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RT-qPCR-based sex identification in *Platynereis dumerilii* juveniles

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Abstract

Platynereis dumerilii has separate sexes, but as juveniles there are no outside morphological characteristics that help determine the sex of individual worms. It is often important to know whether experimental samples are females or males. Previously, we published sex identification markers for worms that are between 40 and 70 segments (40S-70S) (Ribeiro et al, 2025), where we presented reverse transcription polymerase chain reaction (RT-PCR) and gel electrophoresis as an accessible method. In that study, we also performed preliminary results from RT-qPCRs, yielding encouraging data that informed the development of this fully validated approach described here. In this protocol, we present a detailed method for sex identification using reverse transcription quantitative polymerase chain reaction (RT-qPCR), which is more sensitive, and has higher accuracy. Just as the RT-PCR method, this is also based on mRNA expression of sex marker genes from coelomic contents. These samples can be collected without sacrificing the individual worms, thereby allowing sex identification for those samples and designing experiments with sex as a biological variable accounted for. Details of sex-biased genes used here can be found in our previous work (Ribeiro et al., 2025).

Practical Example

P. dumerilii does not have gonads and instead makes clusters of cells (gonial clusters) that are freely floating in the coelomic cavity (Fischer, 1974, 1975; Kuehn et al., 2022; Meisel, 1990). For sex identification, worms can be amputated at their tail, and coelomic contents including the gonial clusters can be collected for sex identification.

In this example, we collected coelomic contents from amputated tails of *P. dumerilii* juveniles at the 50-segment and 60-segment developmental stages (50S and 60S). Total RNA was extracted from these samples, and gene expression levels were measured using RT-qPCR.

For each cDNA sample of a worm, we measured the expression of a male-specific marker (*dmrt1* - doublesex and mab-3 related transcription factor 1) and a female-specific marker (*psmt* - protostome specific methyltransferase). Expression values were reported as cycle threshold (Ct) values (Table 1).

To assign sex, we calculate the **Sex Score** as the difference between the average Ct values of the two markers within the same biological sample: **Sex Score = Avg Ct (*dmrt1*) – Avg Ct (*psmt*)**. Samples with **positive sex scores** are identified as females, whereas samples with **negative sex scores** are as males.

Because sex differentiation relies on the relative expression of the male and female markers in each individual sample, the difference in Average Ct values between *dmrt1* and *psmt* is equivalent to the difference in their ΔCt values (ΔCt is a traditional normalization method in RT-qPCRs). Therefore, normalization to a housekeeping gene using the ΔCt method is not required for sex identification here. Nevertheless, amplification of 18S rRNA was included as a technical positive control to confirm RNA quality and RT-qPCR performance (Table 1).

	A	B	C	D	E	F	G	H	I
	WORM ID	***Avg Ct (dmrt1)***	***Avg Ct (psmt)***	***Avg Ct (18S)***	***ΔCt dmrt1***	***ΔCt psmt***	**Sex Score**	**SEX (RT-qPCR)**	**SEX (RNA-seq)**
	G11	25.8	22.09	23.95	16.34	12.63	3.71	female	female
	G7	27.25	24.61	25.93	13.87	11.23	2.64	female	female
	G10	23.25	30.42	26.83	8.36	15.54	-7.17	male	male
	G12	31.7	28.28	29.99	14.37	10.95	3.42	female	*N/A*
	G3	27.09	22.93	25.01	16.13	11.97	4.15	female	female
	V4	32.41	33.13	32.77	11.8	12.52	-0.72	male	*N/A*
	V3	31.39	35.8	33.59	5.02	9.43	-4.41	male	*N/A*
	V10	21.42	28.83	25.13	3.78	11.19	-7.41	male	male
	V1	24.03	32.28	28.16	5.31	13.56	-8.25	male	male
	V5	30.53	29.31	29.92	13.08	11.87	1.22	female	female
	V9	30.02	28.19	29.11	14.74	12.9	1.83	female	female
	G2	29.92	25.66	27.79	13.48	9.23	4.26	female	female
	V6	29.64	25.89	27.77	12.81	9.05	3.75	female	female
	V8	24.28	30.5	27.39	7.28	13.5	-6.22	male	male
	V7	28.99	27.86	28.42	12.29	11.15	1.14	female	female
	V12	24.61	28.71	26.66	7.47	11.58	-4.1	male	male

Table 1: Results of practical example, showing RT-qPCR Ct values for male (*dmrt1*), female (*psmt*), and housekeeping (18S) markers in individual worm cDNA samples. *dmrt1* primers: Pdu-dmrt.si3.D3-F and Pdu-dmrt.si3.D3-r. *psmt* primers: Pdu.psmt-b1-F and Pdu.psmt-b1-F. 18S primers: 18S-RPR-F and 18S-RPR-R. Primer sequences are provided in Table 2. *N/A*: Samples not sequenced.

