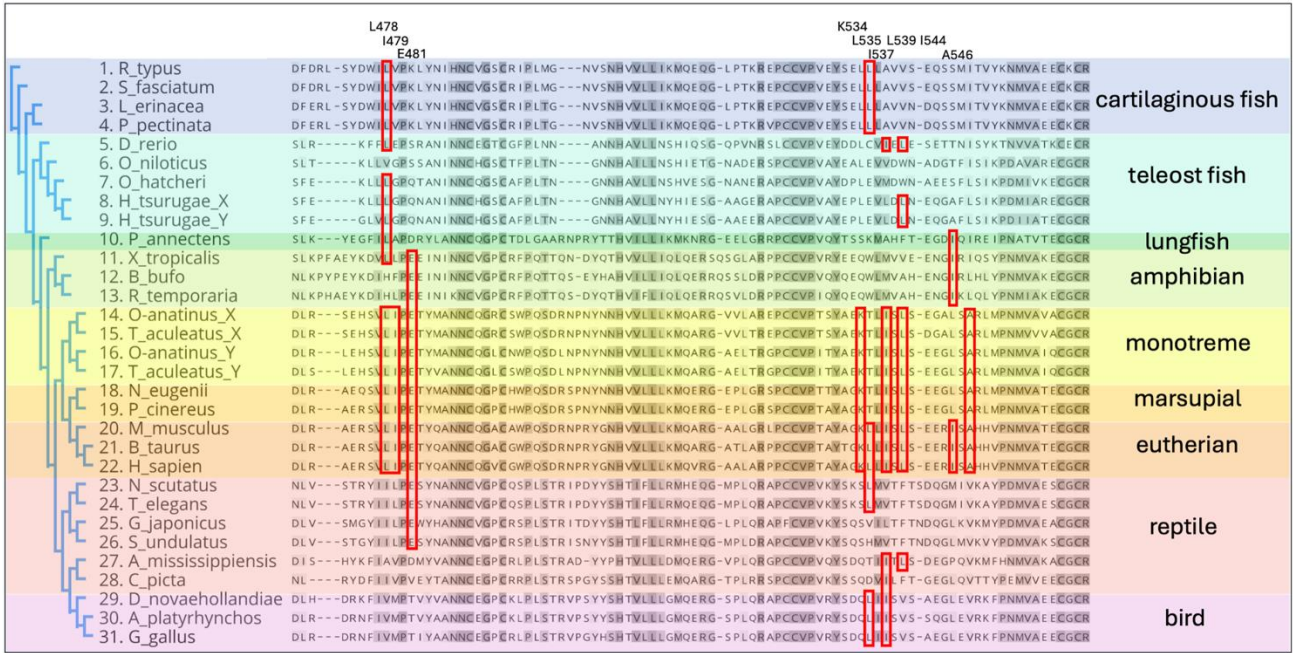


Supplementary Fig. S4 Overexpression of platypus AMHX and AMHY in the chicken Dissected urogenital systems at E9.5 from genetically male and female chicken embryos (determined by PCR). GFP signal indicates expression of the indicated transgenes (GFP-T2A-ORF, platypus proteins, +CC carry the chicken cleavage site). The gonads are outlined (white dotted lines) with the Müllerian ducts where present indicated (black arrows). Scale bar, 1mm.

A

	Residue	Properties	Platypus	Properties	Chicken	Properties	Description
AMHR2	R33	pos charge	R	pos charge	R	pos charge	binds I544 and A546 of AMH
	F62	hydrophobic	F	hydrophobic	I	hydrophobic	hydrophobic interaction with L478, I479, L535, I537, L539
	I64	hydrophobic	I	hydrophobic	I	hydrophobic	hydrophobic interaction with L478, I479, L535, I537, L539
	M76	hydrophobic	M	hydrophobic	V	hydrophobic	hydrophobic interaction with L478, I479, L535, I537, L539
	D81	neg charge	D	neg charge	G	hydrophilic	salt bridge with K534
	E84	neg charge	E	neg charge	G	hydrophilic	salt bridge with K534
	L106	hydrophobic	L	hydrophobic	V	hydrophobic	hydrophobic interaction with L478, I479, L535, I537, L539
AMH	L478	hydrophobic	L	hydrophobic	V	hydrophobic	L478A abolishes signalling and duct regression
	I479	hydrophobic	I	hydrophobic	M	hydrophobic	hydrophobic interaction with F62, I64, M76, L106
	E481	neg charge	E	neg charge	T	polar uncharged	E481R/Y reduces activity 5 fold
	K534	pos charge	K	pos charge	Q	polar uncharged	salt bridge with D81, E84; K534A abolishes signalling
	L535	hydrophobic	T	polar uncharge	L	hydrophobic	hydrophobic interaction with F62, I64, M76, L106
	I537	hydrophobic	I	hydrophobic	I	hydrophobic	hydrophobic interaction with F62, I64, M76, L106
	L539	hydrophobic	L	hydrophobic	V	hydrophobic	hydrophobic interaction with F62, I64, M76, L106
	I544	hydrophobic	L	hydrophobic	L	hydrophobic	binds R33
	A546	hydrophobic	A	hydrophobic	V	hydrophobic	binds R33; A546M abolishes activity

B



Supplementary Fig. S5 Comparative analysis of residues involved in AMH binding to AMHR2 (A) Table showing a comparison of AMHR2 and AMH amino acids involved in receptor binding between human, platypus and chicken. Residues not conserved with human, grey shading. **(B)** Alignment of C-terminal of the AMH TGF- β domain protein sequences from vertebrates showing conservation of amino acids involved in receptor binding defined in the human protein (Hart *et al.*, 2021, red boxes).