Image Attribution

Fig. 1: Typical 2-Temp Amp + Melt RT-qPCR cycle used in this protocol.

Fig. 2: Scheme summarizing interpretation of results.

Materials

	A	B	C	D
	Category	**Item**	**Notes**	**Suggested Vendor / Cat number (Cat.)**
	Consumables	Microcentrifuge tubes	RNase-free	Any laboratory supplier
		RT-qPCR plates	Compatible with qPCR instrument	Bio-Rad or equivalent
		Optical plate seals	Microseal	Bio-Rad/ Cat. MSB1001
		Pipette tips	RNase-free	filtered
	RNA extraction reagents and kits	Arcturus PicoPure RNA isolation Kit	For low-input RNA samples	Applied Biosystems/ Cat. KIT0204
		TRIzol Reagent	For higher-input RNA extraction	Invitrogen/ Cat. 15596026
		Rneasy Mini Kit	For RNA clean up	Qiagen/ Cat. no. 74104
	Enzymes / reagents	RNase-Free DNase Set	On-column DNase treatment	Qiagen/ Cat. no. 79254
	cDNA synthesis kit options	1st Strand cDNA Synthesis Kit for RT-PCR (AMV)	For cDNA synthesis	Sigma-Aldrich/ Cat. no. 11483188001
		High-Capacity RNA-to-cDNA	For cDNA synthesis	Applied Biosystems/ Cat. no. 4387406
	RT-qPCR master mix	SsoAdvanced Universal SYBR Green Supermix	For RT-qPCR reactions	Bio-Rad/ Cat. No. 1725271
	Molecular biology reagent	Nuclease-free water	For reactions	Any laboratory supplier

	A	B	C	D
			and dilutions	
	Equipment	Microcentrifuge	With plate adapters if needed	Any laboratory supplier
		Refrigerated centrifuge	Optional for RNA work	Any laboratory supplier
		qPCR instrument	Capable of melt curve analysis	Bio-Rad or equivalent
		NanoDrop or spectrophotometer	For RNA quantification	Thermo Fisher Scientific or equivalent

Materials required for RT-qPCR-based sex identification in *Platynereis dumerilii* juveniles described in this study. Suggested vendors are provided for reference; equivalent alternatives may be used.

Troubleshooting

Problem

No amplification or Ct ≥39

Solution

Possible cause: Low RNA input or RNA degradation. Solution: Verify RNA concentration and integrity; repeat extraction if needed

Problem

High Ct variability between replicates

Solution

Possible cause: Pipetting error or low template amount Solution: Increase technical replicates; ensure accurate pipetting

Problem

Multiple peaks in melt curve

Solution

Possible cause: Non-specific amplification or primer–dimer formation. Solution: Exclude affected wells; confirm primer specificity and efficiency

Problem

Sex score close to zero

Solution

Possible cause: Low differential expression or early developmental stage Solution: Repeat qPCR, wait animal to reach a more progressed developmental stage, or use additional markers

Problem

RT-qPCR result conflicts with morphology

Solution

Possible cause: Transitional gonial state or biological variability Solution: Re-sample the same animal later or validate using an independent method

Problem

All reactions fail for a gene (NaN Ct values)

Solution

Possible cause: Primer degradation Solution: Aliquot new primers

Safety warnings

- Indeterminate range: If sex score is close to zero (e.g., -0.5< sex score <0.5) classify the sample as indeterminate and repeat sampling or RT-qPCR.Sex identification is more reliable when results are consistent across independent methods (e.g., gonial cluster morphology and RT-qPCR, or RT-qPCR and RNA-seq).
- Gene expression patterns of sex-specific markers may vary with developmental stages (e.g., 40-segment vs. 60–70-segment animals). Younger worms may show reduced differences in the expression of sex marker genes, which can lead to ambiguous results. See Ribeiro et al. (2025) for additional discussion of stage-dependent expression patterns.
- When possible, include additional sex-specific markers to confirm result consistency.
- If feasible, allow worms to develop clear morphological sex characteristics before sampling. For visual confirmation, use general morphological characteristics of gonial clusters and the stages they go through in females and males. Guidelines for visual gonial cluster staging by our lab are under construction, but you can use the previous literature (Fischer, 1974, 1975; Meisel, 1990).

Step-by-Step Protocol

1 Gene Selection

1.1 Minimum requirement: Select one male-specific gene marker and one female-specific gene marker. We suggest using *dmrt1* for males and *psmt* for females.

1.2 Select primers only from the list of RT-qPCR-validated primers provided in Table 2. Primer amplification efficiencies were evaluated under the same RT-qPCR conditions described here (see Step 4) and are reported in Table 3. It is important to note that primers used for RT-qPCR in our previous study (Ribeiro et al., 2025) may show low efficiency in RT-qPCR and should not be used unless validated.

Primer requirements:

- Acceptable efficiency range: 90-110%.
- Expected amplicon size: 70-150 bp depending on primer design.

	A	B	C	D
	Primer	**Sequence**	**Tm**	**Suitability**
	18S-RPR-F	TAGAGTGTTCAAGGCAGGCG	60.04	PCR/ qPCR
	18S-RPR-R	CCACCAACTAAGAACGGCCA	60.25	PCR/ qPCR
	Pdu-actin-RPR-F	AAGATCTTGACCGAGCGTGG	60.11	PCR/ qPCR
	Pdu-actin-RPR-R	GATGGAGCCAAGGCAGTGAT	60.11	PCR/ qPCR
	Pdu-dmrt1-Q1-F	CGCCCCTGATCAAAGGCCAA	63.12	PCR
	Pdu-dmrt1-Q1-R	AAGGCTGAACCGAGCTGCTG	63.07	PCR

	A	B	C	D
	Pdu-dmrt1-Q6-F	TGGCACAAGCATGCAAGCAC	62.7	PCR
	Pdu-dmrt1-Q6-R	GGCCGACGCTCTCAATGGAT	62.65	PCR
	Pdu-psmt-Q1-F	CCAGGGCTGTGCCAACATCT	62.78	PCR
	Pdu-psmt-Q1-R	CGGGATGTGTTCCGCCACTT	63.08	PCR
	Pdu-psmt-Q4-F	GGATGCTTGCCATGGTTGCC	62.58	PCR
	Pdu-psmt-Q4-R	CACTCGGCCGTTGCTAACCT	62.78	PCR
	Pdu-lrcct-Q1-F	ACGGTCTCTCCAACCGACCA	62.99	PCR
	Pdu-lrcct-Q1-R	TCCCGGAAGTCGGAACCAGT	63.01	PCR
	Pdu-lrcct-Q2-F	TCCAAGCAGGAGGGGGAGAC	63.12	PCR
	Pdu-lrcct-Q2-R	AGAAGCCCTGAAGGTCCGGT	63.04	PCR
	Pdu-kctd21-Q4-F	TTGGGCAACTTGGGCAGACA	62.58	PCR
	Pdu-kctd21-Q4-R	CAGGCCCAAGGCTGTTGGTA	62.42	PCR
	Pdu-kctd21-Q5-F	TGGTTGGGCAACTTGGGCAG	63.56	PCR
	Pdu-kctd21-Q5-R	AATGTTCAGCGACAGGCCCA	62.41	PCR

	A	B	C	D
	Pdu.psmt-b1-F	TCTGGTGGATGGTGTGAGGA	60.18	PCR/ qPCR
	Pdu.psmt-b1-R	TAATGGCCACGTCCATCTCC	59.53	PCR/ qPCR
	Pdu.psmt-b2-F	ATGGTGTGAGGAAACAGCCA	59.52	PCR/ qPCR
	Pdu.psmt-b2-R	GGCCACGTCCATCTCCAAA	60	PCR/ qPCR
	Pdu.psmt-si1-m1-F	CATGTCCCTTGAAGTGGCGG	61.31	PCR/ qPCR
	Pdu.psmt-si1-m1-R	GTCGCACAATGTTGCTCAAGT	60	PCR/ qPCR
	Pdu.psmt-si1-m2-F	GTCCCTTGAAGTGGCGGAA	59.93	PCR/ qPCR
	Pdu.psmt-si1-m2-R	GCATGTCGCACAATGTTGCT	60.39	PCR/ qPCR
	Pdu-dmrt.si1.D1-F	CTTGCCACTACGACCTGGAG	60.11	PCR/ qPCR
	Pdu-dmrt.si1.D1-R	AGCCTGTTCTTGGGCATGTT	60.18	PCR/ qPCR
	Pdu-dmrt.si3.D2-F	CGTAATCCGGATGAGAGCCC	60.04	PCR/ qPCR
	Pdu-dmrt.si3.D2-R	TCGTAGTGGCAAGCCTTCAA	60.32	PCR/ qPCR
	Pdu-dmrt.si3.D3-F	CCGTAATCCGGATGAGAGCC	60.04	PCR/ qPCR
	Pdu-dmrt.si3.D3-R	AGCTCGGTTCAGCCTTTTCT	59.61	PCR/ qPCR

Table 2. Primers designed for amplification of target and housekeeping genes.

	A	B	C	D	E	F	G
	Target	**Forward**	**Reverse**	**Ta (°C)**	**Slope**	**Efficiency (%)**	**Best use**
	psmt	Pdu.psmt-b1-F	Pdu.psmt-b1-R	60	-3.5	93.07	RT-qPCR
	psmt	Pdu.psmt-b2-F	Pdu.psmt-b2-R	60	-3.45	94.92	RT-qPCR
	psmt	Pdu.psmt-si1-m1-F	Pdu.psmt-si1-m1-R	60	-3.58	90.25	RT-qPCR
	psmt	Pdu.psmt-si1-m2-F	Pdu.psmt-si1-m2-R	60	-3.47	94.17	RT-qPCR
	dmrt1	Pdu-dmrt.si1.D1-F	Pdu-dmrt.si1.D1-R	60	-3.62	88.90	PCR
	dmrt1	Pdu-dmrt.si3.D2-F	Pdu-dmrt.si3.D2-R	60	-3.57	90.60	RT-qPCR
	dmrt1	Pdu-dmrt.si3.D3-F	Pdu-dmrt.si3.D3-R	60	-3.55	91.29	RT-qPCR
	18S	18S-RPR-F	18S-RPR-R	60	-3.11	110	RT-qPCR
	actin	Pdu-actin-RPR-F	Pdu-actin-RPR-R	60	-3.11	110	RT-qPCR
	dmrt1	Pdu-dmrt1-Q1-F	Pdu-dmrt1-Q1-R	60	0.561	N/A	PCR
	dmrt1	Pdu-dmrt1-Q6-F	Pdu-dmrt1-Q6-R	60	0.357	N/A	PCR
	psmt	Pdu-psmt-Q1-F	Pdu-psmt-Q1-R	60	-2.03	2.11	PCR
	psmt	Pdu-psmt-Q4-F	Pdu-psmt-Q4-R	60	-1.73	2.78	PCR
	lrrct	Pdu-lrcct-Q1-F	Pdu-lrcct-Q1-R	60	0.499	N/A	PCR

	A	B	C	D	E	F	G
	lrrct	Pdu-lrcct-Q2-F	Pdu-lrcct-Q2-R	60	0.667	N/A	PCR
	kctd21	Pdu-kctd21-Q4-F	Pdu-kctd21-Q4-R	60	0.155	N/A	PCR
	kctd21	Pdu-kctd21-Q5-F	Pdu-kctd21-Q5-R	60	0.144	N/A	PCR

Table 3. RT-qPCR primer set efficiency showing annealing temperature (Ta) used in reactions, standard curve slope, amplification efficiency (%), and recommended application for each primer pair. cDNA templates were prepared from RNA isolated from coelomic contents. N/A values represent failure of amplification.

2 Sample collection

2.1 Collection of coelomic contents enriched in gonial clusters and RNA extraction can be performed using the protocols in Ribeiro et al (2025) (file “Gonial Cluster Isolation_RPR.docx”, available at https://github.com/BDuyguOzpolat/Ribeiro-et-al_Sex_Differentiation/blob/main/1.Protocols.zip).

2.2 Animals do not need to be sacrificed. Samples may be obtained by surgical removal of a parapodium and gentle squeezing to release coelomic contents, or via amputation and dissection of the posterior body to remove coelomic contents as described in our previous protocol mentioned in step 2.1.

2.3 Use only animals with ≥ 40 body segments. Animals with fewer than 40 segments may lack sufficient gonial clusters expressing sex-marker genes, and sex identification may not be reliable. Note that individuals with approximately 50 segments can occasionally yield ambiguous results.

2.4 Sample handling parameters:

- Minimum sample amount: depending on gonial cluster abundance; sufficient material should yield ≥1 ng/µL RNA.
- Time from collection to lysis: up to 30 min, room temperature (RT, ~20–22°C).
- Storage conditions and maximum storage time: extract immediately or for long-term storage, preserve cell extract in Pico Pure Extraction Buffer or TRIzol at -70-80°C until RNA extraction.

3 RNA Extraction and cDNA Synthesis

3.1 Extract total RNA following a protocol including on-column DNase treatment. For samples with fewer cells, for example, samples from parapodium removal and squeeze, we recommend protocol described in Ribeiro et al. (2025) (file: "PicoPure_RNA_extraction.docx", https://github.com/BDuyguOzpolat/Ribeiro-et-al_Sex_Differentiation/blob/main/1.Protocols.zip).

Note: For samples with higher cell numbers, for example coelomic contents from cut tails, TRIzol-based extraction protocols work fine, provided RNA quality is preserved. A TRIzol-based protocol is available at https://github.com/BDuyguOzpolat/Ribeiro-et-al_Sex_Differentiation/blob/main/Updated_protocols/Trizol_RNA_DNA_isolation_Jan15-2026.pdf.

3.2 Synthesize cDNA using the RNA templates. We have tested and recommend 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (11 483 188 001, Sigma-Aldrich) or High-Capacity RNA-to-cDNA™ Kit (4387406, Applied Biosystems™). Use manufacturer instructions for cDNA synthesis.

RNA quality control should respect these parameters.
- RNA concentration (minimum): 1ng/µL.
- RNA purity (A260/280): >1.80, measured using a NanoDrop or equivalent spectrophotometer.
- cDNA template: ~2000ng/µL.

4 RT-qPCR

4.1 Perform RT-qPCR for the selected markers using protocol described by Ribeiro et al (2025): file "RT-qPCR with BioRad SsoAdvanced Universal SYBR Green Supermix.docx", https://github.com/BDuyguOzpolat/Ribeiro-et-al_Sex_Differentiation/blob/main/1.Protocols.zip.

4.2 Prepare mix and primer solutions following the manufacturer instructions.

Reaction setup (BioRad SsoAdvanced Universal SYBR Green Supermix):
- Reaction volume: 20 µL.
- Final cDNA amount per reaction: 100ng-100fg.
- Final concentration of each primer: 0.5 µM.

4.3 Mix reactions thoroughly, avoiding bubble formation, and dispense into a compatible RT-qPCR plate.

4.4 Seal the plate using specific microseals. We recommend Microseal ® 'B' (MSB1001, BioRad).

4.5 Briefly centrifuge to collect contents at the bottom of wells, and load into the RT-qPCR instrument.

4.6 Run RT-qPCR using a two-step amplification protocol followed by melt curve analysis (Fig. 1) with the following cycling conditions.

Cycling conditions: 2-step Amp + Melt (Amplification + Melt Curve).

- Amplification settings: 95°C for 3 minutes (initial denaturation); 39 cycles of 95°C for 10 seconds and 60°C for 30 seconds.
- Melt curve settings: 65°C to 95 °C in 0.5°C increments, with a 5-second hold at each step.

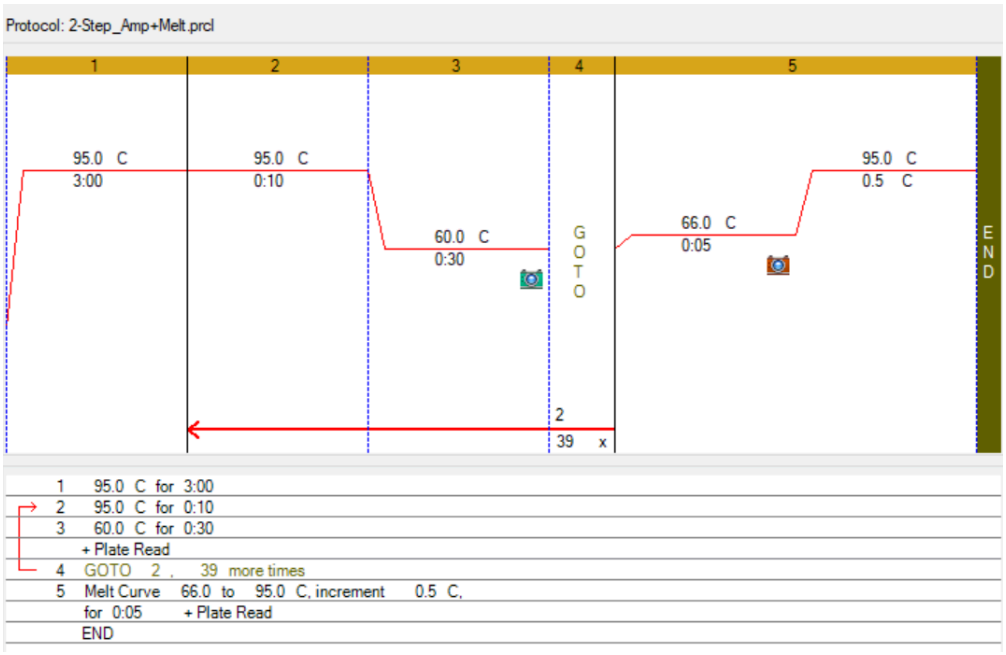

Fig. 1. Typical 2-Temp Amp + Melt RT-qPCR cycle used in this protocol.

4.7 Run at least 2 technical replicates per gene (3 recommended).

4.8 Controls should be run using the same primer sets as experimental samples.

Include the following controls in each run:

- No-template control (NTC)
- No-reverse transcription control (No-RT)

- Positive control samples (known male and female, if available).

5 RT-qPCR analysis

5.1 Retrieve Ct values from your reactions from the RT-qPCR machine.

5.2 For each gene (*dmrt1* and *psmt*) and samples:

1. Exclude failed or non-specific wells based on amplification (e.g. NaN Ct results) and melt curve results (e.g. wells showing multiple peaks or abnormal melt profiles).
2. Calculate the **Average Ct** values from accepted technical replicates.

Replicate acceptance criteria

- Maximum allowed Ct variation between replicates: ≤0.5 cycles
- Consider Ct values ≥39 as not detected (NaN).

6 Identifying sex

6.1 Calculate sex score as:
Sex score = Average Ct (Male marker) − Average Ct (Female marker)

6.2 Interpret results:

- **Negative value:** indicates a **male sample**, because the male marker has a lower Ct (higher expression) than the female marker.
- **Positive value:** indicates a **female sample**, because the female marker has a lower Ct (higher expression) than the male marker.

Only the sign of the sex score is used to assign sex. Because Ct is inversely related to expression level, a lower Ct indicates higher expression, while a higher Ct indicates lower expression. This subtraction directly compares the relative abundance of the male and female markers in the same biological sample (Fig. 2).

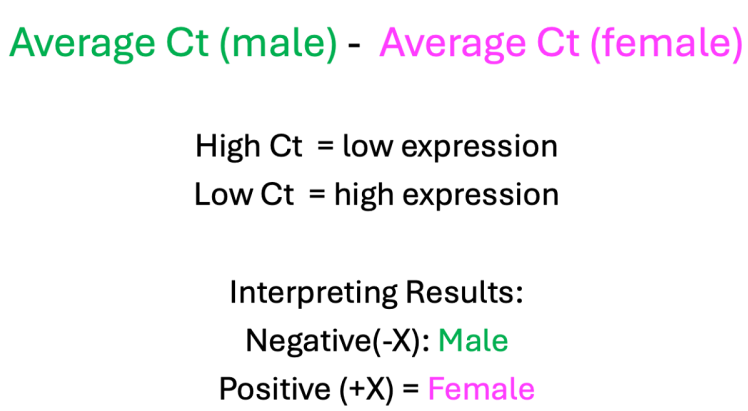

Fig. 2. Scheme summarizing interpretation of results.

Protocol references

Fischer, A. (1974). Stages and stage distribution in early oogenesis in the annelid, *Platynereis dumerilii. Cell and Tissue Research, 156_(1), 35–45. https://doi.org/10.1007/BF00220100*

Fischer, A. (1975). The structure of symplasmic early oocytes and their enveloping sheath cells in the polychaete, *Platynereis dumerilii. Cell and Tissue Research, 160_(3), 327–343. https://doi.org/10.1007/BF00222043*

Kuehn, E., Clausen, D. S., Null, R. W., Metzger, B. M., Willis, A. D., Özpolat, B. D. (2022). Segment number threshold determines juvenile onset of germline cluster expansion in *Platynereis dumerilii. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution, 338_(4), 225–240. https://doi.org/10.1002/jez.b.23100*

Meisel, J. (1990). *Zur Hormonabhängigkeit der Spermatogenese bei Platynereis dumerilii: Licht- und elektronenmikroskopische Befunde sowie experimentelle Untersuchungen in vivo und in vitro_. Ruhr-Universität.*

Ribeiro, R. P., Null, R. W., Özpolat, B. D. (2025). Sex-biased gene expression precedes sexual dimorphism in the agonal annelid *Platynereis dumerilii. Development, 152_(7), dev204513. https://doi.org/10.1242/dev.204513*